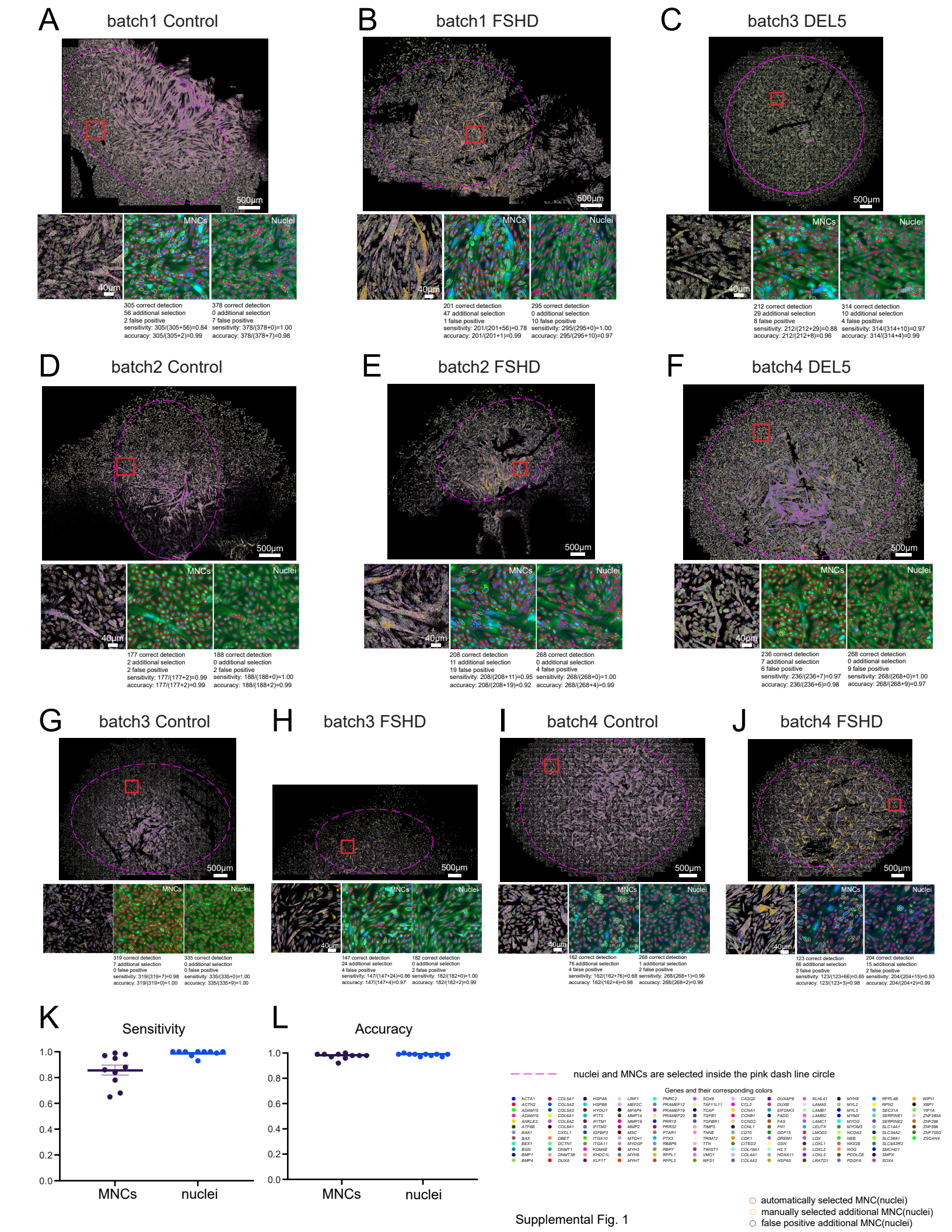
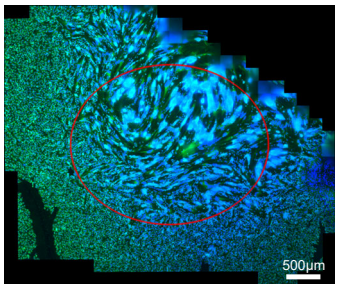


Supplemental Fig. 1

Supplemental Fig. 1. Illustration of spatial overlay of transcripts, and algorithmically segmented mononuclear cells (MNCs) and nuclei, for all the samples used in the study A-J. Top: overall illustration of spatial transcript overlay. All 140 transcript types used in the study are presented with different colors. The dash line circles represent the boundary in which the nuclei and MNCs are selected. The red rectangle represents the area that is going to be magnified below. For each panel; Bottom left: Illustration of spatial transcript overlay inside the magnified area. Bottom middle: Illustration of cellpose selected MNCs inside the magnified region. Bottom right: Illustration of automatically selected nuclei inside the magnified region. For both MNC and nuclei, red circle indicate cellpose detection results, yellow circle indicate manually defined additional selection, and black circle indicate false positive selections (multi-nuclei cells, false MNCs that are part of a multi-nuclei cell, and multiple nuclei detected as a single nuclei). **K.** Sensitivity of cellpose detection for the 10 example patches in A-J. Overall, across the 10 sample regions the sensitivity for MNC is 0.858 ± 0.039; sensitivity for nuclei is 0.989 ± 0.007. **L.** Accuracy of cellpose detection across the 10 example patches in A-J. The accuracy for MNCs are 0.976 ± 0.007, and accuracy for nuclei is 0.986 ± 0.003.

