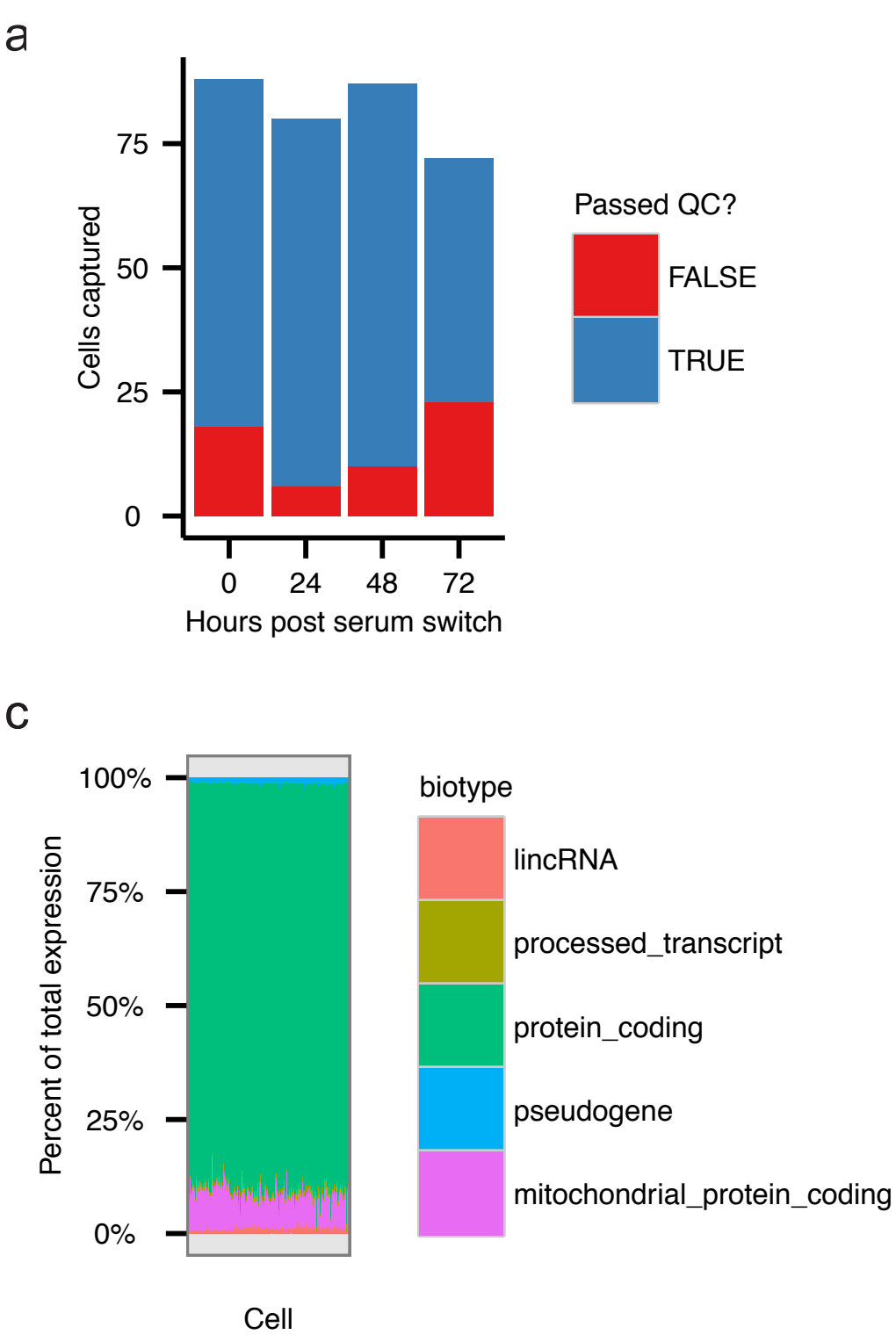
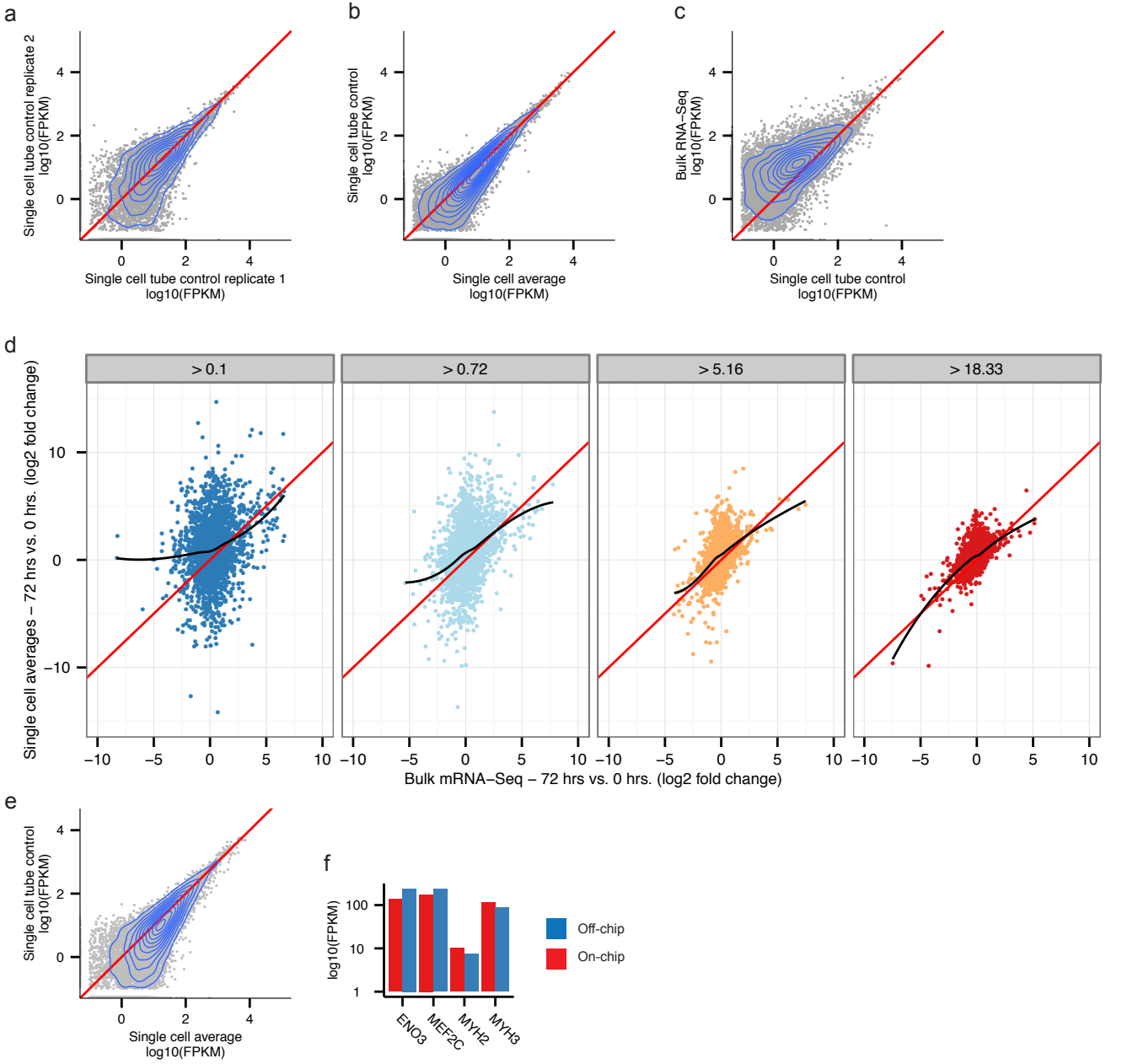
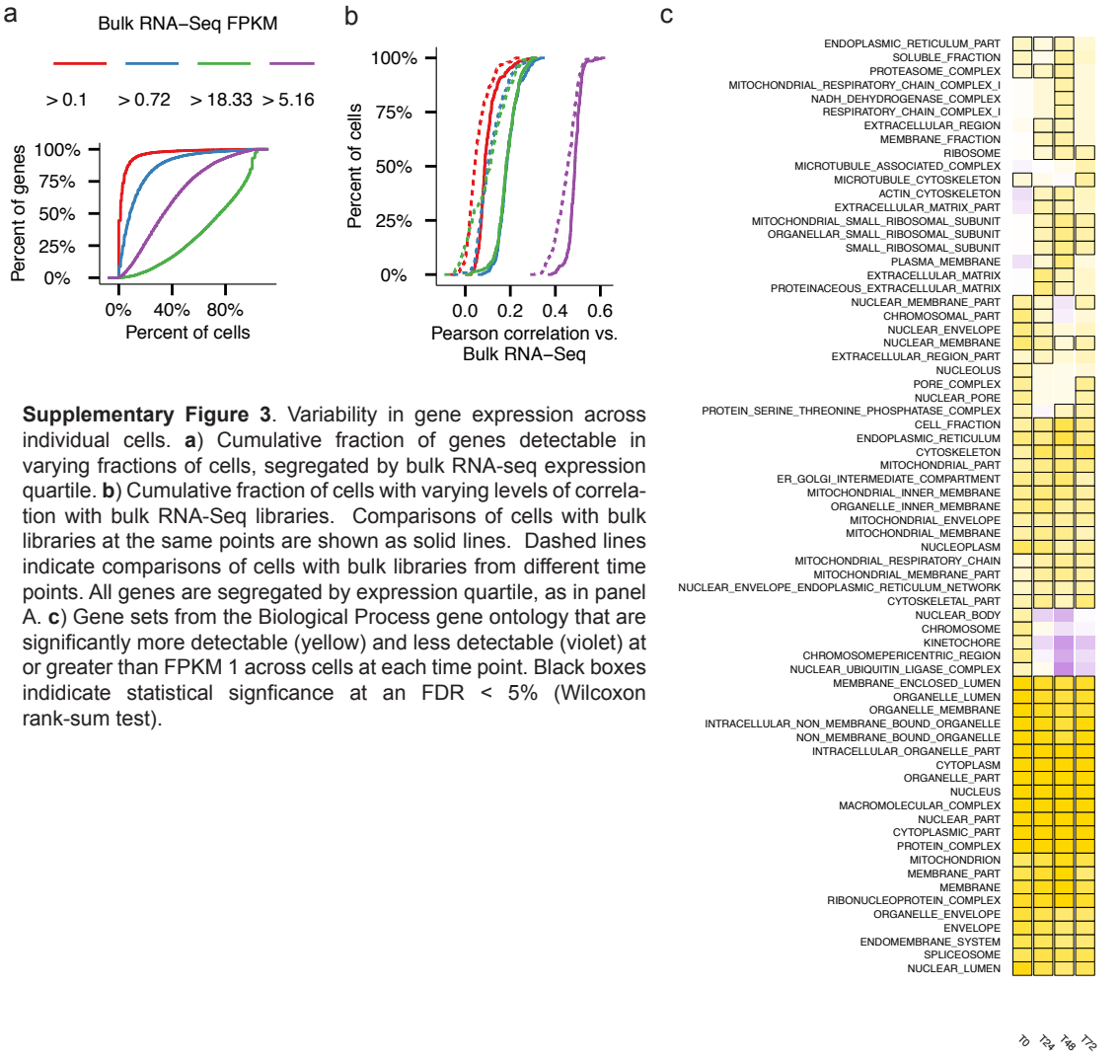


