Muscle stem cells in Duchenne muscular dystrophy exhibit molecular impairments and altered cell fate trajectories impacting regenerative capacity

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Abstract

Satellite cells are muscle-resident stem cells that maintain and repair muscle. Increasing evidence supports the contributing role of satellite cells in Duchenne muscular dystrophy (DMD), a lethal degenerative muscle disease caused by loss of dystrophin. However, whether or not satellite cells exhibit dysfunction due to loss of dystrophin remains unresolved. Here, we used single cell RNA-sequencing (scRNA-seq) to determine how dystrophin deficiency impacts the satellite cell transcriptome and cellular composition by comparing satellite cells from *mdx* and the more severe D2-*mdx* DMD mouse models. DMD satellite cells were disproportionally found within myogenic progenitor clusters and a previously uncharacterized DMD enriched cluster. Despite exposure to different dystrophic environments, *mdx* and D2-*mdx* satellite cells exhibited overlapping impairments indicating cell-autonomous defects due to dystrophin deficiency. When comparing satellite stem cell versus myogenic progenitor populations, we identified unique dysfunctions between DMD and healthy satellite cells including apoptotic cell death and senescence, respectively. Pseudotime analyses revealed differences in cell fate trajectories indicating that DMD satellite cells are stalled in their differentiation capacity. *In vivo* regeneration assays confirmed that DMD satellite cells exhibit impaired myogenic gene expression and cell fate dynamics during regenerative myogenesis. These defects in differentiation capacity are accompanied by impaired senescence and autophagy dynamics. Finally, we demonstrate that inducing autophagy can rescue differentiation of DMD progenitors. Our findings provide novel molecular evidence of satellite cell dysfunction in DMD, expanding on our understanding of their role in its pathology and suggesting pathways to target and enhance their regenerative capacity.

Introduction

Satellite cells, which are muscle-resident stem cells, are responsible for postnatal muscle growth, maintenance of muscle homeostasis, and muscle repair in response to damage[1]. Upon a regeneration-inducing stimulus, satellite cells activate and undergo myogenic commitment, transitioning into proliferative myogenic progenitors (known as myoblasts), and subsequently terminally differentiate into myocytes that undergo fusion to help repair damaged myofibers[2]. This process, known as regenerative myogenesis, is critically dependent on satellite cells[3, 4, 5].

Recent advancement of single-cell transcriptomic technologies has allowed for the characterization of satellite cell populations from resting and regenerating muscle. These studies have not only confirmed the heterogenous nature of the satellite cell population and the myogenic trajectory of satellite cells following injury but have also revealed distinct gene expression signatures that occur due to tissue dissociation procedures[6, 7, 8, 9, 10]. Additionally, we have information pertaining to the impact of aging and muscle degenerative diseases on satellite cell heterogeneity and transcriptional changes at the single-cell level[11, 12, 13, 14, 15, 16].

Duchenne muscular dystrophy (DMD), which impacts one in every 5,000 male births worldwide, is caused by mutations in the X-linked *DMD* gene, which lead to the loss of expression of the protein product dystrophin[17]. The vast heterogeneity of DMD mutations, the large size of the *DMD* gene, and the inefficient transduction of muscle tissue and satellite cells have impeded therapeutic development resulting in a critical need for a cure for this lethal disease. In healthy muscle, dystrophin is an integral component of the dystrophin glycoprotein complex (DGC), a large multimeric protein complex that spans the myofiber membrane, maintaining its integrity and stability[18, 19, 20]. Dystrophin is also expressed in satellite cells, where it mediates the establishment of cell polarity to enable asymmetric stem cell division[21]. Accumulating evidence supports the notion that dystrophin-deficient satellite cells

exhibit intrinsic dysfunction which leads to impaired regenerative capacity[22, 23, 24]. However, whether satellite cells are directly impacted by dystrophin loss or indirectly affected due to the chronic degenerative niche, and the extent to which satellite cell dysfunction contributes to DMD pathology, has yet to be resolved. A question of particular interest is whether dystrophin-deficient satellite cells are fully capable of undergoing regenerative myogenesis and successfully contribute to muscle repair.

We sought to establish how loss of dystrophin impacts satellite cells in both moderate and severe mouse models of DMD. The *mdx* mouse is the most widely used DMD mouse model[25]. However, despite harboring a naturally occurring null mutation in the *Dmd* gene, these mice exhibit a mild dystrophic phenotype and minimal reduction in lifespan. In contrast, D2-*mdx* mice, which are *mdx* mice with a DBA/2 genetic background, exhibit a severe dystrophic pathology and a reduced lifespan of 12-18 months[26, 27]. We performed single-cell RNA-sequencing (scRNA-seq) on satellite cells enriched from *mdx*, D2-*mdx*, and their respective wildtype counterparts to assess the impact of dystrophin-deficiency on the satellite cell transcriptome and stem cell fate.

Interestingly, the majority of differentially expressed genes between the satellite cells of each DMD model and their respective controls overlapped between the two DMD models, indicating molecular impairments that are independent of the dystrophic niche. We identified distinct impairments in DMD satellite cells (apoptosis) and DMD myogenic progenitors (cellular senescence). Additionally, we characterized a DMD-specific satellite cell subpopulation that suggests an alternative, stalled differentiation fate. *In vivo* functional validation assays confirmed that DMD satellite cells exhibit impaired myogenic differentiation along with altered senescence and autophagy dynamics during regenerative myogenesis. Importantly, correcting these satellite cell dysfunctions has the potential to improve regenerative capacity, which is a critical step forward for addressing muscle repair deficits in muscle degenerative diseases.

Results

Altered muscle regeneration and satellite cell profiles in *mdx* and D2-*mdx* mice

To characterize muscle degeneration and the muscle-resident satellite cells in mild and severe DMD mouse models, we isolated tibialis anterior (TA) muscles from three-month-old *mdx* and D2-*mdx* mice, along with their respective wildtype counterparts C57BL/10ScSn (B10) and DBA/2 (DBA). Hematoxylin and eosin staining of TA cross-sections from *mdx* and D2-*mdx* mice revealed signs of chronic muscle degeneration, including the presence of centrally nucleated myofibers and increased variability in myofiber size (Fig. 1A-B).

We performed immunostaining for embryonic myosin heavy chain (eMHC) on TA cross-sections to visualize regenerating myofibers and the canonical satellite cell marker PAX7 to determine satellite cell content. While wildtype muscles exhibited little to no eMHC+ myofibers, *mdx* and D2-*mdx* dystrophic muscles exhibited a significant increase in the percentage of eMHC+ myofibers (Fig. 1C-D). As previously reported, we found that *mdx* TA muscles have an increased number of PAX7+ satellite cells compared to B10 controls[28, 29] (Fig. 1E-F). In contrast, D2-*mdx* mice exhibited significantly lower numbers of PAX7+ satellite cells compared to DBA controls. When comparing *mdx* and D2-*mdx*, D2-*mdx* muscles had significantly reduced satellite cell content compared to *mdx* ($P = 0.019$). Thus, *mdx* muscles exhibit persistent regeneration and enhanced satellite cell numbers, while D2-*mdx* muscles, despite also showing increased regeneration, have reduced satellite cell content compared to their wildtype counterpart.

scRNA-seq reveals differences in satellite cell distribution between healthy and dystrophic muscle

To focus on the satellite cell population, we performed scRNA-seq on prospectively isolated satellite cells from the hindlimb muscles of *mdx* and D2-*mdx* mice, along with their wild-type counterparts (Fig. S1A)[30]. Sequences from the four individual libraries were integrated and cell clustering was performed

and visualized using uniform manifold approximation projection (UMAP) (Fig. S1B-C). Cell clusters were identified by examining highly expressed genes unique to each cluster and comparing these with published scRNA-seq data sets from satellite cells and whole muscle[7, 8, 9, 10] (Fig. S1D). While the majority of our isolated cell population were satellite cells and myogenic progenitors (72.48 - 96.47%), we also detected the presence of pericyte, vascular smooth muscle, glial, Schwann, white blood, and endothelial cells (Fig. S1E-F).

Following the removal of non-myogenic and mature myonuclei cell types, sub-clustering of satellite and myogenic progenitor cells resulted in the identification of seven transcriptionally distinct clusters (Fig. 2A and S2A). Based on their gene expression signature, we designated these clusters as quiescent muscle stem cell (MuSC), stressed MuSC, activated MuSC, cycling progenitor, differentiating myocyte, a population comprised mainly of DMD satellite cells (which we termed DMD enriched), and immunomyoblast (Fig. 2B).

As expected from satellite cells isolated from healthy and homeostatic resting muscle (B10 and DBA), the majority of wildtype satellite cells were found within the quiescent, stressed, and activated MuSC clusters (93.84% in B10 and 94.01% in DBA), which express high levels of *Pax7* and *Myf5* (Fig. 2B-D). Cells within the quiescent MuSC cluster express high levels of genes associated with satellite cell quiescence (*Cd34, Notch3, Hes1*) (Fig. 2B)[31, 32, 33]. The early activated MuSC clusters resembled quiescent satellite cells, but additionally expressed stress response genes (*Fos*, *Junb*, *Jund*) and genes associated with satellite cell activation (*Ccnd1*, *Sdc4*) caused by the tissue dissociation and cell isolation procedure[6].

Not surprisingly, we observed a shift in DMD satellite cells from quiescent and activated states (63.07% in *mdx*; and 65.70% in D2-*mdx*) towards myogenic progenitor states (Fig. 2C-D). These

myogenic progenitor clusters included a cycling progenitor population that expresses cell cycle genes (*Cdk6*, *Mki67*) as well as the myogenic differentiation regulatory factor *Myod1*, and a differentiating myocyte population that expresses the late differentiation marker *Myog* (Fig. 2B). Cell cycle analysis confirmed that the cycling progenitor population had the highest percentage of cells in G2/M and S phase (Fig. 2E). Interestingly, a significant proportion of *mdx* and D2-*mdx* cells were found in the DMD enriched cluster (20.83% *mdx*; and 15.03% D2-*mdx*) compared to their healthy counterparts (1.25% B10 and 1.46% DBA) (Fig. 2C-D). These cells express a combination of quiescence and stress response genes, and uniquely express high levels of immunoglobulin superfamily containing leucine-rich repeat (*Islr*) and the cyclin dependent kinase inhibitor 1C (*Cdkn1c*) (Fig. 2B). As previously reported, we noted a minor population of immunomyoblasts (0.22-2.45%) that are enriched in immune genes (*Ifit1*, *Ifit3*, *Isg15*) (Fig. 2B-D)[8].

We utilized gene expression scores from published studies and gene ontology (GO) terms to validate our cell clusters (Fig. S2B, Table S1). When we assessed the expression of these various scores across the different mouse strains, we confirmed that wildtype B10 and DBA cells are enriched for the "genuine quiescence"[31], "satellite cell"[34], and "Notch GO" scores (Fig. S2C). In contrast, *mdx* and D2-*mdx* cells exhibit higher expression of the "myoblasts/myocytes"[34], "primed core"[31], and "core stress signature"[6] scores. Using a DMD gene signature established from young DMD patients[35], we also confirm that DMD satellite cells, and in particular the DMD enriched cluster are specifically expressing DMD-associated genes (Fig. S2B-C).

DMD satellite cells exhibit distinct dysfunctions between stem cells and progenitors

Principal component analysis revealed that the most significant source of variation between the four data sets is between disease (DMD) and healthy cells suggesting that loss of *Dmd* expression is driving the most significant changes between samples and not differences in the genetic background (Fig. 3A).