A

batch1 Control



Fusion level: 0.38

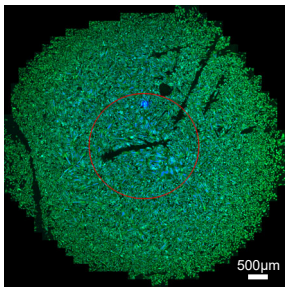
B

batch1 FSHD

Fusion level: 0.33

C

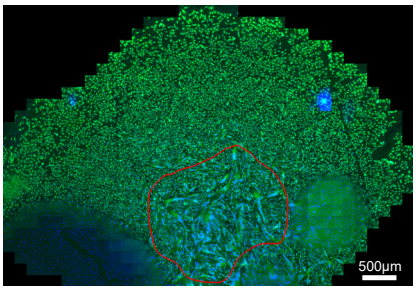
batch3 DEL5



Fusion level: 0.42

D

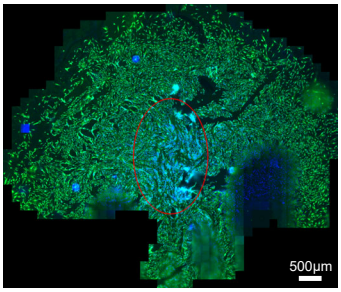
batch2 Control



Fusion level: 0.46

E

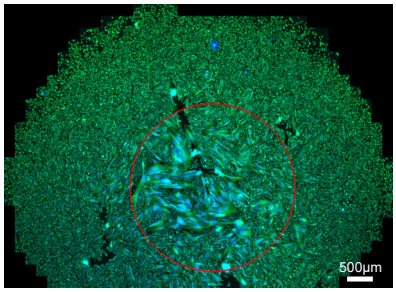
batch2 FSHD



Fusion level: 0.35

F

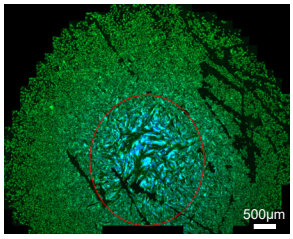
batch4 DEL5



Fusion level: 0.66

G

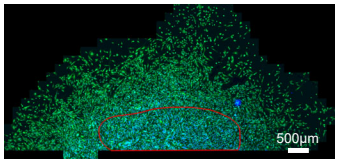
batch3 Control



Fusion level: 0.50

H

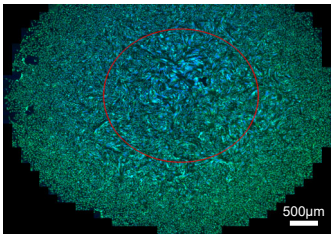
batch3 FSHD



Fusion level: 0.44

I

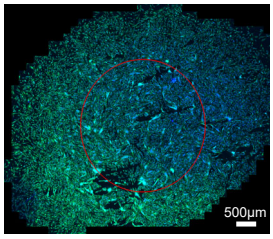
batch4 Control



Fusion level: 0.48

J

batch4 FSHD

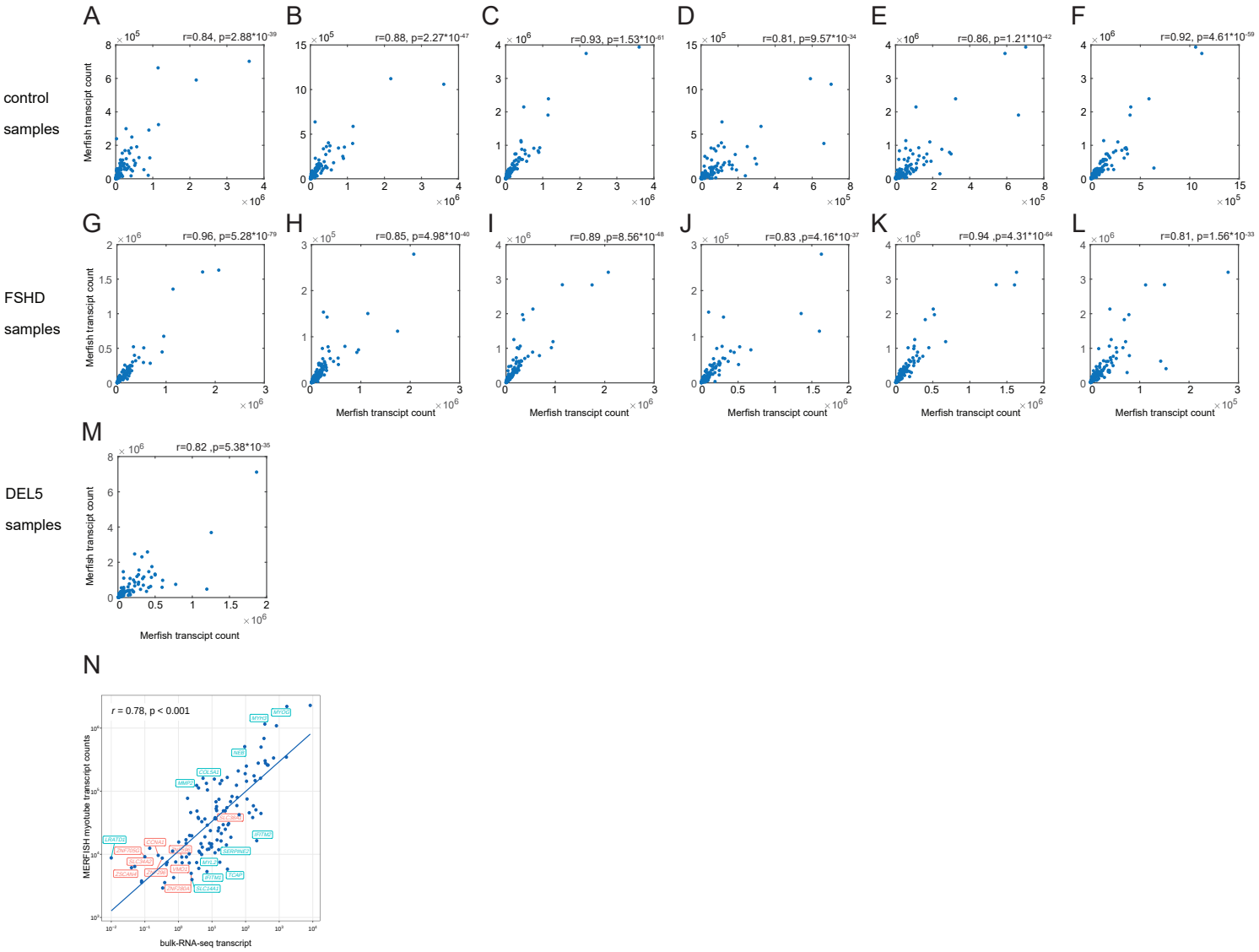


Fusion level: 0.45

Supplemental Fig. 2

Supplemental Fig. 2. Estimation of fusion level of multi-nuclei muscle cells among samples use in the study

A-J. Fluorescence staining image of the 10 samples used in the study. Red circles represent the area for fusion level estimation. Fusion level is estimated by (total number of nuclei – intra-MNC nuclei)/(total number of nuclei) in the selected area