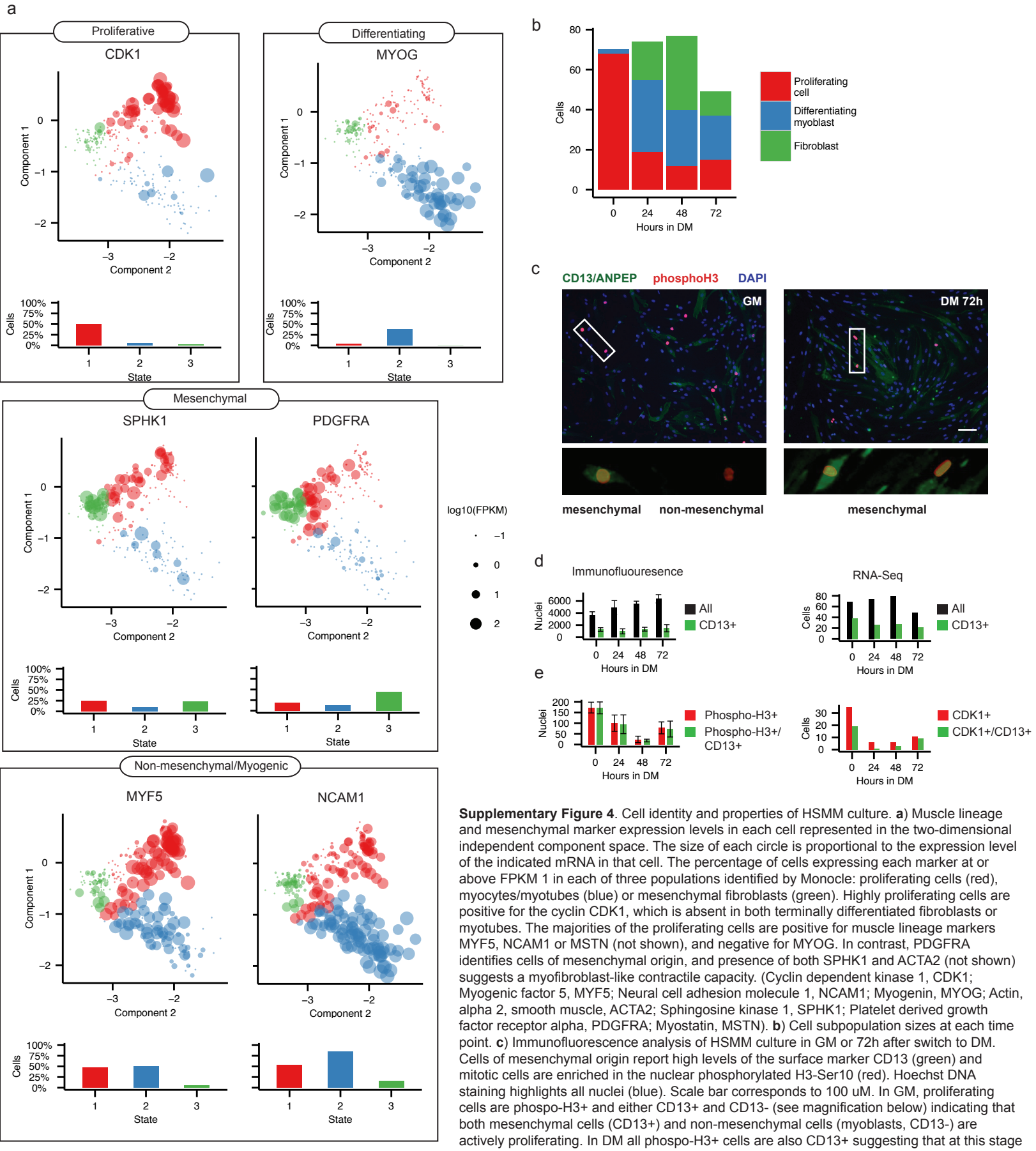
Supplementary Figure 1. Global properties of single-cell RNA-Seq libraries. **a**) Number of cells captured and passing quality control filters (methods) per Fluidigm C1 chip. **b**) Mapped fragments per cell. **c**) Fraction of per-cell cumulative expression, by biotype. **d**) Mean standardized distribution of log-transformed FPKMs across cells (black) compared to the standard normal distribution (red).



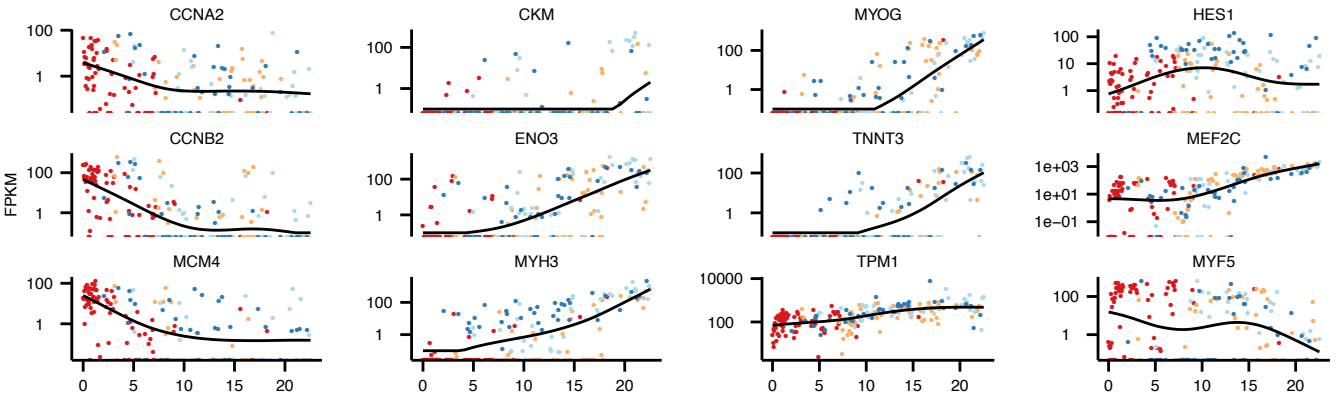
Supplementary Figure 2. Concordance of FPKMs between bulk and single-cell RNA-Seq libraries. **a**) Gene expression values from two replicate off-chip control libraries prepared from ~250 cells collected in GM (time zero). **b**) The cross-cell average expression level from single cell-libraries collected at time zero compared against the off-chip control library. **c**) The off-chip control library compared against the bulk RNA-Seq library average. **d**) Log-tranformed fold changes in gene expression between zero and 72 hours obtained from bulk RNA-Seq compared against those from the single-cell averages at the corresponding time points. Facets correspond to the four quartiles of gene expression (in FPKM determined by bulk RNA-Seq). e) The cross-cell average expression level from single cell-libraries collected after 48 hours in DM compared against the off-chip control library prepared from ~250 cells collected at the same time. f) On-and off-chip measurements of four genes expressed at high levels in large, multinucleated myotubes.