Dystrophin-deficiency in DMD is known to impact the expression of components within the dystrophin glycoprotein complex (DGC)[36]. Thus, we assessed the expression profile of DGC genes within our scRNA-seq data set. We confirm the expression of DGC components in healthy B10 and DBA satellite cells, including dystrophin, utrophin, dystroglycan, sarcoglycans, sarcospan and syntrophin (Fig. 3B). While most DGC genes were downregulated in DMD satellite cells, *Sntb2* was an exception to this trend and exhibited increased expression (Fig. 3B).

We performed differential gene expression analysis between DMD and healthy satellite cells (Fig. 3C-D,) and between the DBA and B10 genetic backgrounds (Fig. S3A-B). We found 742 upregulated genes in *mdx* vs B10 and 774 upregulated genes in D2-*mdx* vs DBA (Fig. 3E). Surprisingly, more than half of these genes (481 genes) are overlapping between the *mdx* and D2-*mdx* sets, suggesting that despite differences in disease severity, the majority of differentially expressed genes are common to both *mdx* and D2-*mdx*. Pathway and process enrichment analysis of the upregulated genes in both *mdx* and D2-*mdx* revealed "extracellular matrix organization", "cytoskeleton in muscle cells", "muscle structure development", and "mitotic cell cycle" as the top enriched terms (Fig. 3F-G). We observed 730 downregulated genes in *mdx* vs B10 and 868 downregulated genes in D2-*mdx* vs DBA, with 411 overlapping between the two comparisons (Fig. 3H). The top downregulated pathways included "extracellular matrix organization", "cell-cell adhesion", and "negative regulation of cell population proliferation" (Fig. 3I-J).

In our comparison between the DBA and B10 backgrounds (Fig. S3A-B), "cell cycle", "cell cycle phase transition" and "mitotic cell cycle process" pathways were upregulated in D2-*mdx* vs *mdx* (Fig. S3C-E). "Skeletal muscle cell differentiation" was downregulated in DBA vs B10, while "muscle tissue development" was downregulated in both D2-*mdx* vs *mdx* and DBA vs B10 (Fig. S3F-H). These

results suggest that muscle regeneration pathways are upregulated in B10/*mdx* compared to DBA/D2-*mdx*[37].

To focus on DMD-specific gene expression differences related to cellular health that would impact myogenic differentiation, we evaluated the top 100 enriched pathways and processes (Fig. S4) and noted "positive regulation of programmed cell death" (apoptosis) and "cellular senescence", which were increased in both DMD models compared to controls (Fig. 4A-B). To determine which satellite cell clusters were most significantly impacted by apoptosis and senescence we assessed which clusters exhibited the most enrichment for these GO terms. Interestingly, the expression of genes related to apoptosis (apoptosis GO) were mostly expressed in the quiescent and stressed MuSC clusters, while expression of genes related to senescence (senescence GO) were enriched in the cycling progenitors and DMD enriched clusters (Fig. 4C and S5A-C). These findings indicate unique cellular dysfunctions between early satellite cell and later myogenic progenitor states.

To examine if DMD satellite cells are indeed undergoing cell death by apoptosis, we performed terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) on freshly isolated myofibers from extensor digitorum longus (EDL) muscles from B10 and *mdx* mice (Fig. S5D). In agreement with our scRNA-seq data, we observed the presence of TUNEL+ and PAX7+ satellite cells in *mdx* myofibers (Fig. 4D). *Mdx* myofibers have more PAX7+ satellite cells compared to control fibers (Fig. S5E) and we found that 23% of these were TUNEL+ (Fig. 4E), indicating double stranded DNA breaks, a hallmark of apoptosis. In contrast, we did not observe any TUNEL+ satellite cells in control B10 fibers.

DMD myogenic progenitors exhibit increased senescence, which has been reported in DMD muscles[13, 23, 38]. We assessed the expression of senescence genes *Cdkn1a* ($p21^{Cip1}$), *Cdkn2a* ($p16^{Ink4a}$), and *Cdkn2b*

(p15^{Ink4b}) in our scRNA-seq data set and confirmed increased expression and percentage of cells expressing senescence genes in DMD satellite cells compared to healthy controls (Fig. 4F). We performed quantitative droplet digital PCR (ddPCR) from freshly isolated satellite cells from B10, *mdx*, DBA, and D2-*mdx* mice and confirmed significantly increased expression of *Cdkn1a*, *Cdkn2a*, and *Cdkn2b* in both *mdx* and D2-*mdx* compared to wildtype controls (Fig. 4G). In addition, we performed senescence-associated β-galactosidase (SA-β-Gal) staining in TA cross sections from B10, *mdx*, DBA and D2-*mdx* mice, which showed significantly increased numbers of senescent nuclei per mm^2 of tissue in DMD muscles compared to wildtype controls (Fig. 4H-I).

Identification of an impaired myogenic differentiation gene signature in DMD satellite cells

Previous studies indicate that satellite cells in DMD exhibit impaired asymmetric cell division and myogenic commitment[21, 22]. We assessed the expression of components of the cell polarity complex as well as the myogenic commitment factor *Myf5* and found reduced expression of *Mark2, Prkci,* and *Myf5* in both *mdx* and D2-*mdx* satellite cells compared to controls (Fig. 5A-B). Expression of genes within the "cell polarity establishment and maintenance" GO term was also reduced in DMD satellite cells (Fig. S2C).

In addition to *Myf5*, we assessed expression levels of canonical satellite cell and myogenic differentiation markers in our scRNA-seq dataset (*Pax7*, *Myod1*, and *Myog*). *Pax7* and *Myf5* levels were highest in B10 and DBA cells and reduced in *mdx* and D2-*mdx* (Fig. 5B). In contrast, *Myod1* and *Myog* levels were higher in *mdx* and D2-*mdx* compared to wildtype controls. These scRNA-seq results were validated by ddPCR analysis of freshly isolated satellite cells from B10, *mdx*, DBA, and D2-*mdx* mice (Fig. 5C). The reduced expression of satellite stem cell (*Pax7*) and early myogenic commitment (*Myf5*) markers and increased expression of myogenic differentiation markers (*Myod1* and *Myog*) would suggest that DMD satellite cells exist in a more differentiated state. However, when we assessed

the expression of genes involved in regulating myogenic differentiation, we found that DMD satellite cells express higher levels of anti-differentiation genes (*Id3*, *Runx1*, *Jdp2*) and lower levels of pro-differentiation genes (*Igfbp5*, *Meg3*, *Megf10*) compared to their wildtype counterparts (Fig. 5D). This suggests an impairment of the normal myogenic program in DMD satellite cells.

To infer myogenic differentiation trajectories, we performed pseudotime analyses of our scRNA-seq dataset. Pseudotime analysis revealed two main trajectories, one from quiescence towards the stressed MuSC cluster (Fig. 5E-F). The other trajectory is representative of a canonical myogenic differentiation program, from quiescence through activated MuSC and towards the cycling progenitor cluster, which represents the furthest pseudotime point from quiescence. The canonical trajectory included two branch points, one going from activated MuSC directly towards differentiating myocyte, and another branch ending in the DMD enriched cluster. Altogether, these trajectories resulted in four possible end points (stressed MuSC, cycling progenitor, differentiating myocyte, and DMD enriched), representing distinct cell fates. Visualization of these pseudotime trajectories on the UMAP for each mouse strain (Fig. 5G) revealed that the majority of B10 and DBA cells are found in the stressed MuSC fate. In contrast, *mdx* and D2-*mdx* cells were clearly skewed towards the cycling progenitor, differentiating myocyte, and DMD enriched fates. The trajectory towards the DMD enriched cluster suggested that DMD satellite cells exhibit an altered fate, which led us to interrogate if this represents an impaired differentiate state.

DMD satellite cells exhibit impaired *in vivo* regenerative myogenesis

Genes that are highly expressed and unique to the DMD enriched cluster include several genes that have been associated with DMD (*Col1a1*, *Col1a2*, *Islr*, *Mest*, *S100a11*, *Postn*)[35, 39, 40, 41]. In addition to these genes, cells in this cluster also have increased expression of the negative cell cycle regulator *Cdkn1c* ($p57^{kip2}$), which has not previously been associated with DMD (Fig. 6A). We validated the increased expression of *Cdkn1c* in freshly isolated DMD satellite cells by ddPCR (Fig. 6B). Using a

recently published single nuclei transcriptomic data set from muscle biopsies collected from DMD patients as well as age and gender-matched controls[42], we confirmed that *CDKN1C* expression is higher in DMD patient satellite cells compared to healthy controls and that expression correlates with disease severity (Fig. S6A). *Cdkn1c* expression in healthy myogenic progenitors coordinates the switch between proliferation and cell-cycle arrest during terminal differentiation[43], and its increased expression in DMD satellite cells within this cluster suggests that these cells are non-proliferating. The DMD enriched cluster also exhibits higher *Pax7* expression and lower *Myod1* and *Myog* in comparison to the differentiating myocyte cluster (Fig. S6B), suggesting that these cells are stalled in their myogenic differentiation.

To assess the differentiation potential of DMD satellite cells we utilized an *in vivo* muscle regeneration approach to induce differentiation within the context of a dystrophic niche. We performed intramuscular injury on the TA and gastrocnemius muscles of B10 and *mdx* mice and then prospectively isolated satellite cells from these mice at one- and three-days post-injury (1 and 3 DPI). Gene expression analyses by ddPCR from these samples were compared to satellite cells isolated from non-injured (NI) muscles. B10 satellite cells followed the expected trends of satellite cells transitioning from quiescence (NI) to activation (1 DPI) and proliferation (3 DPI). Expression of markers of quiescence (*Cd34*, *Calcr, Cxcr4*) as well as satellite stem cells (*Pax7*) were highest in NI cells and were reduced upon activation (Fig. 6C, S6C). *Myod1* expression was highest in B10 1 DPI activated cells, while *Myog* levels increased from 1 to 3 DPI. Interestingly, *mdx* satellite cells did not display the same dynamic expression of myogenic regulatory genes. While *Pax7* and *Myf5* expression was lower in homeostatic *mdx* cells compared to B10, expression of these factors was higher in *mdx* compared to control cells at 1 and 3 DPI (Fig. 6C). We also observed a significant reduction in *Myod1* expression at 1 DPI in *mdx* cells compared to B10. In contrast, *Myog* and *Cdkn1c* levels were elevated in *mdx* cells

compared to B10 at all time points. Altogether these results indicate the dysregulation of myogenic transcription factor expression during regenerative myogenesis of DMD satellite cells.

While ddPCR provides a quantitative readout for transcript levels, myogenic gene expression levels do not always correlate with cell fate. Thus, we also assessed myogenic protein expression and cell fate dynamics using the gold standard *ex vivo* EDL myofiber assay. Rather than isolating the myofibers and subsequently culturing them *ex vivo*, we performed intramuscular injury on the TA muscle and then collecting the adjacent EDL muscle at five-, seven-, and nine-days post injury (5, 7, and 9 DPI) to examine satellite cell differentiation that was induced *in vivo*. Quantification of myogenic cells on B10 injured myofibers revealed an expected increase in the number of MYOG+ cells from 5 to 9 DPI (Fig. 6D-E). In contrast, we did not observe an increase in MYOG+ cells in *mdx* injured fibers and the number of MYOG+ cells were significantly reduced in *mdx* fibers at 9 DPI. The number of PAX7+ cells per fiber was increased over all time points in *mdx* compared to B10 injured fibers. When representing the data as a ratio of MYOG+ to PAX7+ cells, *mdx* myofibers have significantly reduced MYOG+/PAX7+ ratios at 7 and 9 DPI. Most strikingly, we observed the presence of large clusters of cells in *mdx* fibers (Fig. 6F) at all time points that were virtually absent in B10 fibers except for at 9 DPI (Fig. 6G, S6D). These cell clusters contained myogenic cells expressing nuclear PAX7, MYOG as well as $p57^{Kip2}$ (protein nomenclature for *Cdkn1c*) (Fig. 6F). The numbers of large cell clusters (>1000um^2) as well as the area of the observed cell clusters were increased in *mdx* injured myofibers compared to B10 controls (Fig. 6G). Altogether, our scRNA-seq data along with our *in vivo* regenerative myogenesis analyses of DMD satellite cells indicate that DMD satellite cells exhibit impaired myogenic differentiation.