Supplemental Fig. 3

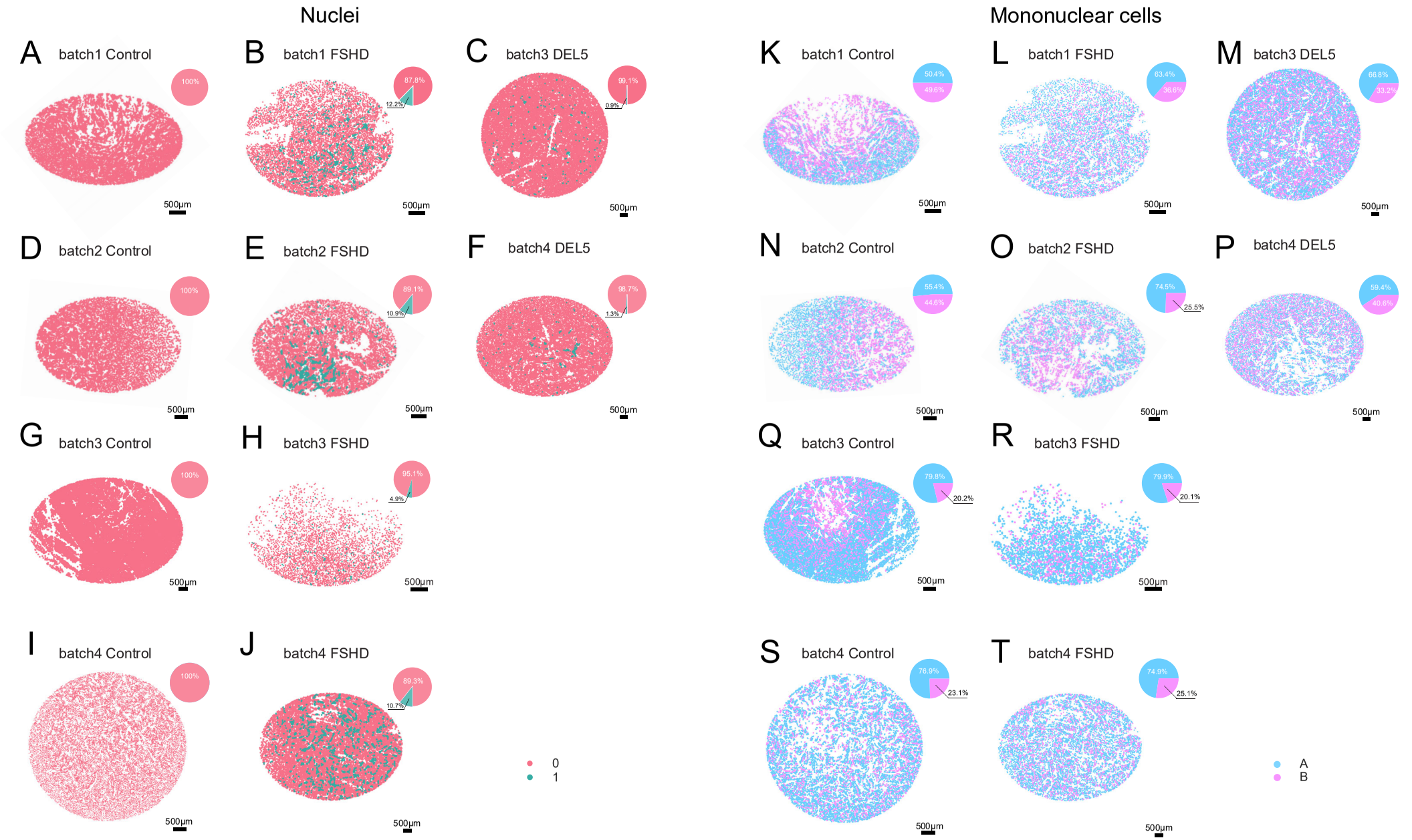
Supplemental Fig. 3. Comparison of counts for all individual genes across samples of the same genotype

A-F: Scatter plots representing the relationship of transcript counts between each pair of control samples. Transcript counts are calculated as the total counts of each of the 140 gene inside the sample range illustrated in supplemental figure 1. Overall transcript counts between control samples shows significantly high correlation (pearson $r>0.8$, $p<0.001$)

G-L: Scatter plots representing the relationship of transcript counts between each pair of FSHD samples. Overall transcript counts between control samples shows significantly high correlation (pearson $r>0.8$, $p<0.001$)

M: Scatter plot representing the relationship of transcript counts between the two DEL5 samples. Overall transcript counts between control samples shows significantly high correlation (pearson $r>0.8$, $p<0.001$)

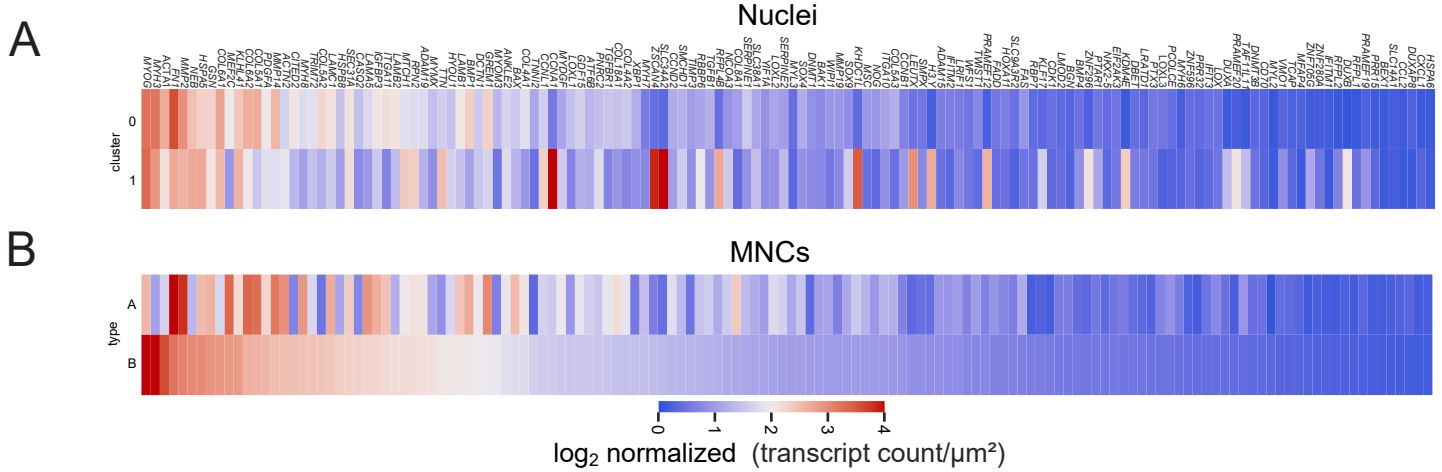
N: Correlation plot of MERFISH myotubes and bulk RNA seq (see Fig 1H), with outliers (blue) and DUX4 target genes (red) labeled.



Supplemental Fig. 4

Supplemental Figure 4. Spatial profile of the nuclei and mononuclear cell subpopulations demonstrated in Figure 2.

A-J: Distribution of nuclei subpopulations across samples in each sample (red: cluster 0; green: cluster 1). Nuclei are selected from the annotated center regions of each sample. Pie charts on the right-top illustrate the preposition of the two subpopulations in each sample.
K-T: spatial distribution of cell soma subpopulations in each sample (blue: cluster A; pink: cluster B). MNCs are selected from the annotated center regions of each sample, same as nuclei. Pie charts on the right-top illustrate the preposition of the two subpopulations in each sample.