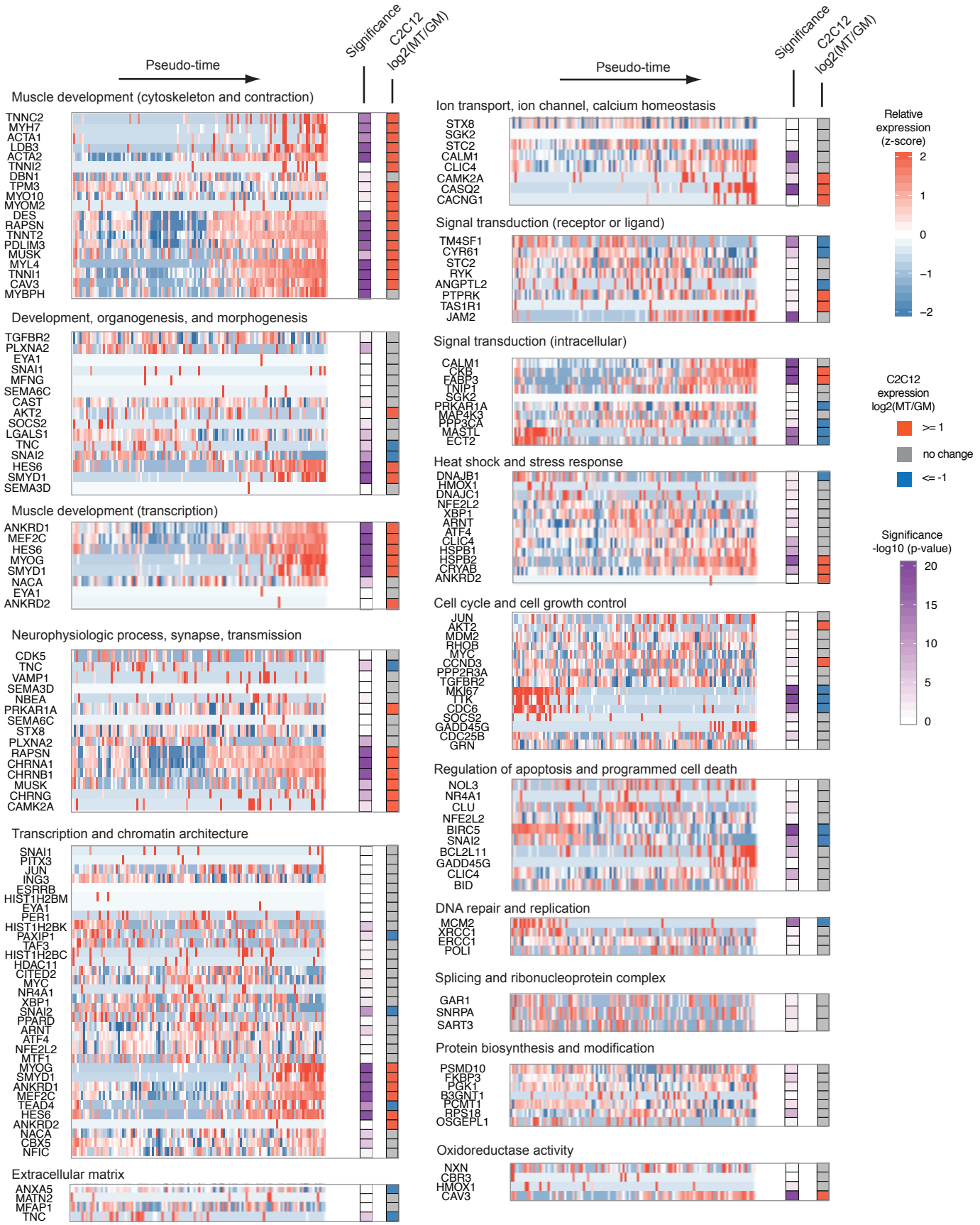
Supplementary Figure 3. Variability in gene expression across individual cells. **a**) Cumulative fraction of genes detectable in varying fractions of cells, segregated by bulk RNA-seq expression quartile. **b**) Cumulative fraction of cells with varying levels of correlation with bulk RNA-Seq libraries. Comparisons of cells with bulk libraries at the same points are shown as solid lines. Dashed lines indicate comparisons of cells with bulk libraries from different time points. All genes are segregated by expression quartile, as in panel A. **c**) Gene sets from the Biological Process gene ontology that are significantly more detectable (yellow) and less detectable (violet) at or greater than FPKM 1 across cells at each time point. Black boxes indidicate statistical signficance at an FDR < 5% (Wilcoxon rank-sum test).



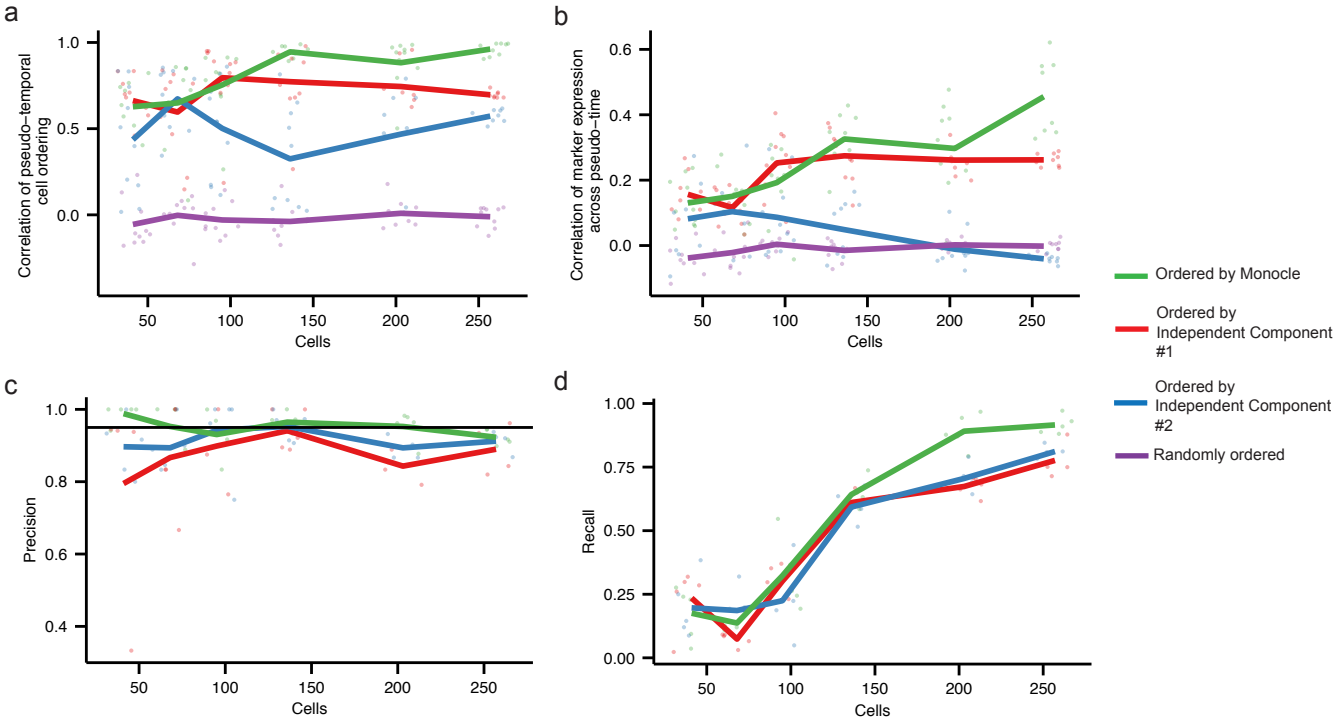
Supplementary Figure 4. Cell identity and properties of HSMM culture. **a**) Muscle lineage and mesenchymal marker expression levels in each cell represented in the two-dimensional independent component space. The size of each circle is proportional to the expression level of the indicated mRNA in that cell. The percentage of cells expressing each marker at or above FPKM 1 in each of three populations identified by Monocle: proliferating cells (red), myocytes/myotubes (blue) or mesenchymal fibroblasts (green). Highly proliferating cells are positive for the cyclin CDK1, which is absent in both terminally differentiated fibroblasts or myotubes. The majorities of the proliferating cells are positive for muscle lineage markers MYF5, NCAM1 or MSTN (not shown), and negative for MYOG. In contrast, PDGFRA identifies cells of mesenchymal origin, and presence of both SPHK1 and ACTA2 (not shown) suggests a myofibroblast-like contractile capacity. (Cyclin dependent kinase 1, CDK1; Myogenic factor 5, MYF5; Neural cell adhesion molecule 1, NCAM1; Myogenin, MYOG; Actin, alpha 2, smooth muscle, ACTA2; Sphingosine kinase 1, SPHK1; Platelet derived growth factor receptor alpha, PDGFRA; Myostatin, MSTN). **b**) Cell subpopulation sizes at each time point. **c**) Immunofluorescence analysis of HSMM culture in GM or 72h after switch to DM. Cells of mesenchymal origin report high levels of the surface marker CD13 (green) and mitotic cells are enriched in the nuclear phosphorylated H3-Ser10 (red). Hoechst DNA staining highlights all nuclei (blue). Scale bar corresponds to 100 uM. In GM, proliferating cells are phospo-H3+ and either CD13+ and CD13- (see magnification below) indicating that both mesenchymal cells (CD13+) and non-mesenchymal cells (myoblasts, CD13-) are actively proliferating. In DM all phospo-H3+ cells are also CD13+ suggesting that at this stage fibroblasts are the only proliferating cells. **d**) Bar plots showing the proportion of fibroblasts along the differentiation trajectory measured by expression of CD13 by immunofluorescence (left panel) and RNA-seq (right panel). **e**) Bar plots showing the total proliferating cells with respect to proliferating mesenchymal cells measured by immunofluorescence (left panel - phospho-H3 positive cells with respect to double positive CD13/phospho-H3 cells) and RNA-seq (right panel - CDK1+ cells with respect to CDK1+/CD13+ cells).