Altered senescence and autophagy dynamics in DMD satellite cells

Cellular senescence was enriched in DMD compared to wildtype progenitors (Fig. S5C). Using the senescence GO score, we confirmed that senescence is highest in the cycling progenitors and DMD enriched clusters (Fig. 4C and 7A). We then assessed the expression of senescence-associated genes *Cdkn1a*, *Cdkn2a*, and *Cdkn2b* in satellite cells following injury by ddPCR and found that in B10 satellite cells, the expression of senescence genes is increased during early activation (Fig. 7B). In contrast, DMD satellite cells exhibit significantly higher baseline senescence gene expression in non-injured satellite cells and these levels are maintained during regeneration, thereby indicating a chronic persistence of senescence gene expression in DMD satellite cells, which we hypothesize is impairing the regenerative capacity of DMD satellite cells[38, 44].

In healthy quiescent satellite cells, cellular autophagy, a nutrient recycling pathway, has been shown to prevent senescence and thus maintain stemness and regenerative capacity[45]. To examine if cellular autophagy is dysregulated in DMD satellite cells we performed ddPCR analysis to assess the expression of a panel of autophagy genes (*Atg9a*, *Atg14*, *Map1lc3b*) in satellite cells freshly isolated from B10, *mdx*, DBA and D2-*mdx* mice. Expression of autophagy genes in homeostatic satellite cells were all downregulated in DMD satellite cells compared to wildtype controls (Fig. 7C). Next, we assessed the expression of autophagy genes (*Atg9a*, *Atg14*, *Map1lc3b*, *Sqstm1*, and *Gabarap*) during injury-induced myogenesis. In wildtype satellite cells, expression levels were highest during quiescence and reduced upon activation (Fig. 7D). This trend was opposite of what we observed with respect to the expression of senescence genes following injury (Fig. 7B). Moreover, this reduction in autophagy gene expression during regenerative myogenesis was absent in *mdx* satellite cells at 1 and 3 DPI suggesting impaired autophagy dynamics in *mdx* satellite cells following injury (Fig. 7D).

We assessed the expression of senescence and autophagy gene expression scores in the single nuclei human DMD transcriptomic data set[42]. Here we observed a similar trend in human DMD satellite cells

as we found in our murine data from *mdx* and D2-*mdx* satellite cells. Human DMD satellite cells exhibited enhanced senescence in the stable patient group, while autophagy was reduced in both stable and declining patient groups (Fig. S7A). These data from DMD patient samples support our findings in mouse satellite cells, which are from a niche that is representative of an early disease state due to their less severe phenotype.

We next asked if modulating autophagy would improve the differentiation capacity of DMD satellite cells. To address this, we used 3-methyladenine (3MA), a class III phosphoinositide kinase inhibitor that inhibits autophagy, and Tat-Beclin 1 (D11), an autophagy promoting peptide that contains 11 amino acids from the autophagy inducing protein Beclin 1[46, 47]. We treated primary myogenic progenitors isolated from *mdx* mice with either 3MA or D11 and assessed their differentiation capacity (Fig. 7E, S7B-C). While inhibiting autophagy with 3MA did not have a significant impact on differentiation, inducing autophagy with D11 resulted in enhanced differentiation of DMD progenitors (Fig. 7F). Thus, inducing autophagy in DMD progenitors improves the differentiation capacity of DMD progenitor cells. Altogether, our results support the notion that counteracting these DMD progenitor-specific impairments (ie. reduced autophagy) can improve their endogenous regenerative capacity.

Discussion

Single cell sequencing technologies have provided the ability to parse out cellular heterogeneity and identify transcriptomic differences within complex cell populations. Here, we used scRNA-sequencing to elucidate the mechanisms by which satellite cells in DMD in particular are impacted by loss of dystrophin. Recent studies have used both single cell and single nucleus transcriptomic approaches to assess whole muscle tissue from various mouse and rat DMD models[13, 14, 48]. While these approaches have identified significant alterations in tissue-wide cellular diversity in DMD, our study uniquely focused on sequencing of the satellite cell population to gain sufficient sequencing depth to identify

distinct satellite cell populations within the heterogenous satellite cell pool. By adequately capturing these subpopulations, we were able to elucidate molecular pathways that differ between DMD and healthy satellite cells (apoptosis) and myogenic progenitors (senescence) and identify a DMD-specific satellite cell subpopulation.

We compared satellite cells from two DMD mouse models, the mildly dystrophic *mdx* mouse and the more severely dystrophic D2-*mdx* mouse. Of note, *mdx* and D2-*mdx* mice exhibit differences in satellite cell content. While *mdx* muscles have higher satellite cell numbers compared to their control counterpart, D2-*mdx* mice have reduced satellite cell numbers (Fig. 1F), supporting previous studies demonstrating that satellite cells have reduced regenerative capacity in DBA/2 muscles compared to C57BL/6, thereby impacting their ability to maintain a sufficient satellite cell pool within the context of chronic degeneration[26]. The altered muscle regenerative potential in DBA and D2-*mdx* is supported by our pathway enrichment analyses, which indicated reduced muscle tissue development and skeletal muscle differentiation when comparing satellite cells from the DBA versus B10 background (Fig. S3G). Whereas an increase in satellite cell numbers has been reported in human DMD tissue[28], these differences in satellite cell number and muscle regeneration should be considered when interpreting data from the D2-*mdx* model. Despite these differences in disease severity and thus satellite cell niche, we found the two DMD models shared many overlapping differentially expressed genes and molecular dysfunctions as compared to their healthy counterparts (Fig. 3G, J).

Prior to the discovery that dystrophin is expressed in the satellite cell, it was generally thought that the loss of dystrophin had minimal impact on satellite cells and their progeny, the myogenic progenitors. While earlier studied have shown no difference in or even accelerated myogenic differentiation kinetics in cultured DMD satellite cells, we propose that *in vitro* culturing selects for those cells capable of proliferating and surviving in culture rather than those vulnerable to cell death[49, 50]. There is indeed

evidence that there is a cell-autonomous impairment of DMD satellite cells and myogenic progenitors that impair their regenerative capacity, thereby leading to an inability to support dystrophic muscle repair [21, 22, 24, 51, 52]. In keeping with this, we identified a DMD enriched cell cluster that uniquely expresses genes associated with DMD, expresses high levels of the cell cycle inhibitor *Cdkn1c*, and has a distinct cell fate trajectory (Fig. 5F, 6A). This DMD enriched cluster paradoxically expresses higher levels of stem cell and myogenic commitment markers *Pax7* and *Myf5* and lower levels of differentiation markers *Myod1* and *Myog* compared to the progenitor and myocyte clusters (Fig. S6B).

While DMD satellite cells are skewed towards progenitor populations (Fig. 2C), as expected in a continually regenerating tissue, we propose that at least a subpopulation of DMD satellite cells is stalled in differentiation and exhibit dysregulated myogenic capacity. In line with this, less than 50% of DMD satellite cells are expressing the "primed core"[31] and "myoblast/myocyte"[34] scores (Fig. S2C). Our *in vivo* regeneration assays revealed an impairment in regenerative myogenesis in *mdx* satellite cells compared to B10 controls (Fig. 6C-G). Interestingly, while *mdx* satellite cells express lower levels of *Pax7* transcripts and higher levels of *Myog* transcripts, we show that the level of gene expression does not correlate with cell number as we observe increased PAX7+ cells and reduced MYOG+ cells in *mdx* fibers following injury. Our results suggest that *mdx* mice have a higher number of cells that are PAX7+ which each have low *Pax7* transcript, and a low number of MYOG+ cells that expressing high levels of *Myog.* In addition, we found previously undescribed large cell clusters uniquely in injured *mdx* myofibers (Fig. 6F-G), suggesting an impairment in cell fusion. Further experiments to isolate and characterize these cells by flow cytometry would provide important insight into this DMD enriched cell population.

We found that DMD satellite cells exhibit enrichment in programmed cell death pathways (Fig. 4A-C). This is the first reporting of increased apoptosis in DMD satellite cells. In contrast, DMD myogenic

progenitors exhibit enrichment in cellular senescence (Fig. 4A-C). We found that healthy satellite cells express increased levels of senescence-associated genes following injury (Fig. 7B). Recent studies have indicated that senescent cells are a normal component within the regenerating muscle niche[11, 16, 53], however DMD satellite cells exhibit increased baseline expression of senescence-associated genes suggesting a chronic and dysregulated senescent state (Fig. 7B). The chronic presence of these senescent cells would hinder muscle regeneration[16, 38].

The nutrient recycling macroautophagy pathway has previously been shown to be dysregulated in DMD muscle and satellite cells[54, 55, 56]. Consistent with these findings, we found that the expression of autophagy genes is universally lower in DMD satellite cells (Fig. 7C) and do not exhibit a dynamic downregulation during regenerative myogenesis (Fig. 7D). We propose that this impairment in autophagy dynamics would also impact the regenerative capacity of DMD satellite cells. Indeed, we found that stimulating autophagy with Tat-Beclin D11, which induces autophagy independently of inhibiting the master metabolic regulator mammalian target of rapamycin, improved the differentiation capacity of *mdx* progenitor cells (Fig. 7E-F)[46]. Our results support earlier work indicating that autophagy reduction in DMD muscle tissue correlates with reduced muscle function and that inducing autophagy with agents such as rapamycin and the AMPK agonist AICAR can improve regeneration and pathology in *mdx* mice[54, 57, 58].

In conclusion, our study provides additional molecular evidence that satellite cells are dysfunctional in DMD and that their reduced regenerative potential contributes to the characteristic progressive muscle wasting of the disease. The work described here serves as a critical resource characterizing the transcriptomes of two widely used DMD mouse models, *mdx* and D2-*mdx*, along with their wildtype counterparts. Importantly, transcriptomic data from satellite cells from DMD patients supports our findings of dysregulated cell death, senescence, and autophagy pathways. Our results indicate that

DMD satellite cells are heterogenous with respect to their myogenic regenerative potential and their exhibition of impaired cellular pathways. We propose that gene therapies aimed at transducing satellite cells should examine the benefits and consequences of targeting specific satellite cell populations. Overall, our study provides a molecular map that illustrates how satellite cell subpopulations are impacted by the loss of dystrophin and will inform therapeutic strategies aimed at improving the regenerative capacity of DMD muscle.

Methods

Mice

Housing, husbandry and all experimental protocols for mice used in this study were performed in accordance with the guidelines established by the McGill University Animal Care Committee, which is based on the guidelines of the Canadian Council on Animal Care. C57BL/10ScSnJ (B10, #000476), C57BL/10ScSn-*Dmd*^{mdx}/J (mdx, #001801), DBA/2J (DBA2, #000671), and D2.B10-*Dmd*^{mdx}/J (D2-*mdx*, #013141) were purchased from The Jackson Laboratory. Two- to three-month-old male mice were used for all experiments.