C

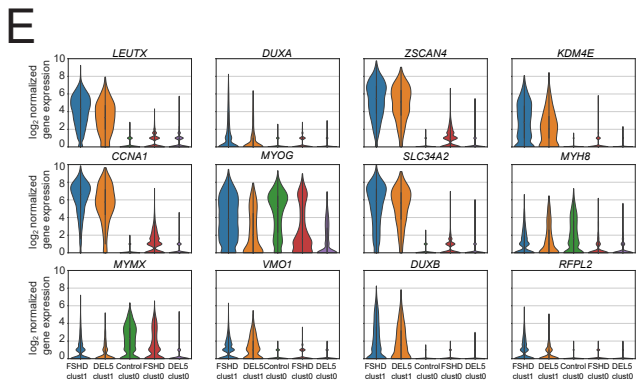
nuclei

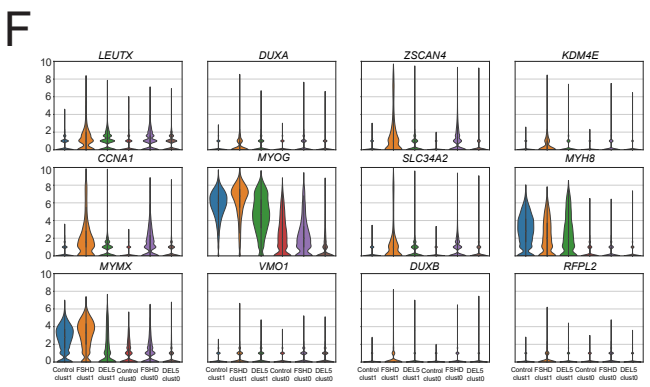
Genotype	total # of nuclei
FSHD	38491
DEL5	47698
Control	69952