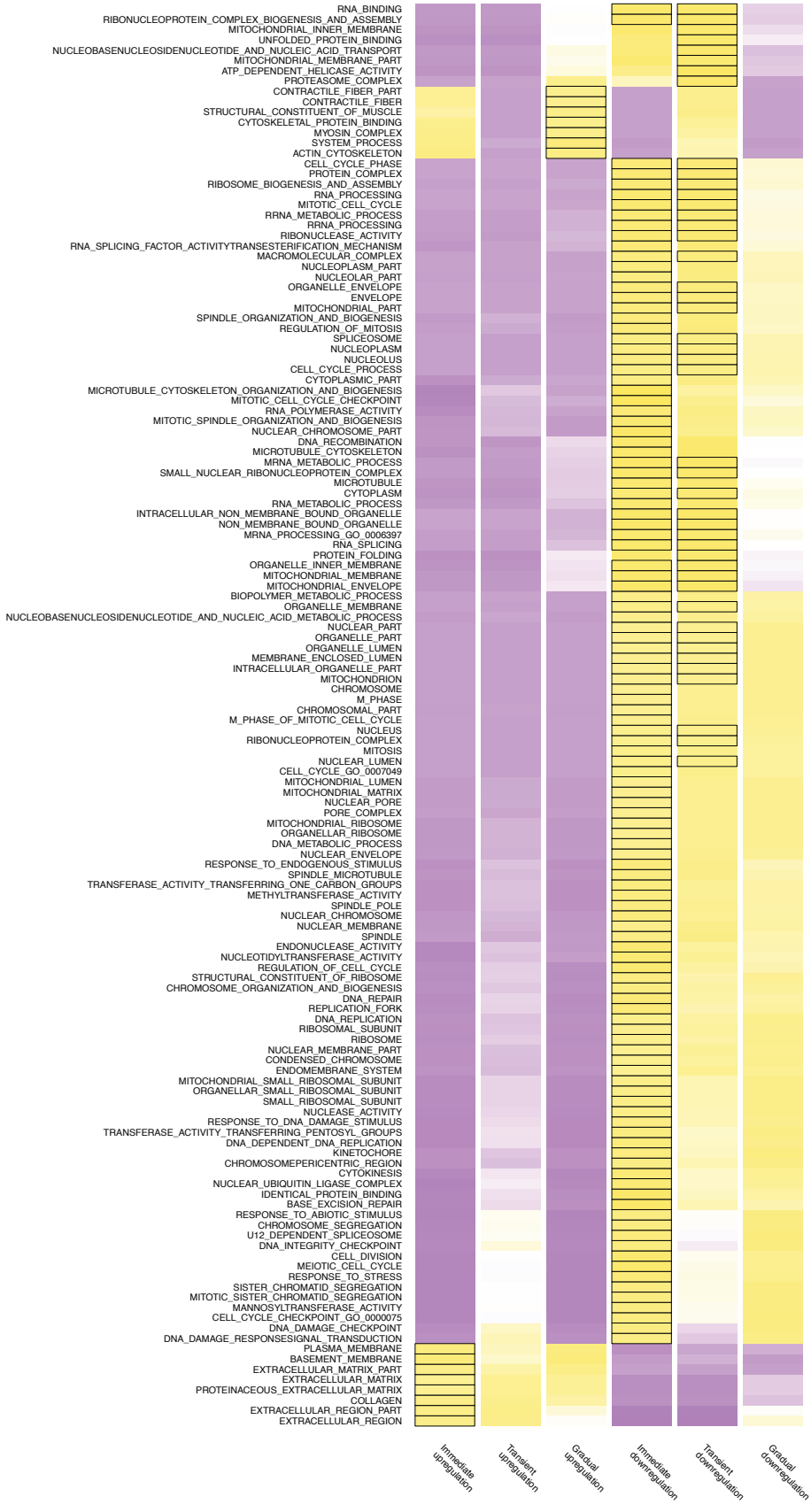
Supplementary Figure 5. Markers of myogenic differentiation in pseudo-time reordered cells. (Cyclin A2, CCNA2; Cyclin B2, CCNB2; Minichromosome maintenance 4, MCM4; Muscle creatine kinase, CKM; Enolase 3, ENO3; Myosin heavy chain 3, MYH3; Myogenin, MYOG; Troponin type 3, TNNT3; Tropomyosin 1, TPM1; Hairy/enhancer-of-split 1, HES1; Myocyte enhancer factor 2 C, MEF2C; Myogenic factor 5, MYF5)



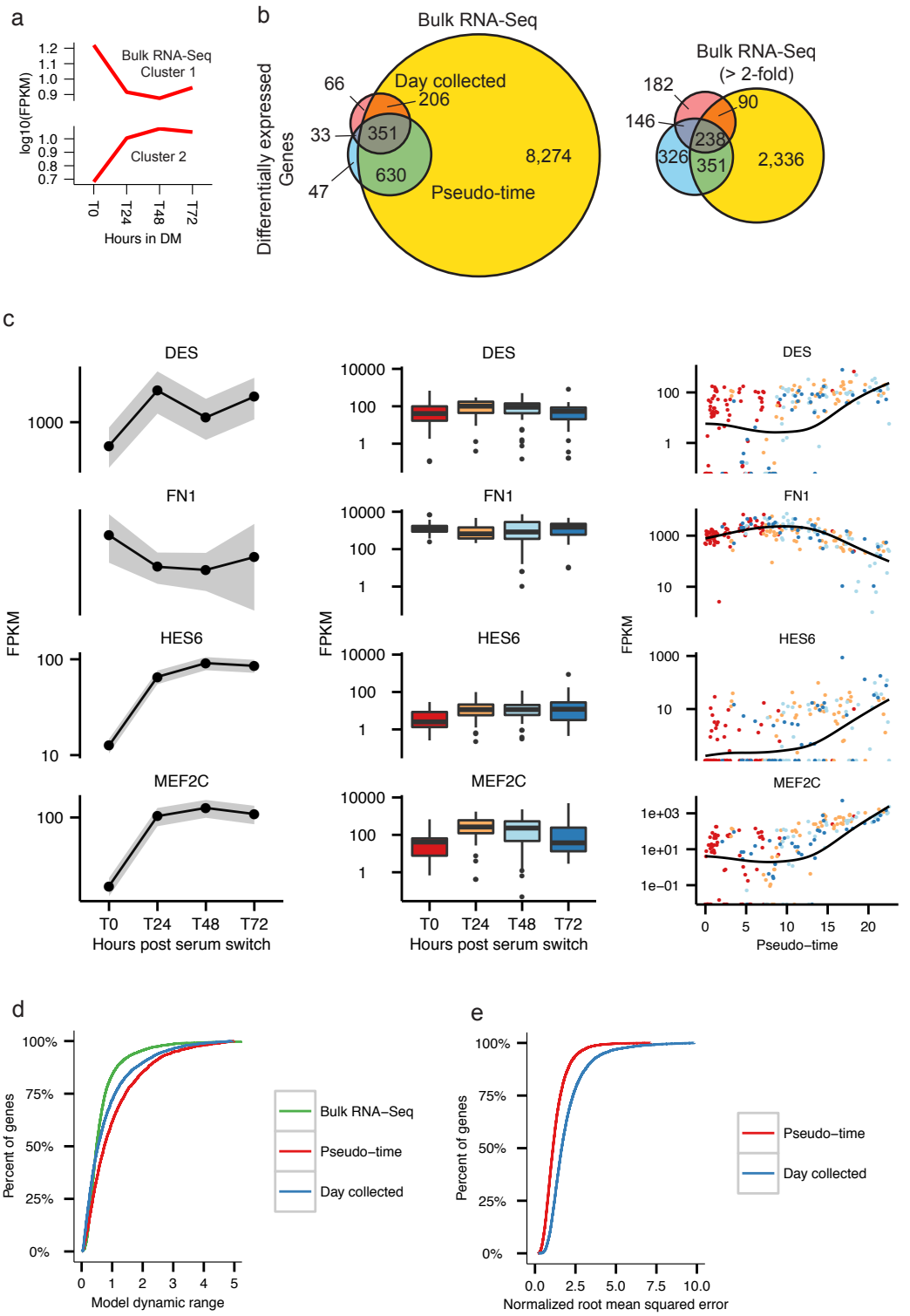
Supplementary Figure 6. Conservation of gene expression dynamics for key molecular markers of myogenesis. Each heatmap shows a block of genes previously reported by Blais et al to be targets of Myod, Myog, or a member of the Mef2 family of transcription factors. The change in expression between C2C12 mouse myoblasts and their derived myotubes is shown in the right column for each block. The heatmap shows the human expression level for each gene in each cell, with columns corresponding to cells placed in pseudo-time order. The statistical significance of pseudo-time dependent changes in expression (as reported by a likelihood ratio test, see Methods) are reported as log-transformed p-values in the middle column. Genes are grouped according to their function and discussion in the study by Blais et al.