Muscle histology

Cryostat cross-sections of tibialis anterior (TA) muscle of 10μm were used for hematoxylin and eosin (H&E) staining, senescence-associated β-galactosidase staining and immunofluorescent (IF) labelling. Sections for H&E were fixed with 4% paraformaldehyde (PFA) and stained with Mayer's modified hematoxylin solution (Abcam) and washed for five minutes followed by differentiation with 1% HCl in 10% ethanol. Sections were stained with Eosin Y working solution (0.25% Eosin disodium salt (Sigma) in 80% ethanol with 0.5% glacial acetic acid), dehydrated and mounted with xylene mounting media (Fisher).

For PAX7 IF labelling, sections were fixed with 2% PFA for two minutes, followed by permeabilization for 15 minutes (0.1% triton X-100 + 0.1M Glycine in PBS) and blocking for one hour (5% donkey serum (DS) (Sigma) + 2% bovine serum albumin (BSA) + Mouse on Mouse Blocking Reagent (Vector Laboratories) in phosphate buffered saline (PBS)). Primary antibody incubation was done overnight at 4°C with pure PAX7 antibody with 5% DS + 2% BSA. Secondary incubation in labelling buffer (5% DS + 2% BSA in PBS) was done for one hour at room temperature, followed by Hoechst staining for 5 minutes. Slides were mounted with ProLong™ Gold antifade reagent (Thermo Fisher).

For eMHC labelling, TA cryosections were fixed with 4% PFA for 10 minutes then permeabilized and blocked for one hour (0.1M glycine + 5% DS + 0.25% triton-X-100 in tris buffered saline (TBS)). Subsequently, cryosections were incubated overnight at 4ºC with primary antibody solution (1% DS + 0.25% triton-X-100 in TBS). Secondary antibody incubation and mounting were performed as above.

Sections were imaged using a Zeiss Axio Observer 7 at 20X, except for eMHC-stained cryosections which were imaged at an EVOS M5000 microscope using a 10X objective.

Senescence-associated β-galactosidase staining

Tibialis anterior (TA) sections were fixed for four minutes with 1% PFA and 0.2% Glutaraldehyde in PBS, followed by incubating for 30 minutes in PBS at pH 5.5. Sections were then incubated with X-gal staining solution (4mM potassium ferricyanide + 4mM potassium ferrocyanide + 2mM $MgCl_2$ +1 mg/mL X-gal and 0.04% IGEPAL (Sigma) in PBS at pH 5.5) for 48 hours at 37°C. Sections were subsequently washed in PBS for 10 minutes and fixed again with 1% PFA diluted in PBS for 10 minutes. Sections were counterstained with Hoechst. The stained sections were mounted with ProLong™ Gold antifade reagent and imaged using a Zeiss Axio Observer microscope.

Single myofiber isolation

Single myofibers were isolated from the extensor digitorum longus (EDL) muscle of mice[59] that were either uninjured or mice that received injections of 1.2% $BaCl_2$ to the TA (30ul at 1.2%) five, seven or nine days prior to isolation to induce injury and thus activate satellite cells. After isolation, single myofibers were collected and fixed immediately in 2% PFA.

IF and TUNEL staining on fibers

Fixed myofibers were permeabilized for 10 minutes (0.1M glycine + 0.1% triton X-100 in PBS) then blocked for two hours (2% BSA + 2.5% DS + 2.5% goat serum (GS) in PBS). Primary antibody incubation was done overnight labelling solution (0.5% DS + 0.5% GS in PBS) at 4°C. Secondary antibody incubation was done for one hour at room temperature in labelling solution, followed by Hoechst staining for one minute. Fibers were mounted on slides with ProLong™ Gold antifade reagent.

TUNEL staining was done with the In Situ Cell Death Detection Kit (Roche). Fibers were fixed with 2% PFA, then permeabilized and blocked as previously described. Fibers were then incubated with TUNEL reaction mixture for one hour at 37°C followed by primary antibody incubation overnight at 4°C. Secondary antibody incubation, Hoechst and mounting on slides were performed as described above.

Myofibers were imaged using a Zeiss LSM710 using ZEN 3.2 (blue edition) with the exception of Fig. 6D that was imaged at an EVOS M5000 microscope (software version 1.6.1899.478) using a 20X objective.

Quantification of IF images

All image analysis was done with Fiji ImageJ (version 1.45f)[60]. PAX7+, MYOG+ and SA-Bgal+ nuclei quantification was performed using the cell counter feature. Cross sectional area of myofibers and aspect ratio was obtained using the automated muscle histology analysis algorithm Myosoft (version 14) to define individual fiber ROIs via WGA staining[61]. Using this same software, the number of eMHC+ fibers were determined by automatically measuring mean grey value. These values were then corrected through background subtraction. A negative control was used to determine the range of values that represented "true" negatives and values above this (>75) were considered eMHC+.

Satellite cell isolation

Satellite cells were prospectively isolated from the hind limb muscles of mice by fluorescence-activated cell sorting (FACS) as previously described[62]. Cells were labelled with negative and positive lineage markers (Table S2) and satellite cells were sorted using a BD FACSAriaTM III (BD Biosciences).

Mouse single cell RNA-sequencing and computational analyses

For each mouse strain (B10, *mdx*, DBA, D2-*mdx*), ITGA7+/VCAM+/Lin- satellite cells from two mice were pooled, sorted and captured on the 10X Genomics Chromium platform and subjected to single cell transcriptomic sequencing. Following satellite cell isolation, the single-cell RNA-sequencing libraries were prepared using the 10X Genomics NextGen scRNA 3' V3.1 (10X Genomics, Pleasanton, CA) with approximately 10,000 satellite cells from each strain. Libraries were then sequenced with an Illumina NovaSeq6000 sequencer (Illumina, San Diego, CA). Reads were processed with Cell Ranger (version 3.0.1) and aligned to the mouse reference transcriptome mm10.

Computational analysis was performed using RStudio (version 4.2.1, 2022-06-23), and the data were imported with Seurat (version 5.1.0)[63]. Cells were filtered to remove cells with less than 1,000 or more

than 5,000 genes, as well as cells with more than 8% mitochondrial genes, to eliminate low quality cells. Doublets were removed using DoubletFinder (version 2.0.4)[64]. After normalization and scaling, cells from the different mouse strains were integrated. The resolution was determined using the Clustree function (version 0.5.1) and unsupervised clustering was carried out using the FindClusters function[65]. The first clustering was done with a resolution of 0.1, and the sub-clustering of the myogenic cells (MuSC and myoblast) was done with a resolution of 0.2. Cells expressing high levels mature myonuclei genes (*Acta1*, *Tnnc2*, *Tnnt3*), representing a minor portion of total myogenic cells (0.04 – 0.11%) were removed for final downstream analysis. Differential gene expression and cluster identification were performed using FindAllMarkers function in Seurat. The scores were generated with the AddModuleScore function, using published genes lists. Stacked violin plots were made using the the StackedVlnPlot function[66]. Dot plots and feature plots were created using scCustomize package (version 2.1.2)[67]. Cell cycle analysis was done using the CellCycleScoring function of the Seurat Package.

Human single nuclei RNA-sequencing and computational analyses

Control and DMD human data were generated and kindly shared by Prof. Jordi Diaz-Manera's team[42]. Computational analysis was performed as described above. Cells were filtered to remove cells with less than 100 or more than 5,000 genes, as well as cells with more than 5% mitochondrial genes, and doublets were removed using DoubletFinder (version 2.0.4)[64]. After normalization and scaling, cells from the different samples were integrated with a resolution of 0.1, and the sub-clustering of the myogenic cells (*PAX7*$^{+}$) was done with a resolution of 0.4. The sample groups were generated as described[42].

Principal component analysis (PCA) – Pseudobulk

The processed individual datasets were merged, and the object was converted into a SingleCellExperiment object. Counts were aggregated with aggregateBioVar package (version 1.14.0)[68]. Differential gene expression was performed using DESeq2 Package (version 1.44.0)[69]. The PCA plot was generated using plotPCA function.

Differential gene expression and Gene Ontology (GO) enrichment analysis

Differentially expressed genes lists were generated using the FindMarkers function. Genes with Log2FC more than 1 and adj pVal less than 0.05 were selected for GO enrichment analysis. GO enrichment was performed using Metascape (metascape.org) with multiple gene lists using the Custom Analysis function[70]. GO Biological Processes, KEGG Pathway, Reactome Gene Sets, CORUM, WikiPathways, and PANTHER Pathway were selected. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were grouped into clusters based on their membership similarities. GO terms analysis has been performed with the enrichGO function of the clusterProfiler package version 4.12.0[71]. Volcano plots were generated using the EnhancedVolcano package (version 1.22.0)[72]. Venn Diagrams were generated using VennDiagram package (version 1.7.3)[73].

Pseudotime analysis

The pseudotime analysis was performed using Monocle3 package (version 1.3.7)[74]. The datasets were processed as described previously. The Seurat object has been transformed as a cell data set using the SeuratWrappers package (version 0.3.5). Cells were then clustered using the cluster_cells function. The trajectory was generated using the learn_graph function by adjusting the default setting of minimal_branch_len to 10, euclidean_distance_ratio to 1 and geodesic_distance_ratio to 1. Finally, cells were ordered along the learned trajectory.

Droplet digital PCR (ddPCR)

RNA was extracted from isolated satellite cells using the PicoPure RNA Isolation Kit (Applied Biosystems) and cDNA was generated with the SuperScript III First-Strand Synthesis System (Invitrogen). ddPCR was performed using ddPCR Supermix for Probes (no dUTP) (Biorad) and data collected with the QX100 Droplet Digital PCR System (Biorad) with QuantaSoft (version 1.7.4). A list of primers can be found in Table S3.

Autophagy modulation assays

Primary satellite cell-derived myoblasts were isolated from *mdx* mice as previously described[75]. Cells were seeded in proliferation media (20% FBS, 10% HS, 3% CEE, 10 ng/mL bFGF and 2 mM L-glutamine in Ham's F-10) on collagen-coated 35 mm plates. When cells reached 80-90% confluence, cells were treated for two hours with either 10 µM Tat-Beclin 1 D11 peptide (Novus Biologicals) or Tat-Beclin 1 L11S peptide scramble control (vehicle, Novus Biologicals); 5 mM 3-methyladenine (3-MA, Sigma) or water (vehicle)[46, 47]. Following treatment, cells were induced to differentiate for two days in differentiation medium (50% DMEM, 50% F10, 5% HS). Cells were fixed and immunolabeled with MyHC antibody, and fusion index was determined as previously described[76].

Western blotting

Cell pellets from a replicate of the autophagy modulation assays were collected in lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 mM EDTA, 0.5% Triton X-100, 1X protease inhibitor, and 1X phosphatase inhibitor) and incubated on ice for 30 minutes. Samples were centrifuged at 15,000 rcf at 4°C for 20 minutes and supernatants collected. The protein concentration of the resulting lysates was determined using the Pierce BCA Assay Kit (Thermo Fisher). Lysates were mixed with 4X Laemmli sample buffer, denatured at 95°C for five minutes, and resolved using an 12% SDS-PAGE gel containing 0.5% 2,2,2-Trichloroethanol (TCE). Total protein was determined using the ChemiDoc imaging system (Bio-Rad) via ultra-violet activation. Samples were then transferred to a

polyvinylidene difluoride (PVDF) membrane and blocked for one hour at room temperature in blocking buffer (5% milk + 0.5% Tween in PBS). Membranes were incubated with primary antibody in blocking buffer at 4°C overnight with agitation, then incubated with secondary antibody in blocking buffer for one hour at room temperature. Membrane was incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher) and visualized using the ChemiDoc imaging system (Bio-Rad). Protein band intensity was quantified using Fiji ImageJ.