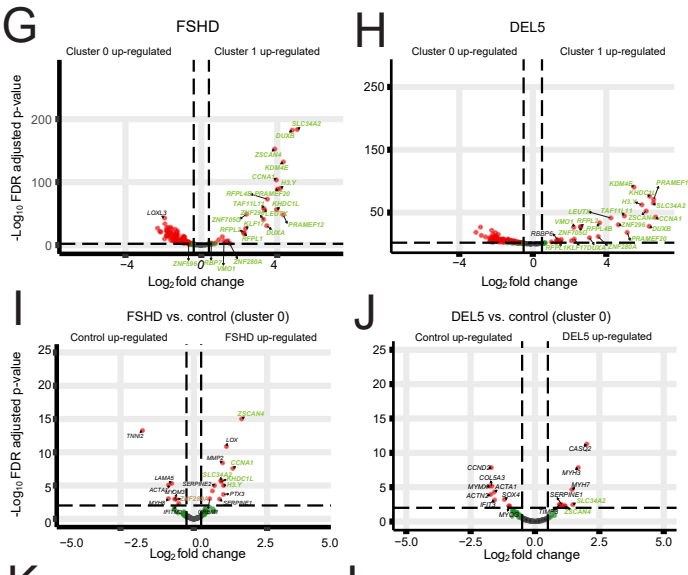
D

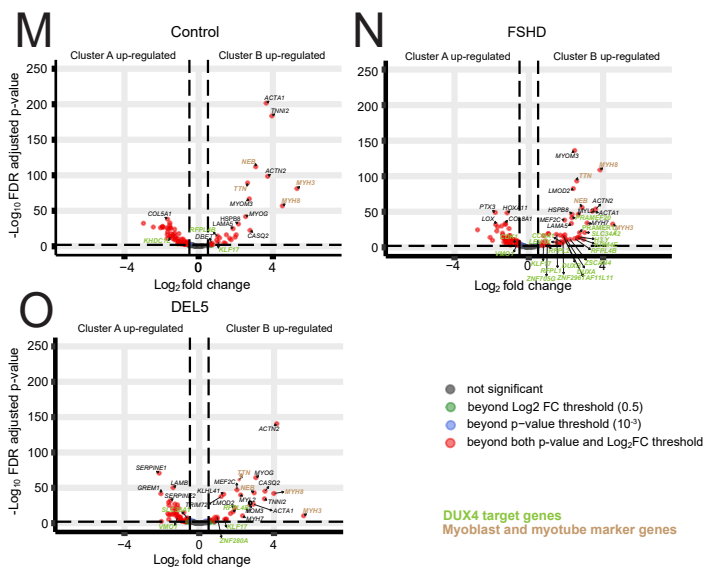
MNCs

Genotype	total # of MNCs
FSHD	23144
DEL5	29264
Control	40397







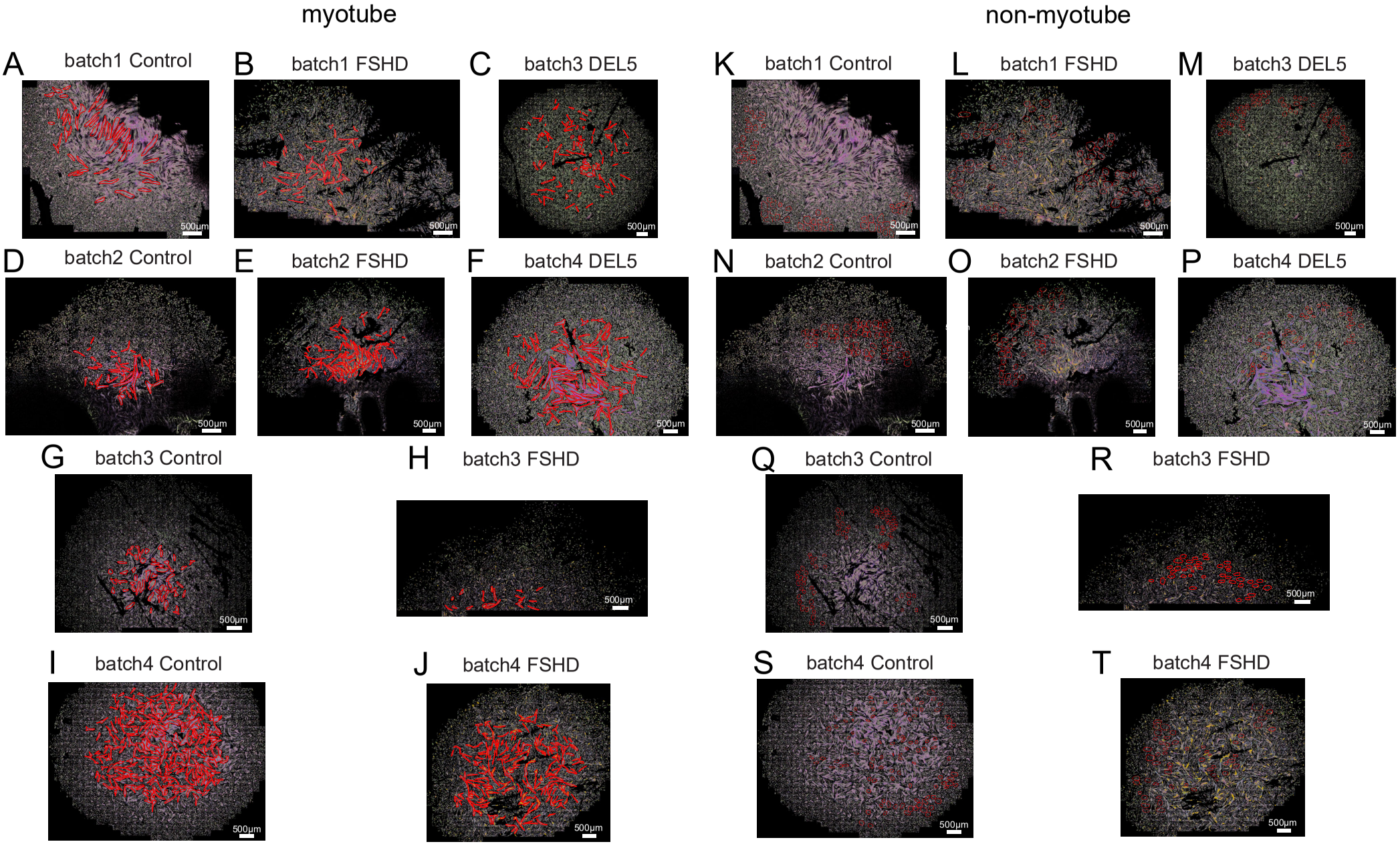


Supplemental Fig. 5

Supplemental Figure 5. Detailed analysis of the nuclei and MNC clusters in Figure 2

A-B: Heatmap illustrating all 140 gene expression patterns of the identified nuclei clusters (**A)** and MNC clusters (**B**). The expression profile for each nuclei cluster is the averaged expression across all its members.
C: Total number of nuclei and percentage of the two nuclei clusters, for each genotype.
D: Statistics of the total number of MNCs, and percentage of the two MNC types, for each genotype.
E-F: Violin plots of 12 selected genes' expression levels (log_2 normalized) between different nuclei clusters (**E**) and MNC clusters (**F**) with specific genotypes.
G-H: Volcano plots illustrate the differential expression between the two identified nuclei clusters across all FSHD samples (**G**) and all DEL5 samples (**H**). ~88% DUX4 target genes show up-regulation in cluster 1 for both FSHD and DEL5 samples (FSHD: 21 of 24 DUX4 target gene up-regulated, DEL5: 20 of 24 DUX4 target gene up-regulated, Log_2FC threshold: 0.5; p-value threshold: 0.001).
I-J: Volcano plots illustrate the differential expression between the cluster 0 nuclei of Control samples and those of FSHD samples (**I**) and DEL5 samples (**J**). Compared with Control, *ZSCAN4* and *SLC34A2*, both DUX4 target genes are up-regulated in cluster 0 nuclei of FSHD and DEL5 samples, while *KDHC1L*, *CCNA1* and *H3.Y* only show up-regulation in FSHD cluster 0 nuclei. (Log_2FC threshold: 0.5; p-value threshold: 0.001).
K-L: Volcano plots illustrate the differential expression between cluster 0 nuclei and cluster 1 nuclei in FSHD samples (**K**) and DEL5 samples (**L**). More than 75% of the DUX4 target genes show up-regulation in cluster 1 for both FSHD and DEL5 samples (FSHD: 19 of 24 DUX target gene up-regulated, DEL5: 20 of 24 DUX target gene up-regulated), while one DUX target gene (ZNF596) shows up-regulation in Control cluster 0 nuclei, compared to DEL5 cluster 1 nuclei. (Log_2FC threshold: 0.5; p-value threshold: 0.001).

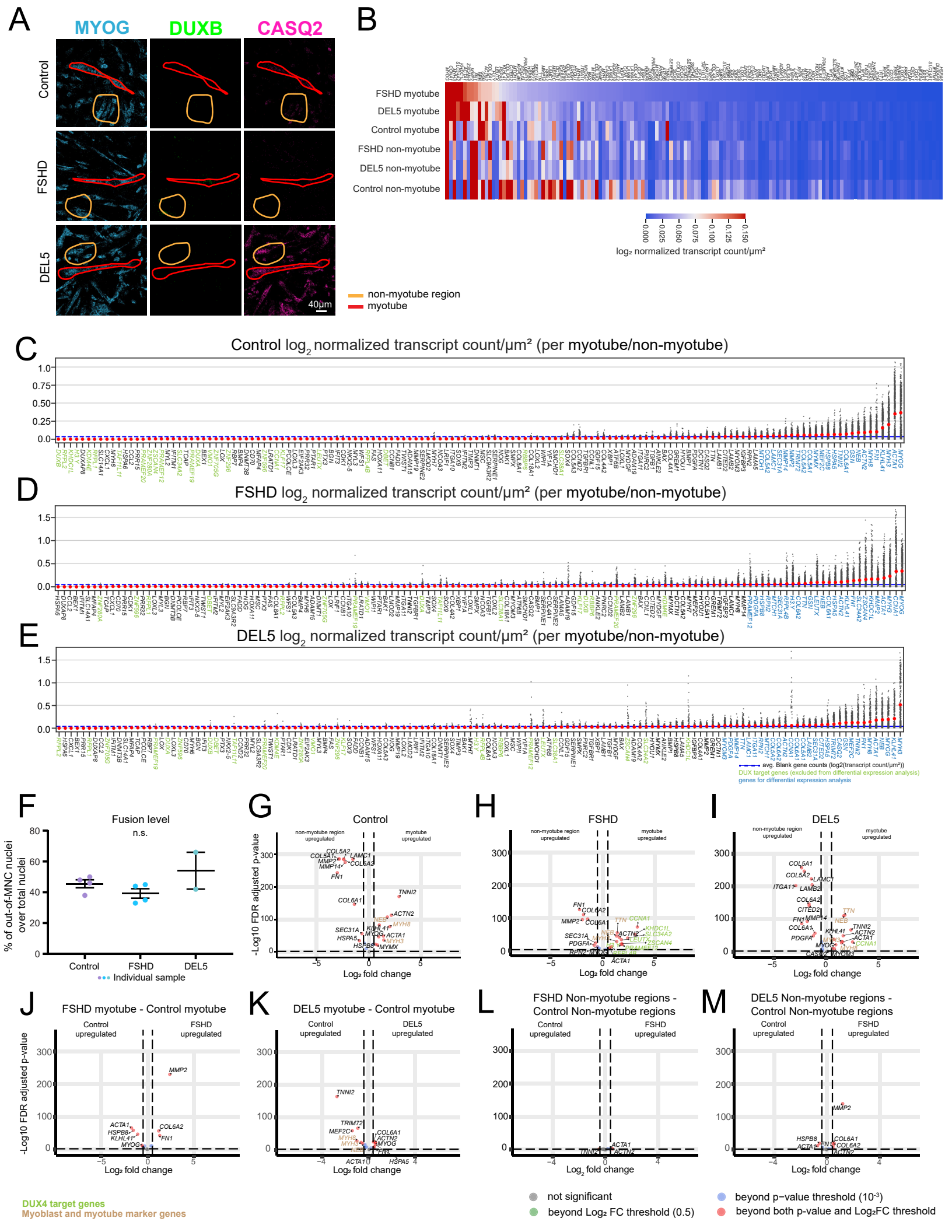
M-O: Volcano plots illustrate the differential expression between the two identified MNC clusters in Control samples (**M**), FSHD samples (**N**), and DEL5 samples (**O**). For DUX target genes, *RFPL4B* shows up-regulation in the type B MNCs of for all three genotypes; Meanwhile, myotube marker genes *MYH8, MYH3, NEB* and *TTN* show up-regulation in type B MNCs across all genotypes (Log_2FC threshold: 0.5; p-value threshold: 0.001).