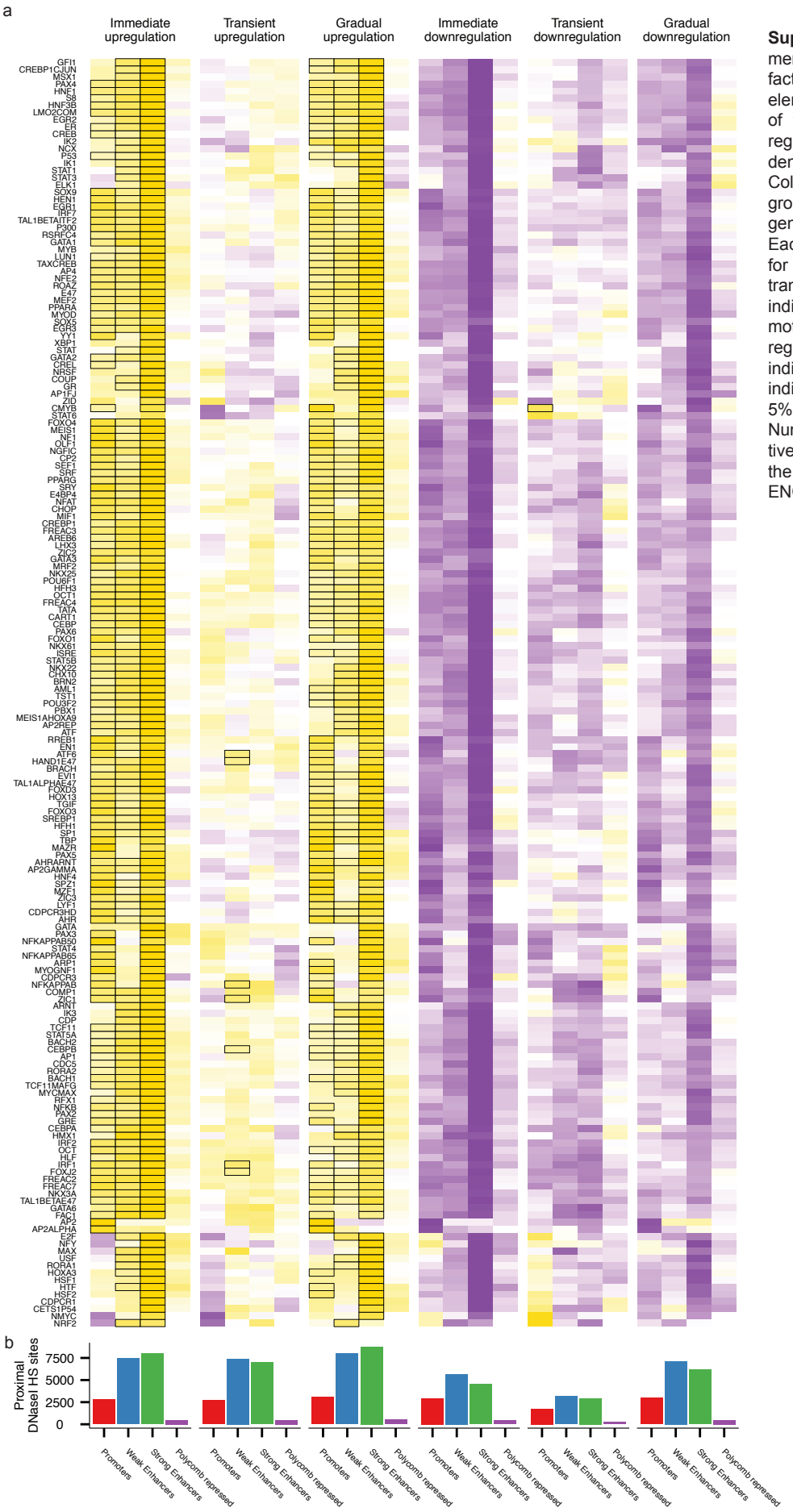
Supplementary Figure 7. Robustness of Monocle as assessed by cross-validation simulation experiments. Randomly sampled subsets of cells were ordered with Monocle as described in the Supplemental Methods. In each panel, the x axis displays the number of cells in the subsets used for cross-validation. Each colored point corresponds to a distinct randomly selected subset, and the colored lines indicate the mean value of the mertric on the vertical axis. **a**) Consistency of pseudotime ordering. The vertical axis shows correlation between the pseudotime values of cells ordered as a subset and ordered as part of the full data set. **b**) Consistency of the trend of expression of genes from Supplemental Figure 6. The vertical axis shows the average correlation between the genes under subset orderings and their expression values ordered as part of the full data set. **c**) Precision of dynamically regulated genes under subset ordering (precision = # true positives / (# true positives + # false positives). **d**) Recall of dynamically regulated genes under subset ordering (recall = # true positives / (# true positives + # false negatives). True positives and true negatives were defined as genes that marked significantly dynamically regulated or not significantly regulated (respectively) by Monocle on the full data set.



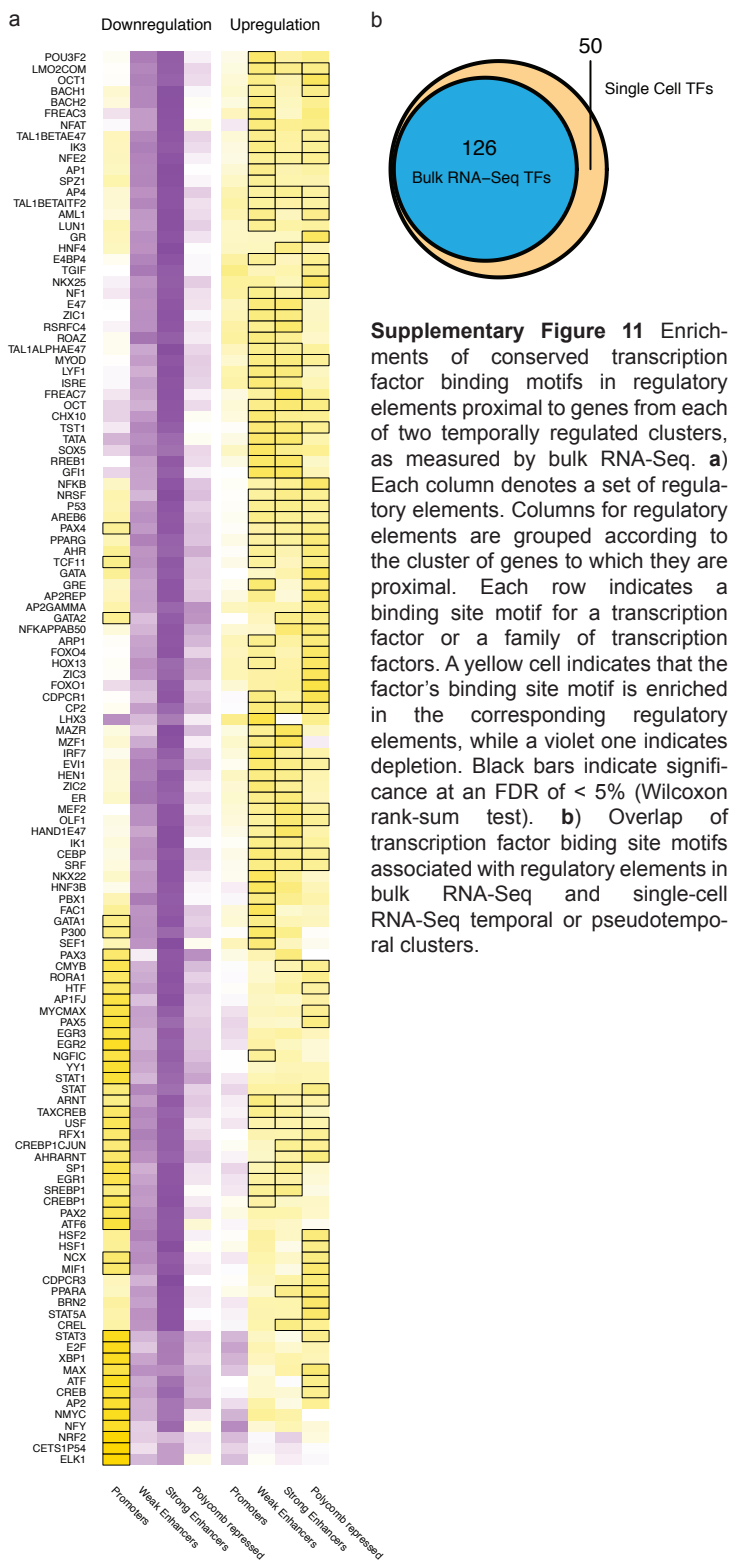
Supplementary Figure 8. Gene ontology enrichments for each distinct cluster of (pseudo) temporally regulated genes. Black boxes indidicate statistical signficance at an FDR < 5% (Wilcoxon rank-sum test).