Statistical analysis

For data not related to scRNA-seq, statistical analyses and visualizations were done in GraphPad Prism (version 10.2.2). Significance was determined through two-tailed unpaired t-tests, two-way analysis of variance (ANOVA) with Šídák's multiple comparisons test, mixed-effects analysis with Šídák's multiple comparisons test, or one-sided Fisher's exact test, as indicated. P values in figure legends for two-way ANOVAs and mixed-effects analysis describe row*column effect unless otherwise noted. Confidence interval = 95% for all tests.

Two-tailed t-tests in Fig. 1 were performed on three biological replicates per group.
For all two-tailed unpaired t-tests of strain comparison ddPCR data, n = 3 for all groups except D2-*mdx* where n = 4. Two-way ANOVA tests which were conducted on NI, 1 DPI and 3 DPI data. Three biological replicates were used for all groups except B10 3DPI, where n = 4. Cluster data in Fig. 6E and G was done using EDL fibers from one biological replicate per condition. Significance was then calculated via mixed-effects analysis due to uneven sample size. β-galactosidase assays were done with three biological replicates per condition and significance calculated via two-tailed unpaired t-tests. TUNEL+ nuclei percentage was calculated from three mice per strain, counting n = 25, 50 and 21 B10 and n = 82, 45 and 69 *mdx* PAX7+ nuclei. A one-sided Fisher's exact test was used as B10 has zero

positives. Autophagy modulation assays were done on three biological replicates per condition and significance determined with two-tailed unpaired t-tests.

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Author contributions

N.C.C. conceived and managed the project. J.A.G., R.R., R.L.F., A.C., S.L. designed and performed experiments, and collected data. J.A.G., R.R., R.L.F., A.C., and N.C.C. analyzed and interpreted the data. M.Y. and J.A.S. contributed bioinformatics analysis. N.C.C. and R.R. wrote the manuscript. All authors carefully reviewed and provided critical insights into the manuscript.

Competing interests

The authors declare no competing interests.

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Figure 1

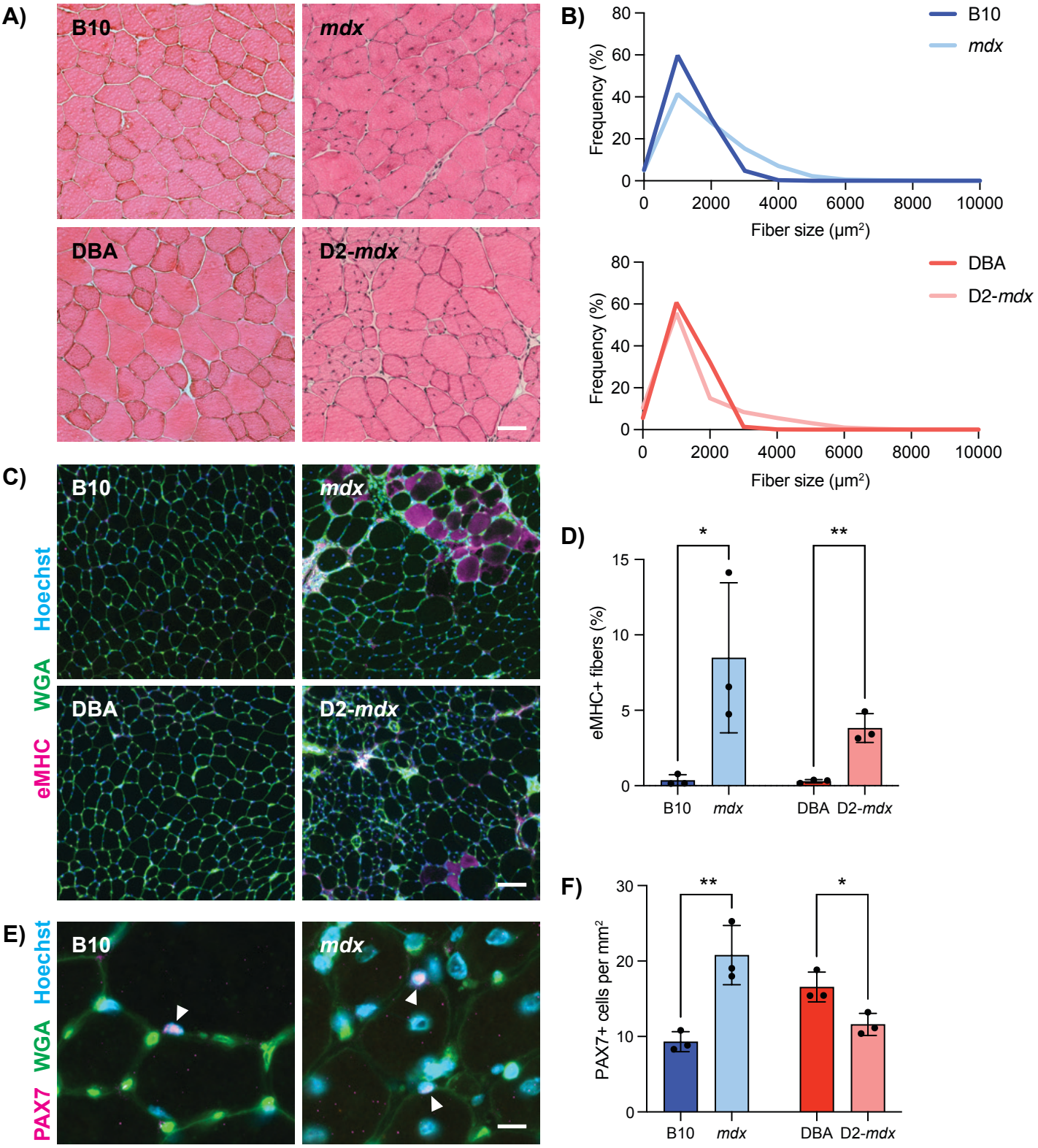
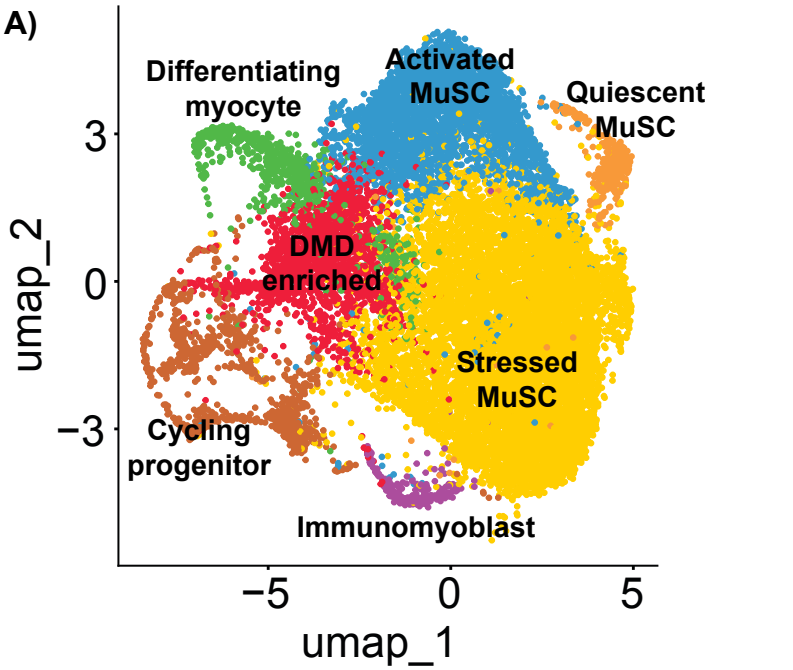
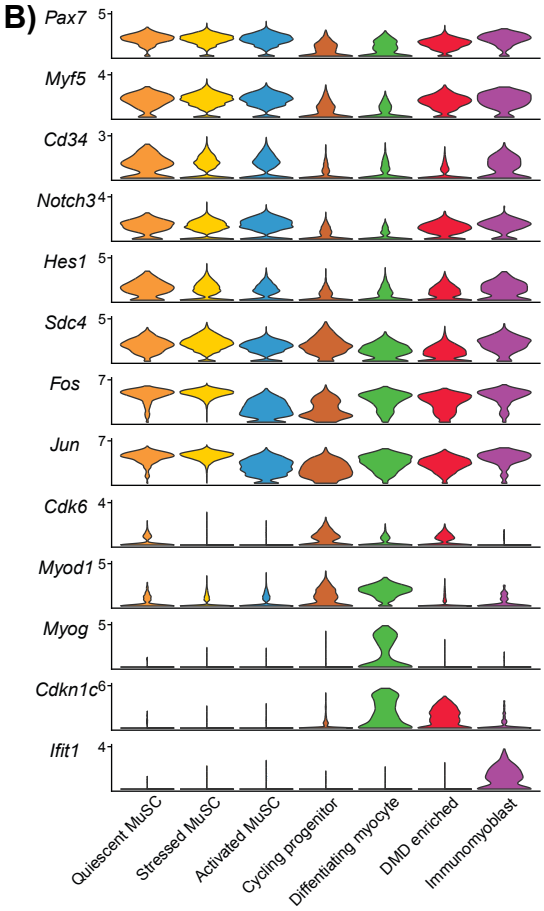
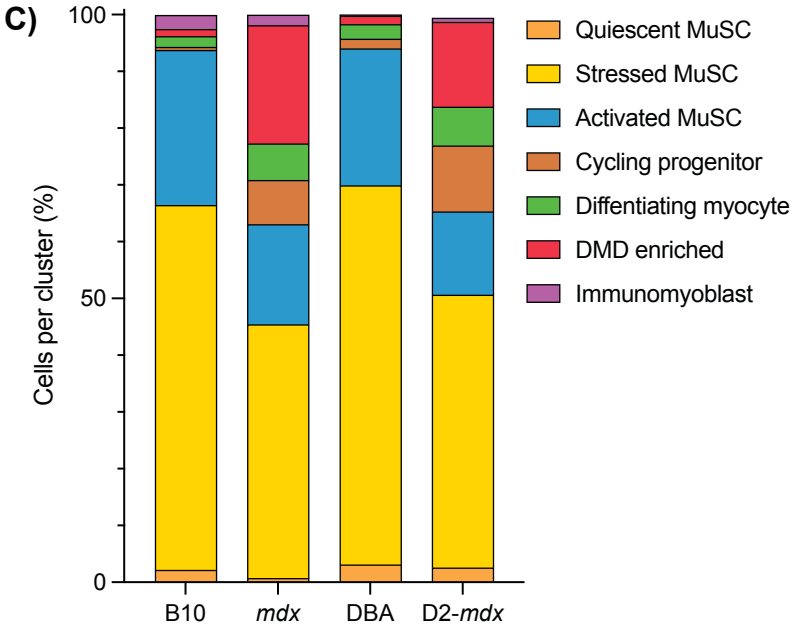
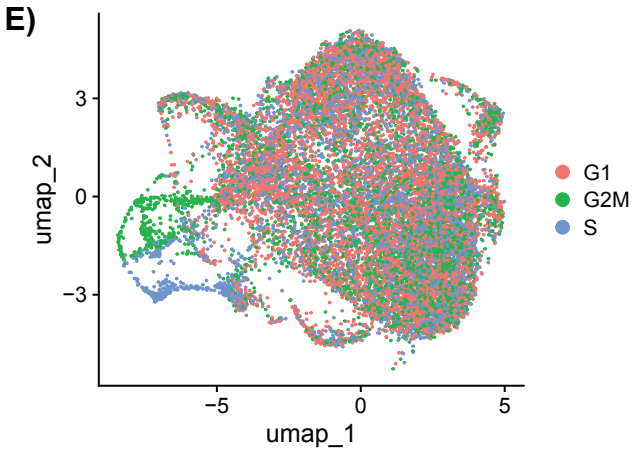