Supplemental Fig. 6

Supplemental Figure 6. Illustration of the manually defined myotubes and non-myotube regions in all samples

A-J: Annotated myotubes in each sample (red). The background image represents the spatial distribution of all 140 genes used in the experiment.
K-T: Non-myotube regions in each sample (red). The background image represents the spatial distribution of all 140 genes used in the experiment.


A
MYOG
DUXB
CASQ2
Control
FSHD
DEL5
40µm
non-myotube region
myotube
B
FSHD myotube
DEL5 myotube
Control myotube
FSHD non-myotube
DEL5 non-myotube
Control non-myotube
0.000
0.025
0.050
0.075
0.100
0.125
0.150
log₂ normalized transcript count/µm²
C
Control log2 normalized transcript count/µm² (per myotube/non-myotube)
D
FSHD log2 normalized transcript count/µm² (per myotube/non-myotube)
E
DEL5 log2 normalized transcript count/µm² (per myotube/non-myotube)
avg. Blank gene counts (log2(transcript count/µm²))
DUX target genes (excluded from differential expression analysis)
genes for differential expression analysis
F
Fusion level
n.s.
% of out-of-MNC nuclei over total nuclei
Control
FSHD
DEL5
Individual sample
G
Control
H
FSHD
I
DEL5
non-myotube region upregulated
myotube upregulated
-Log10 FDR adjusted p-value
Log2 fold change
J
FSHD myotube - Control myotube
K
DEL5 myotube - Control myotube
L
FSHD Non-myotube regions - Control Non-myotube regions
M
DEL5 Non-myotube regions - Control Non-myotube regions
Control upregulated
FSHD upregulated
DEL5 upregulated
DUX4 target genes
Myoblast and myotube marker genes
not significant
beyond Log2 FC threshold (0.5)
beyond p−value threshold (10-3)
beyond both p-value and Log2FC threshold

Supplemental Figure. 7. Detailed gene expression profiles between myotube and non-myotube areas in Control, FSHD, and DEL5 samples

A. Expression of three genes (*MYOG, DUXB,* and *CASQ2*) around and inside an example myotube and a non-myotube region.

B. Heatmap of the $\log_2$ area normalized expression level of all 140 genes used in the study.

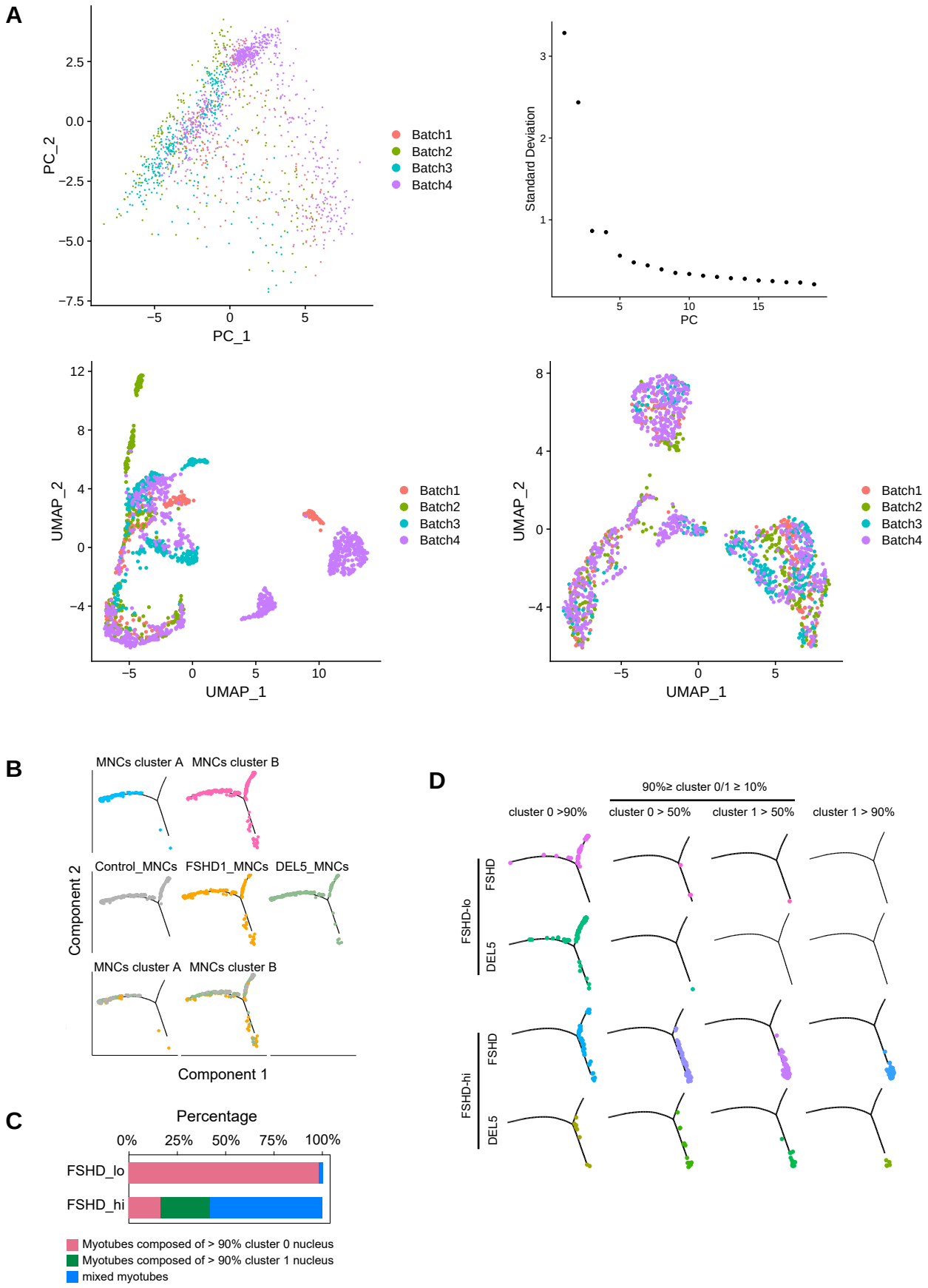
C-E. $\log_2$ area normalized gene counts per myotube or non-myotube region, for control (**C**), FSHD (**D**) and DEL5 (**E**) genotype. Red dots represent the average normalized gene counts for each of the 140 genes. Blue dash line represents the average normalized blank gene counts. Only genes with higher average normalized counts than blank gene level will be used for differential expression analysis.