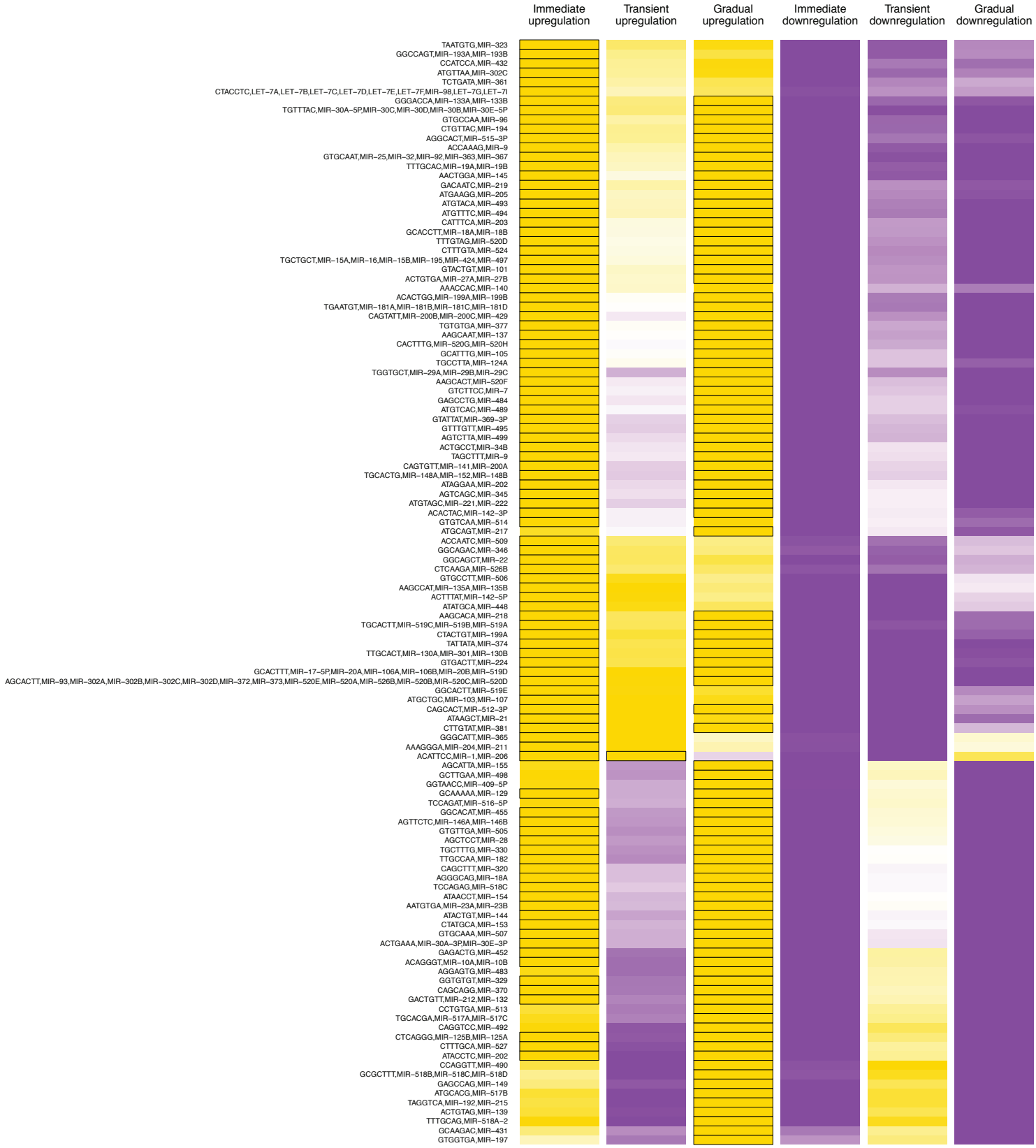
Supplementary Figure 9. Bulk RNA-Seq and single-cell data without reordering compresses the dynamic range of expression. **a**) K-means of bulk RNA-Seq produces two non-redundant trends in gene expression. **b**) DE genes identified by Cuffdiff 2 between any pair of time points using bulk RNA-Seq, compared against DE genes found by Monocle using single cell RNA-Seq. The right venn diagram shows overlap between genes from single-cell RNA-Seq and those with at least a 2-fold change between some pair of time points in bulk RNA-Seq. **c**) Expression levels of selected marker genes measured by bulk RNA-Seq (left), single-cell RNA-Seq ordered by time collected (middle), and pseudo-time reordered single cell RNA-Seq (right). Grey ribbons in the left panel indicate 95% confidence intervals produced by Cuffdiff 2 as part of its model of variability across biological replicates. Boxplots in the middle panel are colored by time point, and these colors are applied to cells in the right panel, indicating the time at which they were collected. Black lines in the left panel indicate a generalized additive model of pseudo-time vs each gene's expression level. **d**) Cumulative distribution of gene expression dynamic range, defined as the maximum FPKM less the minimum FPKM, by three different sources of expression measurement. **e**) Cumulative distributions of normalized root mean squared error (RMSE) of generalized additive models of expression for both unordered and pseudo-time ordered single cell expression profiles



Supplementary Figure 10. Enrichments of conserved transcription factor binding motifs in regulatory elements proximal to genes from each of four distinct (pseudo) temporally regulated clusters. **a**) Each column denotes a set of regulatory elements. Columns for regulatory elements are grouped according to the cluster of genes to which they are proximal. Each row indicates a binding site motif for a transcription factor or a family of transcription factors. A yellow cell indicates that the factor's binding site motif is enriched in the corresponding regulatory elements, while a violet one indicates depletion. Black bars indicate significance at an FDR of < 5% (Wilcoxon rank-sum test). **b**) Number of HSMM DNase hypersensitive sites proximal to genes in each of the six clusters, as classified by ENCODE according to function.