A)
B10
mdx
DBA
D2-mdx
B)
B10
mdx
Frequency (%)
Fiber size (μm²)
DBA
D2-mdx
C)
Hoechst
WGA
eMHC
D)
eMHC+ fibers (%)
B10
mdx
DBA
D2-mdx
E)
Hoechst
WGA
PAX7
F)
PAX7+ cells per mm²

Figure 2









D)

	Quiescent MuSC	Stressed MuSC	Activated MuSC	Cycling progenitor	Diffentiating myocyte	DMD enriched	Immuno-myoblasts
B10	2.16	64.35	27.33	0.54	1.92	1.25	2.45
mdx	0.72	44.69	17.66	7.80	6.43	20.83	1.86
DBA	3.10	66.81	24.10	1.72	2.59	1.46	0.22
D2-*mdx*	2.57	48.35	14.77	11.67	6.88	15.03	0.72

Figure 3

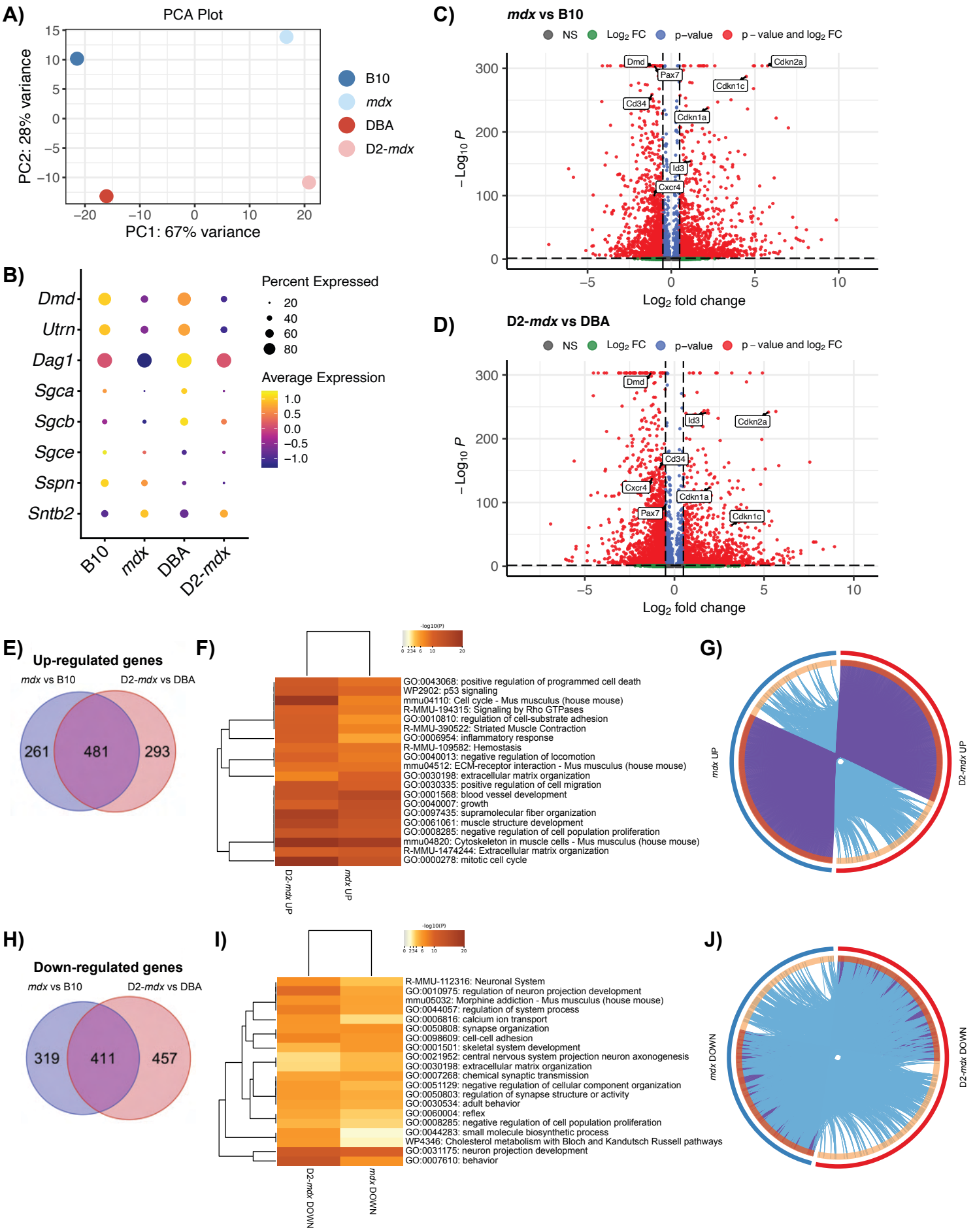

A)
PCA Plot
PC2: 28% variance
PC1: 67% variance
B10
mdx
DBA
D2-mdx
B)
Dmd
Utrn
Dag1
Sgca
Sgcb
Sgce
Sspn
Sntb2
B10
mdx
DBA
D2-mdx
Percent Expressed
20
40
60
80
Average Expression
1.0
0.5
0.0
−0.5
−1.0
C)
mdx vs B10
NS
Log2 FC
p−value
p − value and log2 FC
−Log10 P
Log2 fold change
Dmd
Pax7
Cdkn2a
Cdkn1c
Cd34
Cdkn1a
Id3
Cxcr4
D)
D2-mdx vs DBA
NS
Log2 FC
p−value
p − value and log2 FC
−Log10 P
Log2 fold change
Dmd
Id3
Cdkn2a
Cd34
Cxcr4
Cdkn1a
Pax7
Cdkn1c
E)
Up-regulated genes
mdx vs B10
D2-mdx vs DBA
261
481
293
F)
-log10(P)
GO:0043068: positive regulation of programmed cell death
WP2902: p53 signaling
mmu04110: Cell cycle - Mus musculus (house mouse)
R-MMU-194315: Signaling by Rho GTPases
GO:0010810: regulation of cell-substrate adhesion
R-MMU-390522: Striated Muscle Contraction
GO:0006954: inflammatory response
R-MMU-109582: Hemostasis
GO:0040013: negative regulation of locomotion
mmu04512: ECM-receptor interaction - Mus musculus (house mouse)
GO:0030198: extracellular matrix organization
GO:0030335: positive regulation of cell migration
GO:0001568: blood vessel development
GO:0040007: growth
GO:0097435: supramolecular fiber organization
GO:0061061: muscle structure development
GO:0008285: negative regulation of cell population proliferation
mmu04820: Cytoskeleton in muscle cells - Mus musculus (house mouse)
R-MMU-1474244: Extracellular matrix organization
GO:0000278: mitotic cell cycle
D2-mdx UP
mdx UP
G)
mdx UP
D2-mdx UP
H)
Down-regulated genes
mdx vs B10
D2-mdx vs DBA
319
411
457
I)
-log10(P)
R-MMU-112316: Neuronal System
GO:0010975: regulation of neuron projection development
mmu05032: Morphine addiction - Mus musculus (house mouse)
GO:0044057: regulation of system process
GO:0006816: calcium ion transport
GO:0050808: synapse organization
GO:0098609: cell-cell adhesion
GO:0001501: skeletal system development
GO:0021952: central nervous system projection neuron axonogenesis
GO:0030198: extracellular matrix organization
GO:0007268: chemical synaptic transmission
GO:0051129: negative regulation of cellular component organization
GO:0050803: regulation of synapse structure or activity
GO:0030534: adult behavior
GO:0060004: reflex
GO:0008285: negative regulation of cell population proliferation
GO:0044283: small molecule biosynthetic process
WP4346: Cholesterol metabolism with Bloch and Kandutsch Russell pathways
GO:0031175: neuron projection development
GO:0007610: behavior
D2-mdx DOWN
mdx DOWN
J)
mdx DOWN
D2-mdx DOWN

Figure 4

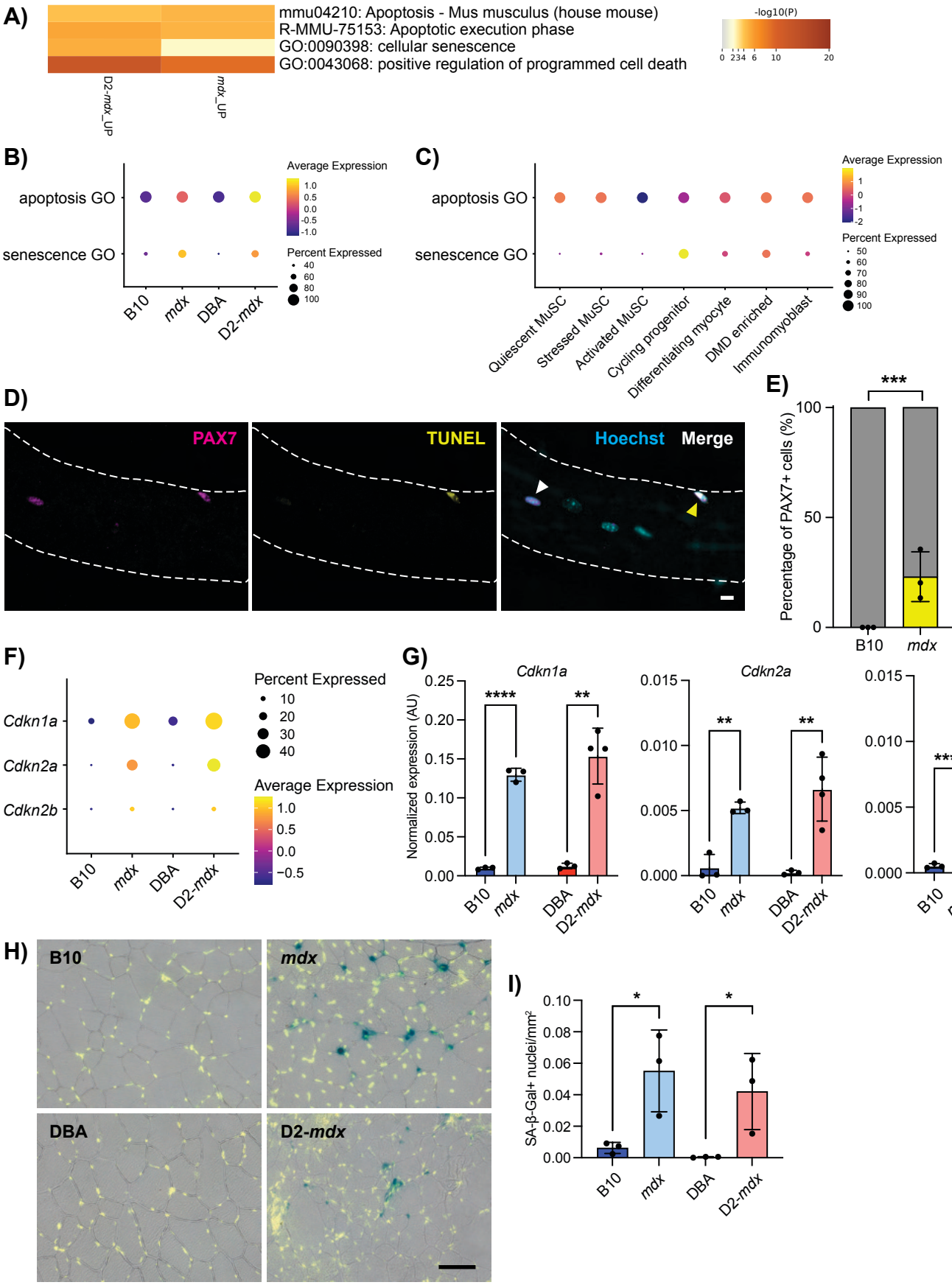

A)
mmu04210: Apoptosis - Mus musculus (house mouse)
R-MMU-75153: Apoptotic execution phase
GO:0090398: cellular senescence
GO:0043068: positive regulation of programmed cell death
D2-mdx_UP
mdx_UP
-log10(P)
0 2 3 4 6 10 20
B)
apoptosis GO
senescence GO
B10
mdx
DBA
D2-mdx
Average Expression
Percent Expressed
C)
apoptosis GO
senescence GO
Quiescent MuSC
Stressed MuSC
Activated MuSC
Cycling progenitor
Differentiating myocyte
DMD enriched
Immunomyoblast
Average Expression
Percent Expressed
D)
PAX7
TUNEL
Hoechst
Merge
E)
***
Percentage of PAX7+ cells (%)
TUNEL+
TUNEL-
B10
mdx
F)
Cdkn1a
Cdkn2a
Cdkn2b
B10
mdx
DBA
D2-mdx
Percent Expressed
Average Expression
G)
Cdkn1a
Cdkn2a
Cdkn2b
Normalized expression (AU)
****
**
B10
mdx
DBA
D2-mdx
H)
B10
mdx
DBA
D2-mdx
I)
SA-β-Gal+ nuclei/mm²
*
B10
mdx
DBA
D2-mdx