F. Fusion level of the selected samples. Fusion level is calculated as (total number of nuclei – intra-MNC nuclei)/(total number of nuclei), which represents the fraction of nuclei that belongs to multi-nuclei muscle cells, inside the selection areas shown in supplemental Figure 2. No significant difference noted between samples across genotypes (Control: 0.4550±0.0263; FSHD: 0.3925±0.0307; DEL5: 0.5400±0.1200, mean ± standard error; Control vs. FSHD: p=0.1143; DEL5 vs. FSHD: p=0.5333, DEL5 vs. Control: p=0.8000, Two-tailed Wilcoxon rank sum test)

G-I. Volcano plots comparing myotube and non-myotube gene expression for control **(G)**, FSHD **(H)**, and DEL5 **(I)** samples. Myotubes and non-myotube areas in each sample are aggregated into pseudobulks for differential gene analysis. Only the blue labeled genes in C-E are used in differential gene analysis. DUX4 target genes are labeled in green. Myotube and myoblast marker genes are labeled in brown. (Log_2FC threshold: 0.5; p-value threshold: 0.001)

J-K. Volcano plots comparing myotube gene expression between Control and FSHD **(J)** or DEL5 **(K)** genotypes. Only the intersection of blue labeled genes in C and D are used for this analysis.(Log_2FC threshold: 0.5; p-value threshold: 0.001)

L-M. Volcano plots comparing non-myotube areas gene expression between Control and FSHD **(L)** or DEL5 **(M)** genotypes. Only the intersection of blue labeled genes in C and E are used for this analysis. (Log_2FC threshold: 0.5; p-value threshold: 0.001)



Supplemental Fig. 8

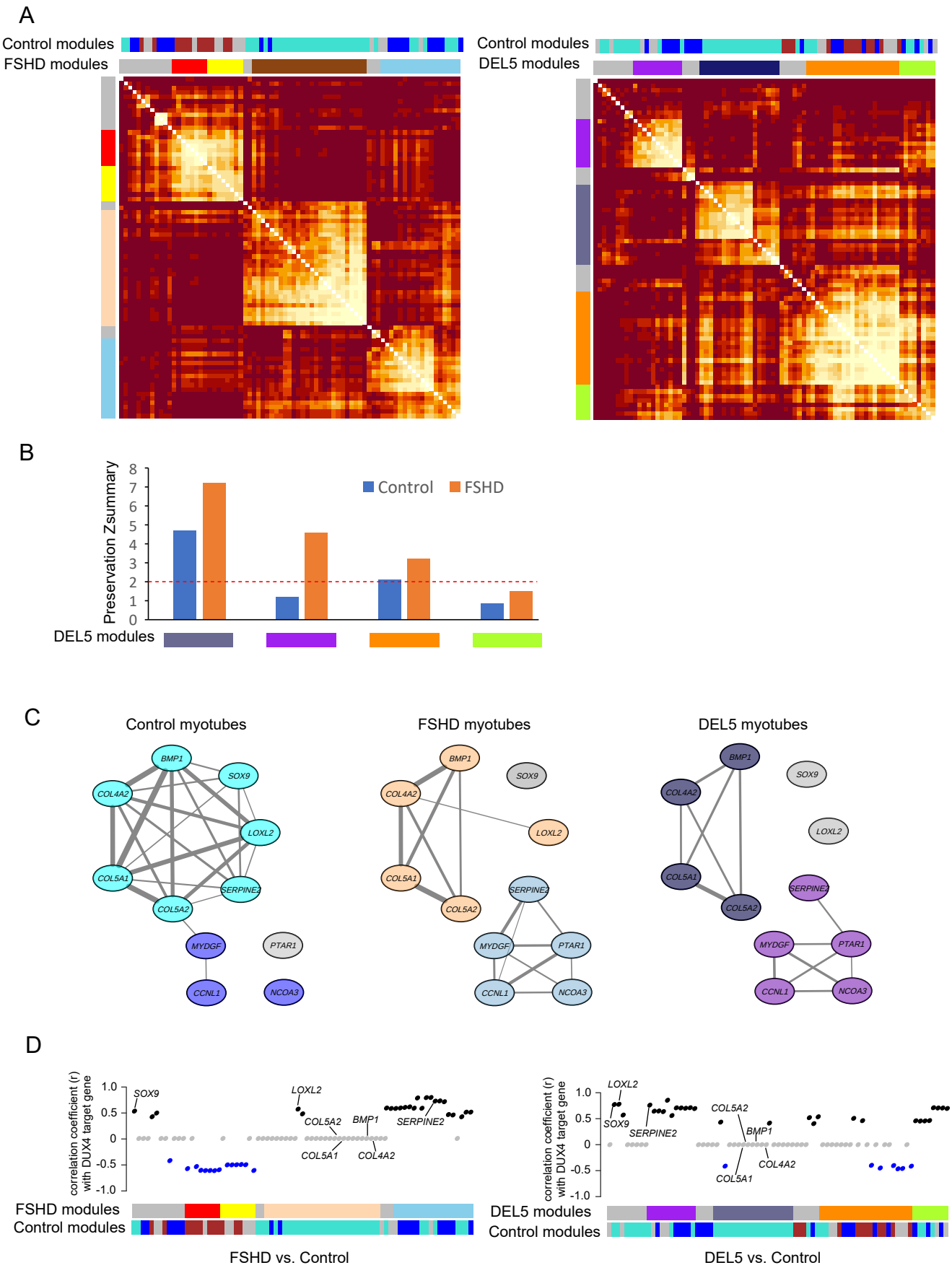
Supplemental Figure 8. Supplemental information regarding pseudotime trajectory analysis

A: (Top) PCA and Elbow plots for combined myotube/non-MT region data. (Bottom) UMAP plots of non-corrected (left) and batch-corrected single myotube/non-MT region MERFISH data (right), as shown in **Figure 4A**, display cells colored by batch.

B: ~1000 MNCs, comprising both cluster A and B as shown in **Figure 3A**, are plotted on the pseudotime trajectory from **Figure 4H**, grouped by clusters (top), cell types (middle), or both (bottom).