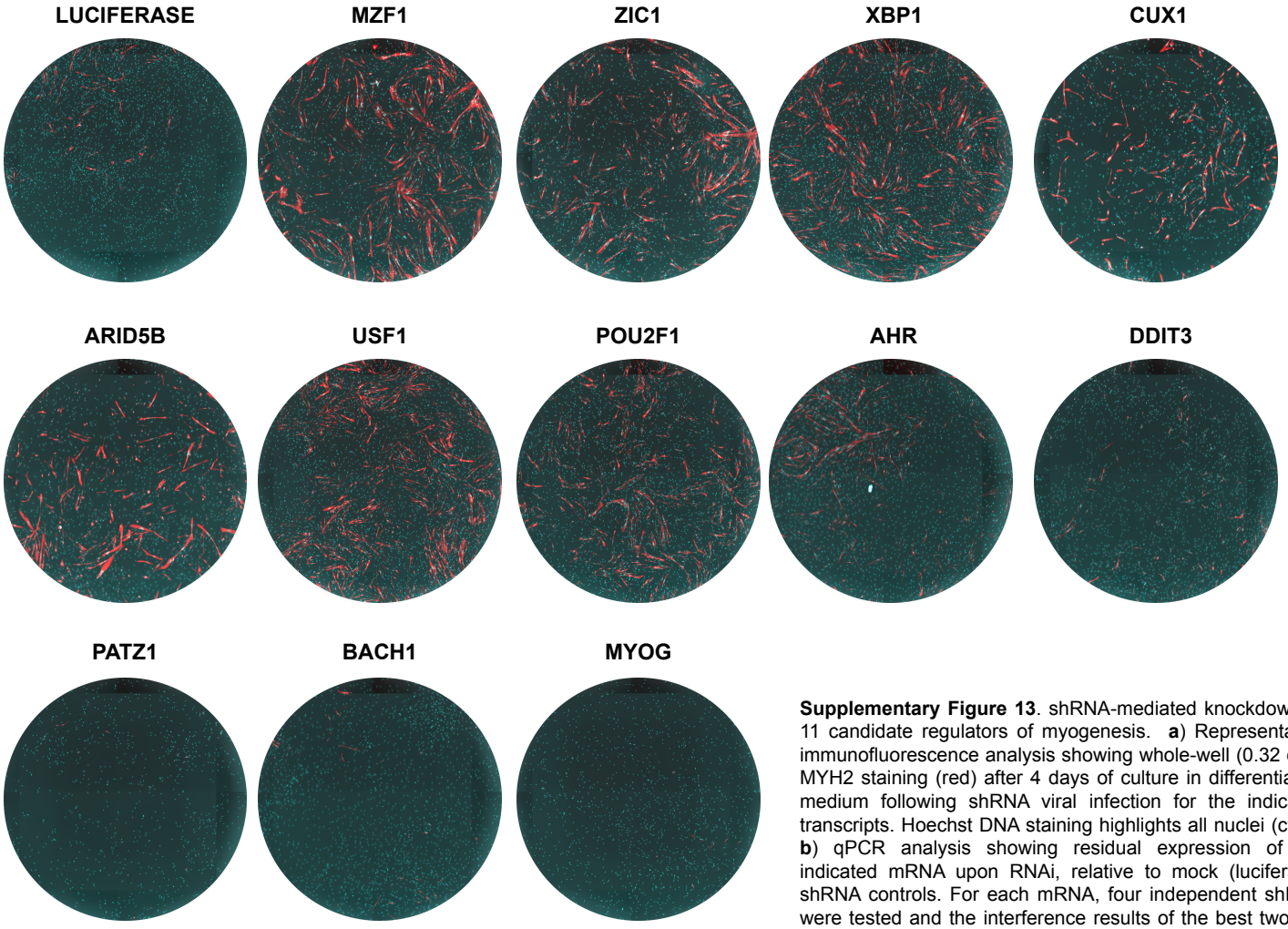


Supplementary Figure 11 Enrichments of conserved transcription factor binding motifs in regulatory elements proximal to genes from each of two temporally regulated clusters, as measured by bulk RNA-Seq. **a**) Each column denotes a set of regulatory elements. Columns for regulatory elements are grouped according to the cluster of genes to which they are proximal. Each row indicates a binding site motif for a transcription factor or a family of transcription factors. A yellow cell indicates that the factor's binding site motif is enriched in the corresponding regulatory elements, while a violet one indicates depletion. Black bars indicate significance at an FDR of < 5% (Wilcoxon rank-sum test). **b**) Overlap of transcription factor biding site motifs associated with regulatory elements in bulk RNA-Seq and single-cell RNA-Seq temporal or pseudotemporal clusters.



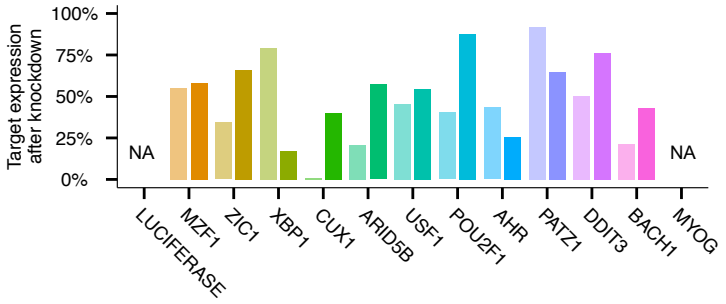
Supplementary Figure 12. Enrichments of microRNA family target sites in genes belonging to each cluster. Black bars indicate significance at an FDR of < 5% (Wilcoxon rank-sum test)

a



Supplementary Figure 13. shRNA-mediated knockdown of 11 candidate regulators of myogenesis. **a**) Representative immunofluorescence analysis showing whole-well (0.32 cm^2) MYH2 staining (red) after 4 days of culture in differentiation medium following shRNA viral infection for the indicated transcripts. Hoechst DNA staining highlights all nuclei (cyan) **b**) qPCR analysis showing residual expression of the indicated mRNA upon RNAi, relative to mock (luciferase) shRNA controls. For each mRNA, four independent shRNA were tested and the interference results of the best two are reported.

b



Supplementary Tables

Table qPCR primers used to verify knockdown efficiency

Gene	Target transcript	Forward primer	Reverse primer
AHR	NM_001621.2	ACAACCACATAGTTCGTTTACCT	AGGACAGTAAAGTTGGTAGGGTG
ARID5B	NM_032199.1	CGATGCTGAAACGCATCCAG	AGGATCTGAGGGTTCCTGCT
BACH1	NM_001186.2	GCCTCAGCTCTGGTTGATGATA	TGTCGGGAAGTTCAGTGGAAA
CUX1	NM_001913.2	TCTCATCGGCCAATCACTCC	CTCTATGGCCTGCTCCACG
DDIT3	NM_004083.4	TCAGAGCTGGAACCTGAGGA	GTCCCGAAGGAGAAAGGCAA
HIVEP2	NM_006734.3	TCCTCACGCTTTGCAGTCAT	GCTCTGTTGCGTTTAGGCTG
MYOG	NM_002479.4	TAAACGCCTTGATGTGCAGC	GCTGGGTGCCATTTAAACCC
MZF1	NM_003422.2	CCGTAGAGAAGGGCAGACAC	GCCTCATAGAGGGTACCAGG
PATZ1	NM_014323.2	TACTTGCGGGCAGCATACAT	AGAGGAGAAACCTCGGTTACAG
POU2F1	NM_002697.2	CTGATTCGTCCCTCTCCAGC	CTGGTGCGTTTCTTCCTCCT
RREB1	NM_002955.4	ATCTGCCCCATGACTAAGGC	CACCACTCCTGGAACACACA
USF1	NM_007122.3	TTGTGCTCCTCTCGCACAAT	CAGGACAAGCCCCAGAGTTT
XBP1	NM_005080.3	AAGCCAAGGGGAATGAAGTGA	AGAGGTGCACGTAGTCTGAG
ZIC1	NM_003412.3	CGCAAACACATGAAGGTCCA	CGGCGAAGGCTGCGA

Table shRNA hairpin sequences used for knockdown

Gene	Clone ID	shRNA	Vector	Forward Oligo Sequence	Reverse Oligo Sequence
AHR	TRCN0000245286	1	pLKO_TRC005	CCGGATCCACAGTCAGCCATAATAACTCGAGTTATTATGGCTGACTGTGGATTTTTTG	AATTCAAAAAATCCACAGTCAGCCATAATAACTCGAGTTATTATGGCTGACTGTGGAT
AHR	TRCN0000021258	2	pLKO.1	CCGGCGGCATAGAGACCGACTTAATCTCGAGATTAAGTCGGTCTCTATGCCGTTTTTG	AATTCAAAAACGGCATAGAGACCGACTTAATCTCGAGATTAAGTCGGTCTCTATGCCG
AHR	TRCN0000245283	3	pLKO_TRC005	CCGGGCGGCATAGAGACCGACTTAACTCGAGTTAAGTCGGTCTCTATGCCGCTTTTTG	AATTCAAAAAGCGGCATAGAGACCGACTTAACTCGAGTTAAGTCGGTCTCTATGCCGC
AHR	TRCN0000245285	4	pLKO_TRC005	CCGGGCAACAAGATGAGTCTATTTACTCGAGTAAATAGACTCATCTTGTTGCTTTTTG	AATTCAAAAAGCAACAAGATGAGTCTATTTACTCGAGTAAATAGACTCATCTTGTTGC
ARID5B	TRCN0000365579	1	pLKO_TRC005	CCGGTACCCTTTAGCTGCTATAAATCTCGAGATTTATAGCAGCTAAAGGGTATTTTTG	AATTCAAAAATACCCTTTAGCTGCTATAAATCTCGAGATTTATAGCAGCTAAAGGGTA
ARID5B	TRCN0000370799	2	pLKO_TRC005	CCGGATAGAGTTAGAAGTCAGTATTCTCGAGAATACTGACTTCTAACTCTATTTTTTG	AATTCAAAAAATAGAGTTAGAAGTCAGTATTCTCGAGAATACTGACTTCTAACTCTAT
ARID5B	TRCN0000365578	3	pLKO_TRC005	CCGGATAGAACGAATACCCTATTTACTCGAGTAAATAGGGTATTCGTTCTATTTTTTG	AATTCAAAAAATAGAACGAATACCCTATTTACTCGAGTAAATAGGGTATTCGTTCTAT
ARID5B	TRCN0000370859	4	pLKO_TRC005	CCGGTGCGATGAGTTTGCGCCAAATCTCGAGATTTGGCGCAAACTCATCGCATTTTTG	AATTCAAAAATGCGATGAGTTTGCGCCAAATCTCGAGATTTGGCGCAAACTCATCGCA