Figure 5

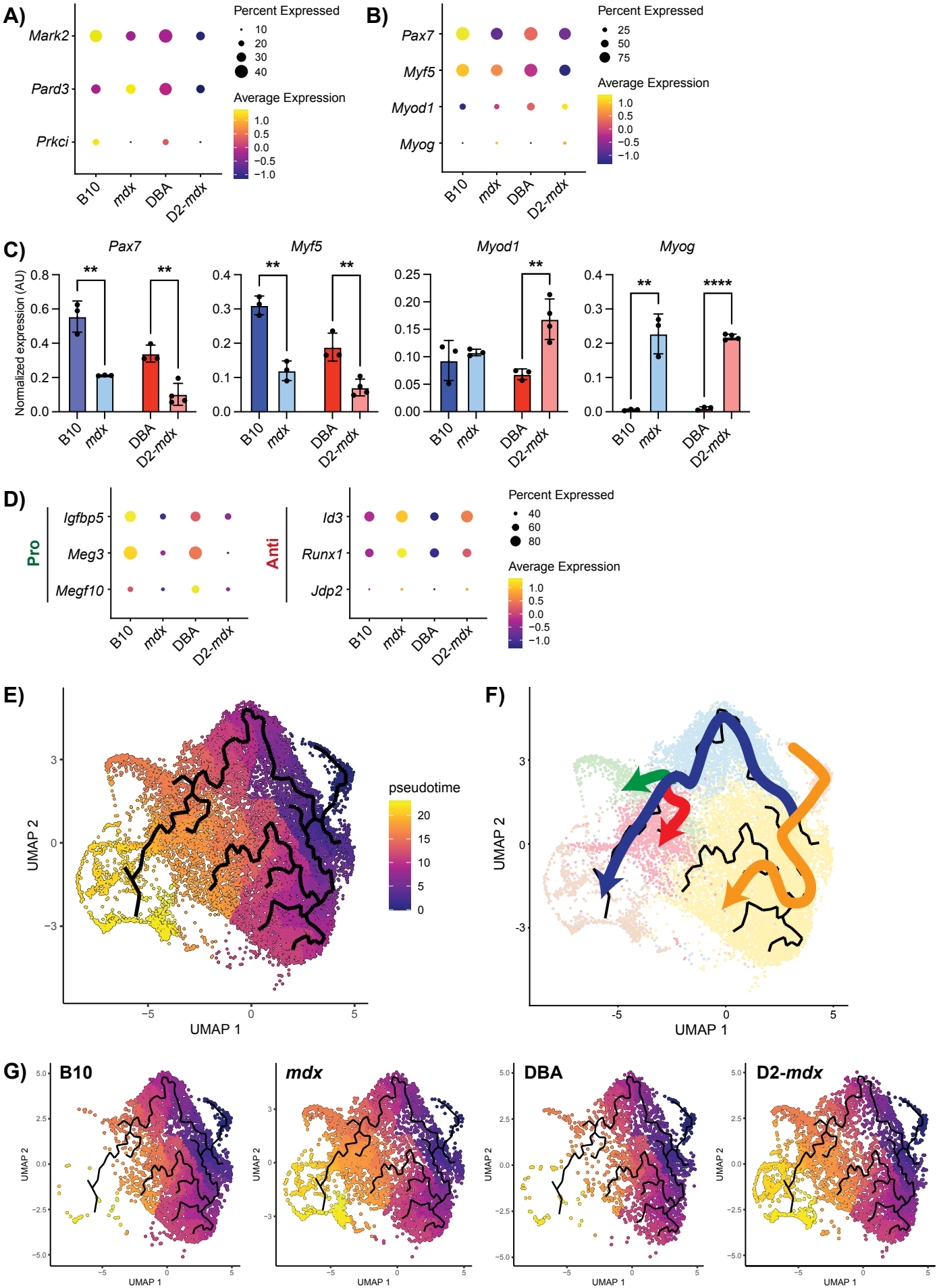

A)
Mark2
Pard3
Prkci
B10
mdx
DBA
D2-mdx
Percent Expressed
10
20
30
40
Average Expression
1.0
0.5
0.0
−0.5
−1.0
B)
Pax7
Myf5
Myod1
Myog
Percent Expressed
25
50
75
C)
Pax7
Myf5
Myod1
Myog
Normalized expression (AU)
**
****
D)
Pro
Anti
Igfbp5
Meg3
Megf10
Id3
Runx1
Jdp2
Percent Expressed
40
60
80
E)
UMAP 1
UMAP 2
pseudotime
20
15
10
5
0
F)
G)
B10
mdx
DBA
D2-mdx

Figure 6

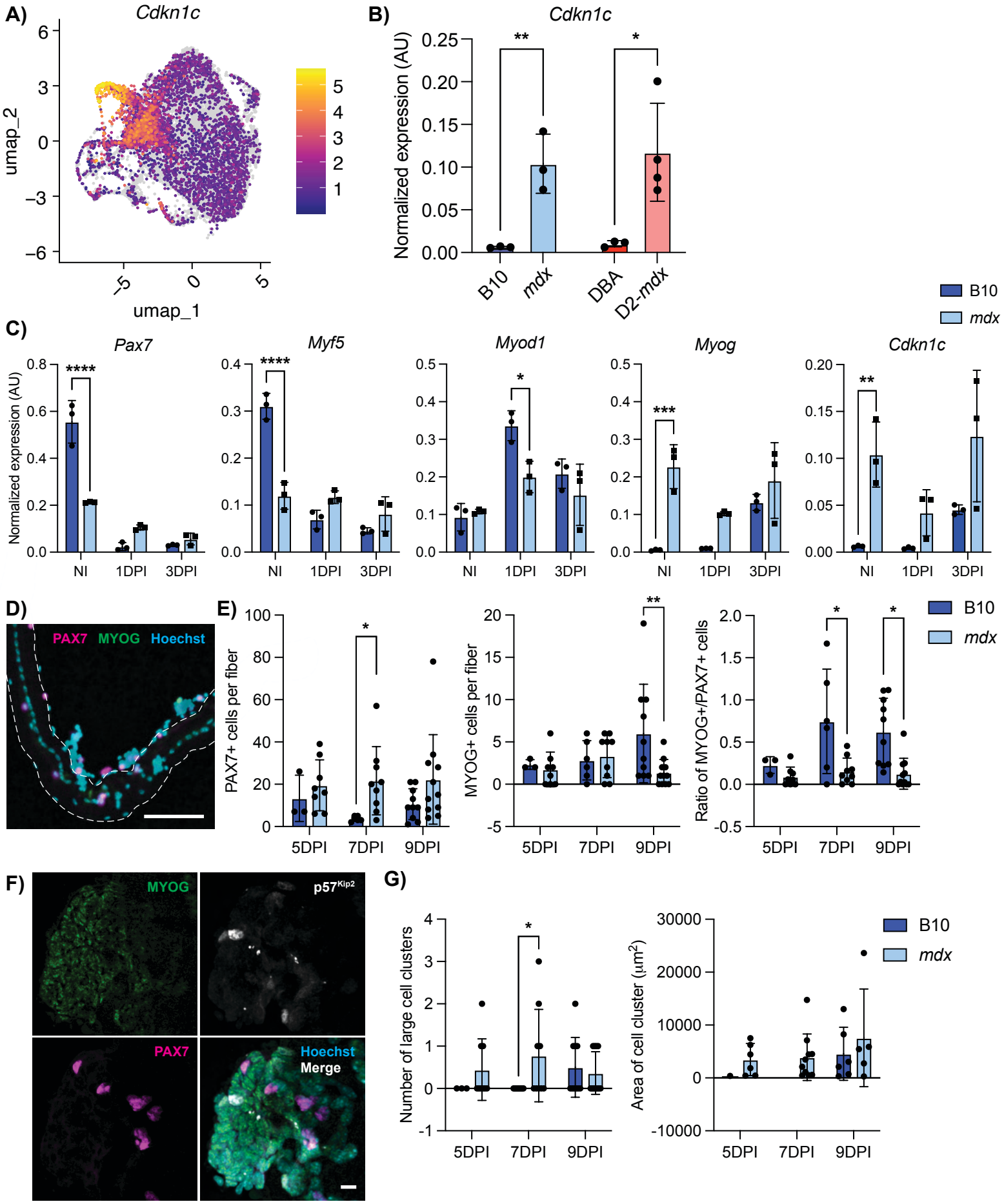

A)
Cdkn1c
umap_2
umap_1
B)
Cdkn1c
Normalized expression (AU)
B10
mdx
DBA
D2-mdx
C)
Pax7
Myf5
Myod1
Myog
Cdkn1c
Normalized expression (AU)
NI
1DPI
3DPI
D)
PAX7
MYOG
Hoechst
E)
PAX7+ cells per fiber
MYOG+ cells per fiber
Ratio of MYOG+/PAX7+ cells
5DPI
7DPI
9DPI
F)
MYOG
p57Kip2
PAX7
Hoechst
Merge
G)
Number of large cell clusters
Area of cell cluster (μm2)