C: Composition of the two types of nuclei, as identified in **Figure 2A**, in both FSHD-hi and FSHD-lo samples.

D: FSHD-hi/lo myotubes from FSHD/DEL5, which consist of cluster 0 or 1 nuclei at varying percentages, are projected to the pseudotime tree shown in **Figure 4H**.



Supplemental Fig. 9

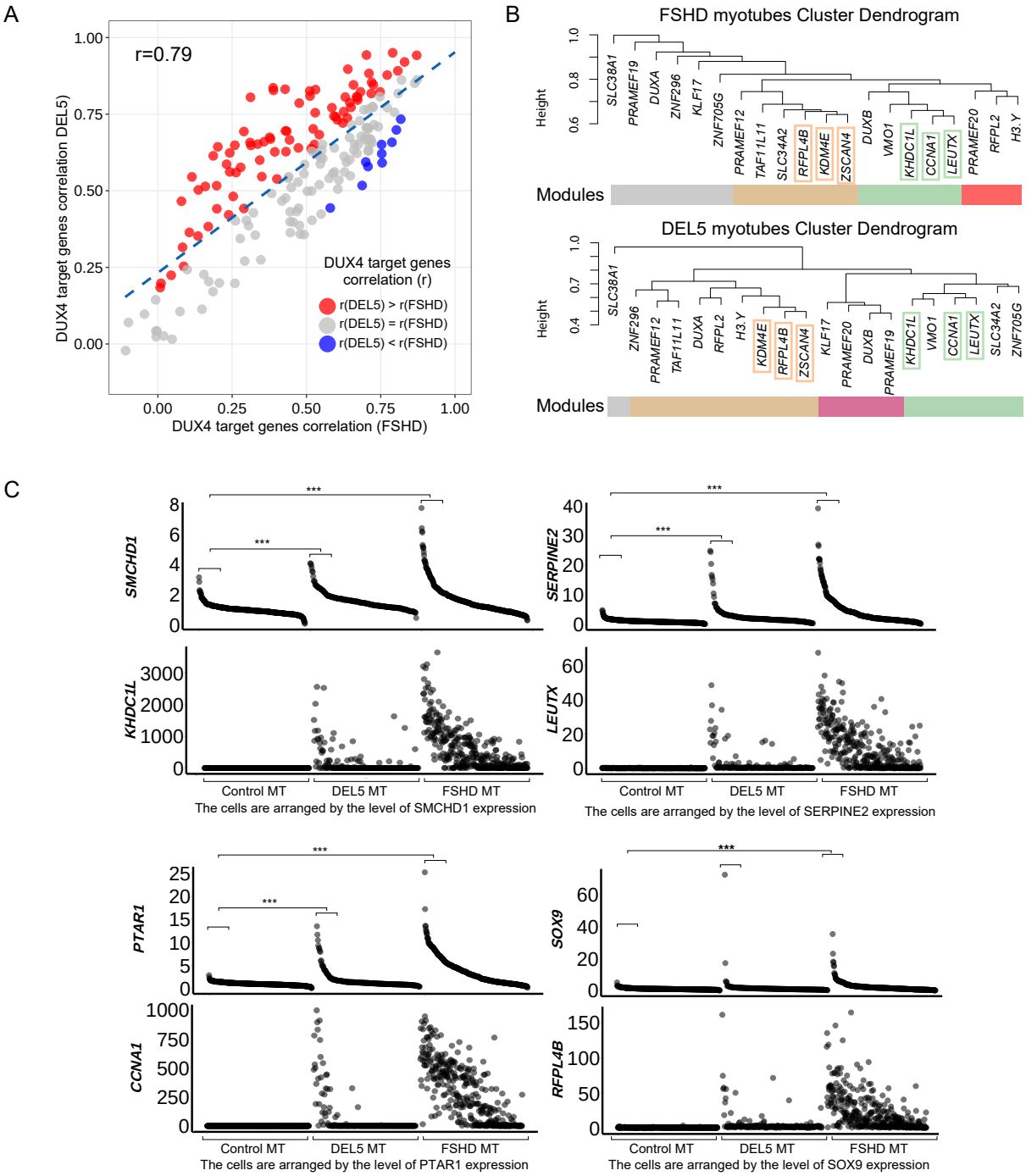
Supplemental Figure 9. Supplemental information regarding non-DUX4 target gene-coexpression analysis

A: The TOM heatmaps of non-DUX4 targets of FSHD (left) and DEL5 (right) myotubes are plotted with the genes ordered based on the gene dendrogram of FSHD or DEL5 myotubes, respectively. The corresponding modules of both Control and FSHD/DEL5 myotubes are visible on the top of the heatmaps, as indicated.

B: The preservation Zsummary values of DEL5 modules in Control and FSHD. The modules in DEL5 have stronger evidence of preservation in FSHD than in Control. The Zsummary value under red dash line is considered as the module is not preserved.

C: A total of eleven genes, including seven genes from the turquoise module, in Control myotubes are analyzed using WGCNA to calculate their connection weights. These weights are visualized using Cytoscape (left). The thickness of the lines represents the strength of the connections, with wider lines indicating higher-weighted connections. Only positively correlated connections with weights larger than 0.06 are displayed. The co-expression networks of the same genes in FSHD myotubes (middle) and DEL5 myotubes (right) are also examined. The modules to which these genes belong are indicated by different colors.

D: The modules of Control and FSHD myotubes (left) as well as DEL5 myotubes (right) are displayed along with the highest correlation coefficient (r) values of each gene pair between the respective genes and DUX4 target genes. In the visualization, dark dots indicate gene pairs with correlation coefficients greater than 0.4, blue dots represent gene pairs with correlation coefficients less than -0.4, and grey dots represent gene pairs that do not meet the threshold, with the values presented as 0. The seven genes from the turquoise module of Control myotubes in (**C**) are labeled.



Supplemental Fig. 10

Supplemental Figure 10. Supplemental information regarding DUX4 target gene-coexpression analysis

A: The correlations between DUX4 target genes show a relatively consistent pattern between FSHD and DEL5. Pearson correlation analysis reveals a strong correlation (r = 0.79) between the correlation values between DUX4 target genes in both cell lines. Out of 190 correlation values, 99 values (shown in grey) do not exhibit significant differences between the two cell lines. The red dots indicate higher values in DEL5, while the blue dots indicate higher values in FSHD.

B: The dendrogram illustrates the clustering of DUX4 target genes in FSHD myotubes (left) and DEL5 myotubes (right).

C: The plot shows the relative expression levels of four genes correlated with DUX4 targets within individual myotubes, normalized by the mean value of Control cells and ordered from high to low in each cell line. The top 20% highest expression levels in Control cells are compared to the top 20% values in DEL5 or FSHD, with *** $p < 0.0001$. The corresponding DUX4 target genes with the highest correlation coefficient (r) are shown beneath the plot.