BACH1	TRCN0000433926	1	pLKO_TRC005	CCGGGCATATCAGACAGCAATTTAACTCGAGTTAAATTGCTGTCTGATATGCTTTTTG	AATTCAAAAAGCATATCAGACAGCAATTTAACTCGAGTTAAATTGCTGTCTGATATGC
BACH1	TRCN0000430446	2	pLKO_TRC005	CCGGGAAATTGGAAACGATGATTATCTCGAGATAATCATCGTTTCCAATTTCTTTTTG	AATTCAAAAAGAAATTGGAAACGATGATTATCTCGAGATAATCATCGTTTCCAATTTC
BACH1	TRCN0000416033	3	pLKO_TRC005	CCGGAGCGTCTTGAAAGCCTAATATCTCGAGATATTAGGCTTTCAAGACGCTTTTTTG	AATTCAAAAAAGCGTCTTGAAAGCCTAATATCTCGAGATATTAGGCTTTCAAGACGCT
CUX1	TRCN0000013749	1	pLKO.1	CCGGGCCGACAACATCAAGCTCTTTCTCGAGAAAGAGCTTGATGTTGTCGGCTTTTTG	AATTCAAAAAGCCGACAACATCAAGCTCTTTCTCGAGAAAGAGCTTGATGTTGTCGGC
CUX1	TRCN0000013751	2	pLKO.1	CCGGCGGCTTCTTCTACACACTGTTCTCGAGAACAGTGTGTAGAAGAAGCCGTTTTTG	AATTCAAAAACGGCTTCTTCTACACACTGTTCTCGAGAACAGTGTGTAGAAGAAGCCG
CUX1	TRCN0000428064	3	pLKO_TRC005	CCGGACCGATTCCAGCTGCGGTTAACTCGAGTTAACCGCAGCTGGAATCGGTTTTTTG	AATTCAAAAAACCGATTCCAGCTGCGGTTAACTCGAGTTAACCGCAGCTGGAATCGGT
CUX1	TRCN0000413385	4	pLKO_TRC005	CCGGAGATCCCAGAGCCCATCAAAGCTCGAGCTTTGATGGGCTCTGGGATCTTTTTTG	AATTCAAAAAAGATCCCAGAGCCCATCAAAGCTCGAGCTTTGATGGGCTCTGGGATCT
DDIT3	TRCN0000364393	1	pLKO_TRC005	CCGGTGAACGGCTCAAGCAGGAAATCTCGAGATTTCCTGCTTGAGCCGTTCATTTTTG	AATTCAAAAATGAACGGCTCAAGCAGGAAATCTCGAGATTTCCTGCTTGAGCCGTTCA
DDIT3	TRCN0000364328	2	pLKO_TRC005	CCGGCTGCACCAAGCATGAACAATTCTCGAGAATTGTTCATGCTTGGTGCAGTTTTTG	AATTCAAAAACTGCACCAAGCATGAACAATTCTCGAGAATTGTTCATGCTTGGTGCAG
DDIT3	TRCN0000368985	3	pLKO_TRC005	CCGGAGGTCCTGTCTTCAGATGAAACTCGAGTTTCATCTGAAGACAGGACCTTTTTTG	AATTCAAAAAAGGTCCTGTCTTCAGATGAAACTCGAGTTTCATCTGAAGACAGGACCT
DDIT3	TRCN0000368983	4	pLKO_TRC005	CCGGAGAGCCCTCACTCTCCAGATTCTCGAGAATCTGGAGAGTGAGGGCTCTTTTTTG	AATTCAAAAAAGAGCCCTCACTCTCCAGATTCTCGAGAATCTGGAGAGTGAGGGCTCT
MYOG	TRCN0000430479	1	pLKO_TRC005	CCGGTGGCCACAGATGCCACTACTTCTCGAGAAGTAGTGGCATCTGTGGCCATTTTTG	AATTCAAAAATGGCCACAGATGCCACTACTTCTCGAGAAGTAGTGGCATCTGTGGCCA
MYOG	TRCN0000426530	2	pLKO_TRC005	CCGGCATTCAGCTGCCTCCTTAGAGCTCGAGCTCTAAGGAGGCAGCTGAATGTTTTTG	AATTCAAAAACATTCAGCTGCCTCCTTAGAGCTCGAGCTCTAAGGAGGCAGCTGAATG
MZF1	TRCN0000329828	1	pLKO_TRC005	CCGGACCAGAGCACCAAGCTCATTCCTCGAGGAATGAGCTTGGTGCTCTGGTTTTTTG	AATTCAAAAAACCAGAGCACCAAGCTCATTCCTCGAGGAATGAGCTTGGTGCTCTGGT
MZF1	TRCN0000329903	2	pLKO_TRC005	CCGGCCGCAGGTCCAGGTAGTGTAACTCGAGTTACACTACCTGGACCTGCGGTTTTTG	AATTCAAAAACCGCAGGTCCAGGTAGTGTAACTCGAGTTACACTACCTGGACCTGCGG
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PATZ1	TRCN0000274379	1	pLKO_TRC005	CCGGTGATCACTTGAACGGACATATCTCGAGATATGT	AATTCAAAAATGATCACTTGAACGGACATATCTCG

				CCGTTCAAGTGATCATTTTTG	AGATATGTCCGTTCAAGTGATCA
PATZ1	TRCN0000274414	2	pLKO_TRC005	CCGGACTATCAGCTCCAAGGTATTTCTCGAGAAATACCTTGGAGCTGATAGTTTTTG	AATTCAAAAAACTATCAGCTCCAAGGTATTTCTCGAGAAATACCTTGGAGCTGATAGT
PATZ1	TRCN0000274416	3	pLKO_TRC005	CCGGCGAGTACTTTGAGTCGGTGTTCTCGAGAACACCGACTCAAAGTACTCGTTTTTG	AATTCAAAAACGAGTACTTTGAGTCGGTGTTCTCGAGAACACCGACTCAAAGTACTCG
PATZ1	TRCN0000274417	4	pLKO_TRC005	CCGGAGATTGTTCAGTCGGCATTTGCTCGAGCAAATGCCGACTGAACAATCTTTTTTG	AATTCAAAAAAGATTGTTCAGTCGGCATTTGCTCGAGCAAATGCCGACTGAACAATCT
POU2F1	TRCN0000232119	1	pLKO_TRC005	CCGGACAACACAGCAACCGTGATTTCTCGAGAAATCACGGTTGCTGTGTTGTTTTTTG	AATTCAAAAAACAACACAGCAACCGTGATTTCTCGAGAAATCACGGTTGCTGTGTTGT
POU2F1	TRCN0000232120	2	pLKO_TRC005	CCGGGCAACTGGGAACCTGGTATTTCTCGAGAAATACCAGGTTCCCAGTTGCTTTTTG	AATTCAAAAAGCAACTGGGAACCTGGTATTTCTCGAGAAATACCAGGTTCCCAGTTGC
POU2F1	TRCN0000232121	3	pLKO_TRC005	CCGGTTTCACTCTGCAGTGTGATTGCTCGAGCAATCACACTGCAGAGTGAAATTTTTG	AATTCAAAAATTTCACTCTGCAGTGTGATTGCTCGAGCAATCACACTGCAGAGTGAAA
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USF1	TRCN0000233478	4	pLKO_TRC005	CCGGCAACAGGTGGAAGATCTTAAACTCGAGTTTAAGATCTTCCACCTGTTGTTTTTG	AATTCAAAAACAACAGGTGGAAGATCTTAAACTCGAGTTTAAGATCTTCCACCTGTTG
XBP1	TRCN0000277990	1	pLKO_TRC005	CCGGGAACAGCAAGTGGTAGATTTACTCGAGTAAATCTACCACTTGCTGTTCTTTTTG	AATTCAAAAAGAACAGCAAGTGGTAGATTTACTCGAGTAAATCTACCACTTGCTGTTC
XBP1	TRCN0000278051	2	pLKO_TRC005	CCGGGCCTGTCTGTACTTCATTCAACTCGAGTTGAATGAAGTACAGACAGGCTTTTTG	AATTCAAAAAGCCTGTCTGTACTTCATTCAACTCGAGTTGAATGAAGTACAGACAGGC
XBP1	TRCN0000278050	3	pLKO_TRC005	CCGGGACCCAGTCATGTTCTTCAAACTCGAGTTTGAAGAACATGACTGGGTCTTTTTG	AATTCAAAAAGACCCAGTCATGTTCTTCAAACTCGAGTTTGAAGAACATGACTGGGTC
XBP1	TRCN0000277989	4	pLKO_TRC005	CCGGAGATCGAAAGAAGGCTCGAATCTCGAGATTCGAGCCTTCTTTCGATCTTTTTTG	AATTCAAAAAAGATCGAAAGAAGGCTCGAATCTCGAGATTCGAGCCTTCTTTCGATCT
ZIC1	TRCN0000421779	1	pLKO_TRC005	CCGGGGCCAGTTTCTTGTCGAATTGCTCGAGCAATTCGACAAGAAACTGGCCTTTTTG	AATTCAAAAAGGCCAGTTTCTTGTCGAATTGCTCGAGCAATTCGACAAGAAACTGGCC
ZIC1	TRCN0000418658	2	pLKO_TRC005	CCGGCAATAGACCCAGGACGAGTAACTCGAGTTACTCGTCCTGGGTCTATTGTTTTTG	AATTCAAAAACAATAGACCCAGGACGAGTAACTCGAGTTACTCGTCCTGGGTCTATTG

ZIC1	TRCN0000435544	3	pLKO_TRC005	CCGGGACTGATCACACAC GTATACACTCGAGTGTATA CGTGTGTGATCAGTCTTT TTG	AATTCAAAAAGACTGAT CACACACGTATACACTC GAGTGTATACGTGTGTG ATCAGTC