Figure 7

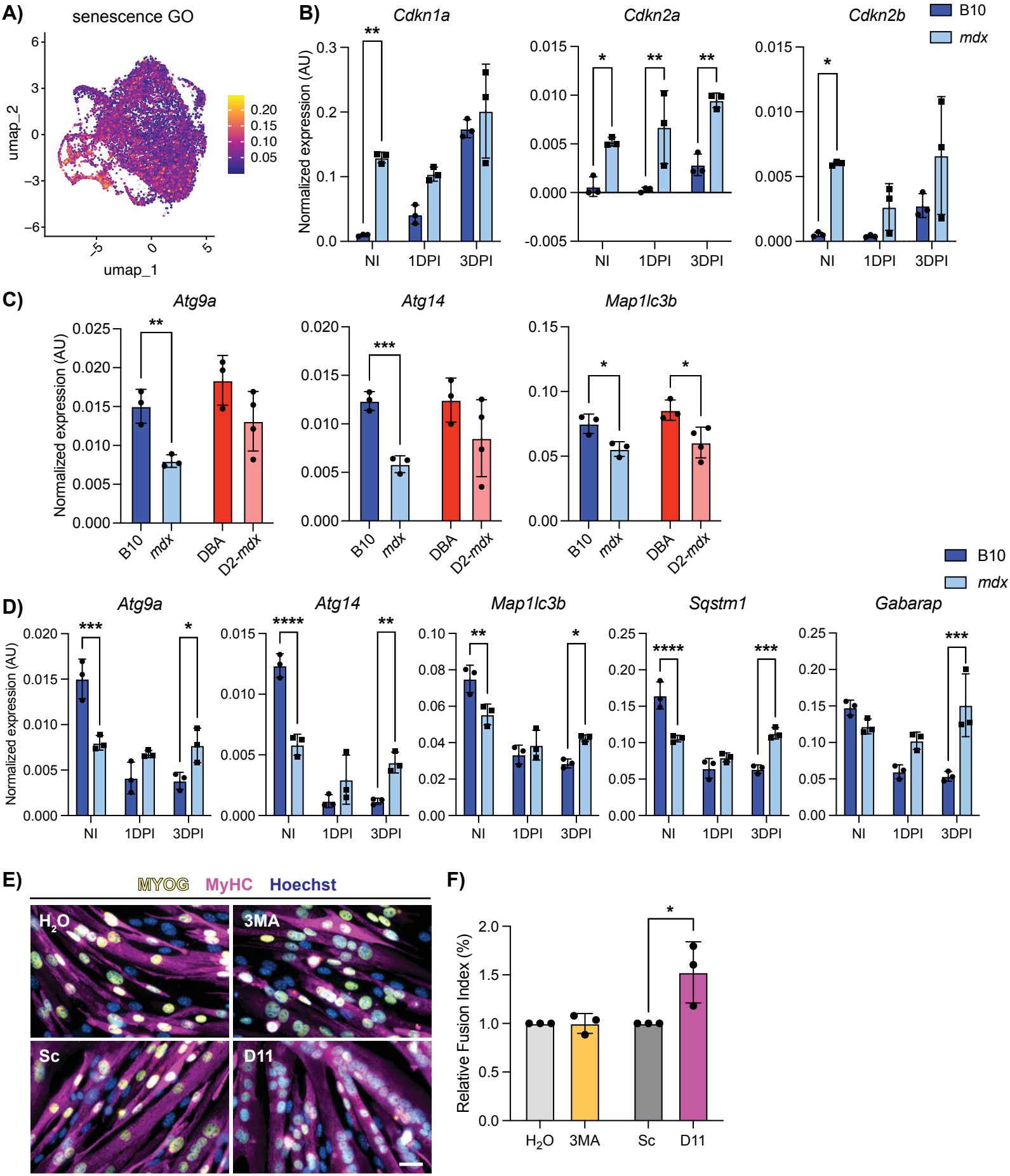

A)
senescence GO
umap_2
umap_1
0.20
0.15
0.10
0.05
B)
Cdkn1a
Cdkn2a
Cdkn2b
B10
mdx
Normalized expression (AU)
NI
1DPI
3DPI
C)
Atg9a
Atg14
Map1lc3b
Normalized expression (AU)
B10
mdx
DBA
D2-mdx
D)
Atg9a
Atg14
Map1lc3b
Sqstm1
Gabarap
Normalized expression (AU)
B10
mdx
NI
1DPI
3DPI
E)
MYOG
MyHC
Hoechst
H2O
3MA
Sc
D11
F)
Relative Fusion Index (%)
H2O
3MA
Sc
D11

Figure Legends

Figure 1. Altered regenerative and satellite cell profiles in *mdx* and D2-*mdx* mice. **A)** H&E images from TA cross-sections of three-month old male DMD models (*mdx* and D2-*mdx)* and their respective controls (B10 and DBA), showing morphological changes in the DMD models such as centralized nuclei and changes in fiber cross sectional area. **B)** Quantification of fiber cross sectional area, demonstrating changes in DMD models compared to controls. **C)** Representative WGA (green) and eMHC (magenta) IF labelling of TA cross-sections which, as quantified in **D)** shows a significant increase in eMHC+ fibers in DMD models compared to controls. **E)** Representative IF labelling of TA cross-sections for WGA (green) and PAX7 (magenta), showing PAX7+ satellite cells (arrows). **F)** Quantification of number of PAX7+ cells per mm^2 of TA, showing a significant increase in *mdx* and a decrease D2-*mdx* compared to their respective controls. Scalebars = 50 µm (**A, C**) and 10 µm (**E**), *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed unpaired t-test, n = 3 biological replicates per strain). Data are expressed as frequency distribution (**B**) or mean ± SD (**D, F**).

Figure 2. Single cell RNA-seq analysis of DMD satellite cells. **A)** UMAP representation after unsupervised clustering of myogenic cells showing the mapping of cells into seven distinct clusters. **B)** Violin plot of highly expressed markers used to define the identity of each myogenic cluster. **C)** Percentage of cells belonging to each cluster by strain, summarized in **D)**, showing a decrease in quiescent, stressed and activated muscle stem cells (MuSC) and increase in cycling, differentiating and "DMD enriched" clusters in the DMD models *mdx* and D2-*mdx* compared to their respective controls. **E)** Feature plot of cell cycle stage, showing that cycling progenitors are in G2M or S phase while quiescent, stressed, activated, and differentiating cells have higher levels of cells in G1 phase.

Figure 3. Differential gene expression in DMD satellite cells. **A)** PCA plot showing separation of DMD models versus healthy controls across PC1 and mouse genetic background on PC2. **B)** Dot plot

of the expression of genes which comprise the dystrophin-glycoprotein complex, showing decreased expression of all components except *Sntb2* in DMD models vs respective controls. **C, D)** Volcano plots of differentially expressed genes in *mdx* versus B10 (**C**) and D2-*mdx* versus DBA (**D**). **E, H)** Venn diagram of up-regulated (**E**) and down-regulated (**H**) genes from *mdx* versus B10 (blue) and D2-*mdx* versus DBA (pink) and their overlap and **F, I)** heatmaps of top 20 enriched terms, coloured by p-value, related to these gene lists. **G, J)** Circos plots representing overlap between up-regulated (**G**) and down-regulated (**J**) gene lists. Outer circle represents the gene list for *mdx* (blue) and D2-*mdx* (red). Inner circle represents gene lists, where hits are arranged along the arc. Genes that hit multiple lists are colored in dark orange, and genes unique to a list are shown in light orange. Purple curves link identical genes between gene lists. Blue curves link genes that belong to the same enriched ontology term.

Figure 4. Apoptosis in DMD satellite cells and senescence in DMD myogenic progenitors. **A)** Selected terms related to apoptosis and senescence from top 100 terms derived from differentially upregulated genes in *mdx* and D2-*mdx* satellite cells compared to controls. Dot plot of the expression of apoptosis and senescence GO-terms comparing expression by **B)** strain and by **C)** cell cluster. **D)** Representative IF labelling of an uninjured *mdx* EDL myofiber for PAX7 (magenta) and TUNEL (yellow) showing a nucleus positive for PAX7 only (white arrow) and a nucleus double positive for both PAX7 and TUNEL (yellow arrow). **E)** Quantification of number of total PAX7+ nuclei that are positive for TUNEL, visualized as a percentage, showing their presence in *mdx* only (n = 3 biological replicates). **F)** Dot plot of scRNA-seq data showing an increase in senescence-associated genes *Cdkn1a*, *Cdkn2a* and *Cdkn2b* in DMD models as compared to controls and **G)** verification of this increase in *Cdkn1a*, *Cdkn2a*, and *Cdkn2b*, with ddPCR from prospectively isolated satellite cells (n = 3 or 4 biological replicates as indicated). **H)** SA-β-Gal (blue) and Hoechst (yellow) staining of TA cross-sections showing senescence in DMD models and **I)** quantification of SA-β-Gal+ nuclei showing significant

increase in DMD muscle as compared to controls. Scalebars = 10 µm (**D**), 50 µm (**H**). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (two-tailed unpaired t-test (**G, I**), one-sided Fisher's exact test (**E**)). Data are expressed as mean ± SD.

Figure 5. DMD MuSCs exhibit impaired myogenic differentiation. **A)** Dot plot of cell polarity genes and **B)** canonical myogenic regulatory factors from scRNA-seq data. **C)** Digital PCR validation of myogenic regulatory factors from prospectively isolated satellite cells, showing lowered expression of early satellite cell factors like *Pax7* and increased expression of late differentiation factors such as *Myog.* **P < 0.01, ****P < 0.0001 (two-tailed unpaired t-test)). Data are expressed as mean ± SD. **D)** Dot plot of scRNA-seq data for regulators of differentiation showing a decrease in pro-differentiation and an increase in anti-differentiation genes in DMD models compared to controls. **E)** Pseudotime analysis of satellite cells showing cell fate trajectories with **F)** four distinct cell fates, and **G)** visualization by strain showing a preference for the "DMD enriched cluster" fate in DMD compared to control satellite cells.

Figure 6. DMD satellite cells exhibit impaired *in vivo* regenerative myogenesis. **A)** Feature plot of *Cdkn1c* showing increased expression in areas representing DMD-enriched and differentiating myocyte clusters. **B)** ddPCR validation of *Cdkn1c* expression from prospectively isolated satellite cells showing significant increase in DMD models compared to their respective controls (F = 18.09, P = 0.0002). **C)** Expression of myogenic factors and *Cdkn1c* in satellite cells from non-injured (NI), one-day post-injury (1DPI) and three-day post-injury (3DPI) showing dysregulation in *mdx* mice during myogenesis. (P < 0.0001 for *Pax7, Myf5* and *Myod1*, P = 0.0028 for *Myog*, P = 0.0002 for *Cdkn1c*) **D)** Representative IF labelling of an *mdx* EDL myofiber isolated 7-days post-injury for PAX7 (magenta) and MYOG (green), showing myogenic clusters along the fiber. **E)** Quantification of PAX7+ and

MYOG+ nuclei at 5, 7 and 9 DPI showing more PAX7+ and less MYOG+ nuclei in *mdx*. (Strain*DPI = n.s., strain factor P = 0.0124 for PAX7, P = 0.038 for MYOG) **F)** A large myogenic cluster labelled for MYOG, $p57^{Kip2}$ and PAX7 and **G)** quantification of the number of large clusters (> 1000 μm^2) and the area of these clusters. Scalebars = 100 µm (**D**), 10 µm (**F**). *P < 0.05, **P < 0.01, ****P < 0.0001 (two-tailed unpaired t-test (**B, E, G**), two-way ANOVA (**C**)). Data are expressed as mean ± SD.

Figure 7. DMD satellite cells have impaired autophagy and senescence. **A)** Feature plot showing cells positive for the senescence GO term, showing higher amounts in the regions where cycling progenitors and DMD enriched cells cluster. **B-D)** ddPCR quantification of gene expression in satellite cells in **B)** senescence-associated genes *Cdkn1a*, *Cdkn2a*, and *Cdkn2b* from non-injured (NI), one day post injury (1DPI) and three days post injury (3DPI) B10 and *mdx* mice showing dysregulation in *mdx*. (P < 0.0001 and P < 0.0001) **C)** Strain-comparison of the expression of autophagy-associated genes showing reduced expression of autophagy genes in both DMD models compared to healthy controls and **D)** changes in autophagy-associated genes in NI, 1DPI and 3DPI B10 and *mdx* satellite cells demonstrating impaired autophagy dynamics in *mdx* cells during regeneration (P = 0.0004, 0.0001, 0.0109, <0.0001, and 0.0001, respectively). **E)** Representative images of IF staining against PAX7, MYOG and MyHC from *mdx* primary myoblasts treated with an autophagy inhibitor (3-MA, 5 mM and H_2O control) or autophagy inducer (Tat-D11, 10 µM and Scramble control) for two hours prior to differentiation for two days. **F)** Fusion index of each condition was determined and shows increased differentiation after Tat-D11 treatment. Scalebars = 25 µM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (two-tailed unpaired t-test (**C, F**) and two-way analysis of variance (**B, D**)). Data are expressed as mean ± SD.