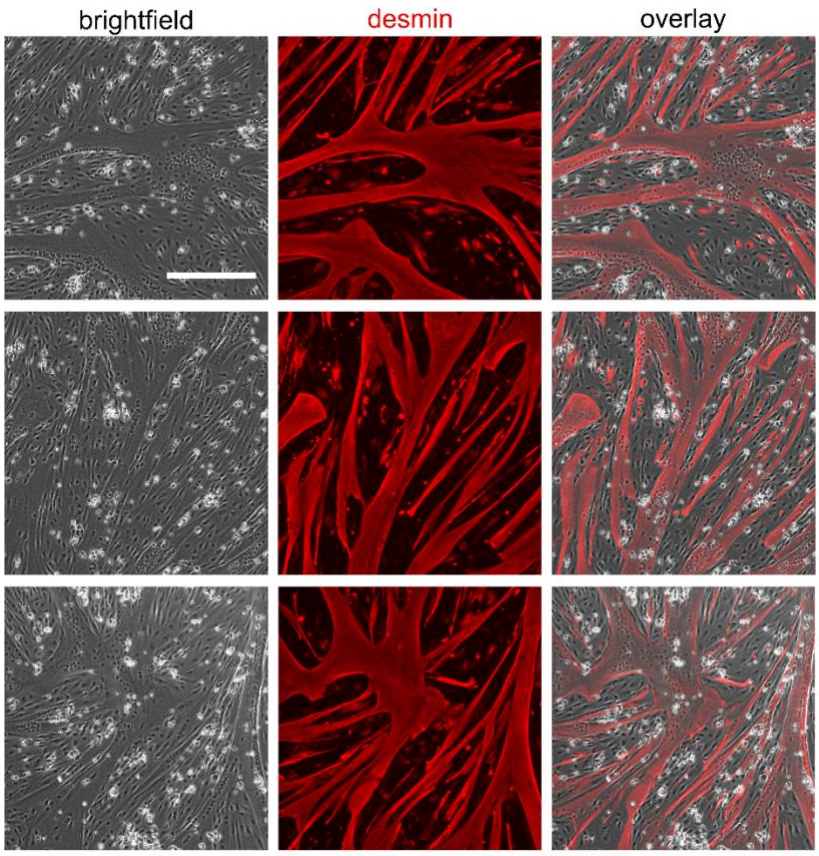
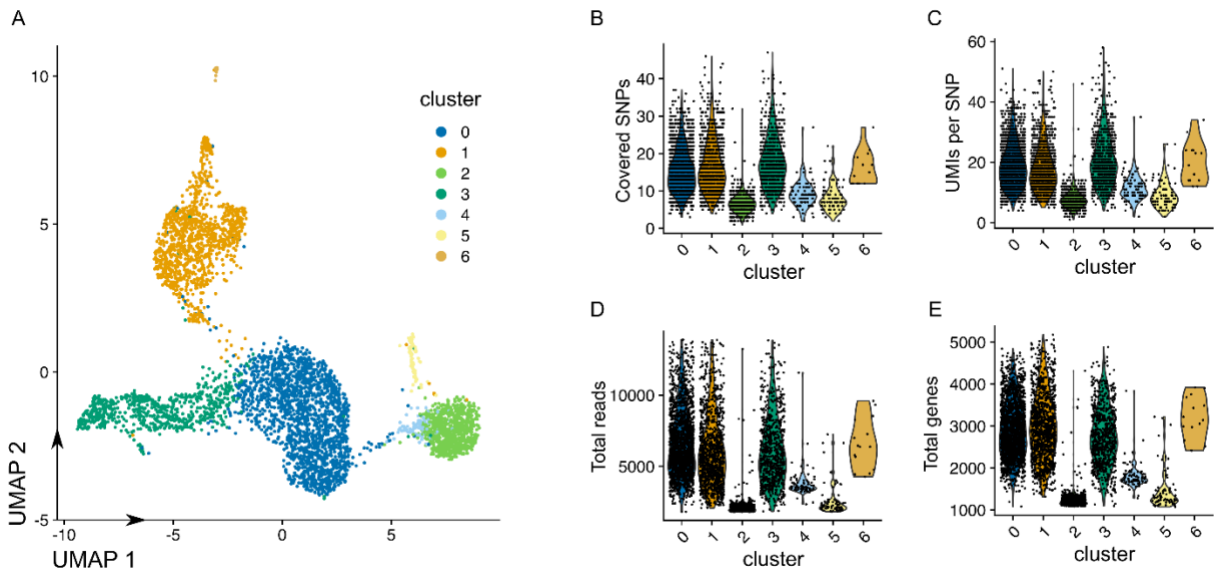


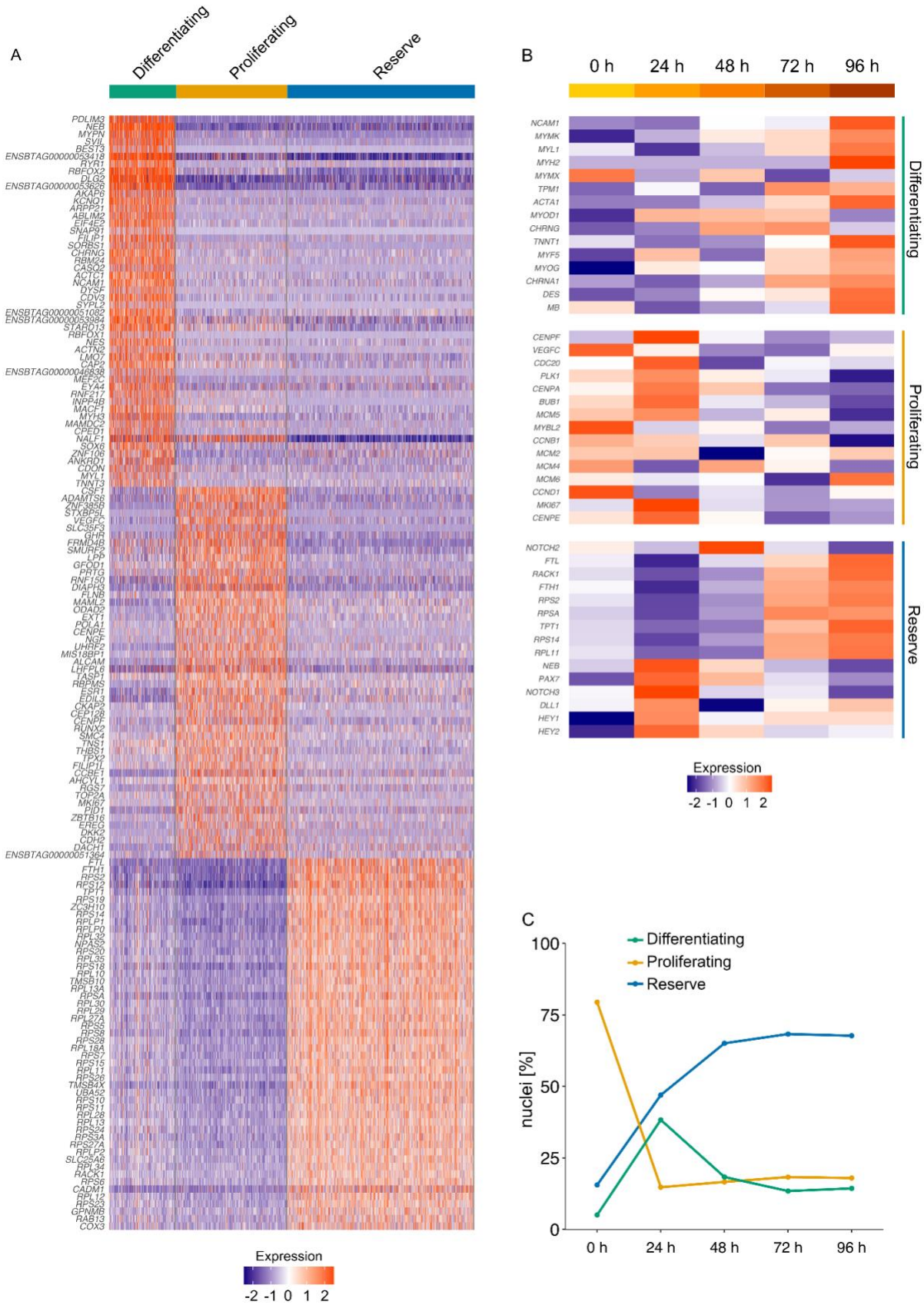
Supplementary Figure 1: Desmin colocalizes with fused myotubes (related to Figure 1)

Overlay of brightfield and desmin-stained immunofluorescence microscopy images. Scale bar, 300 µm.



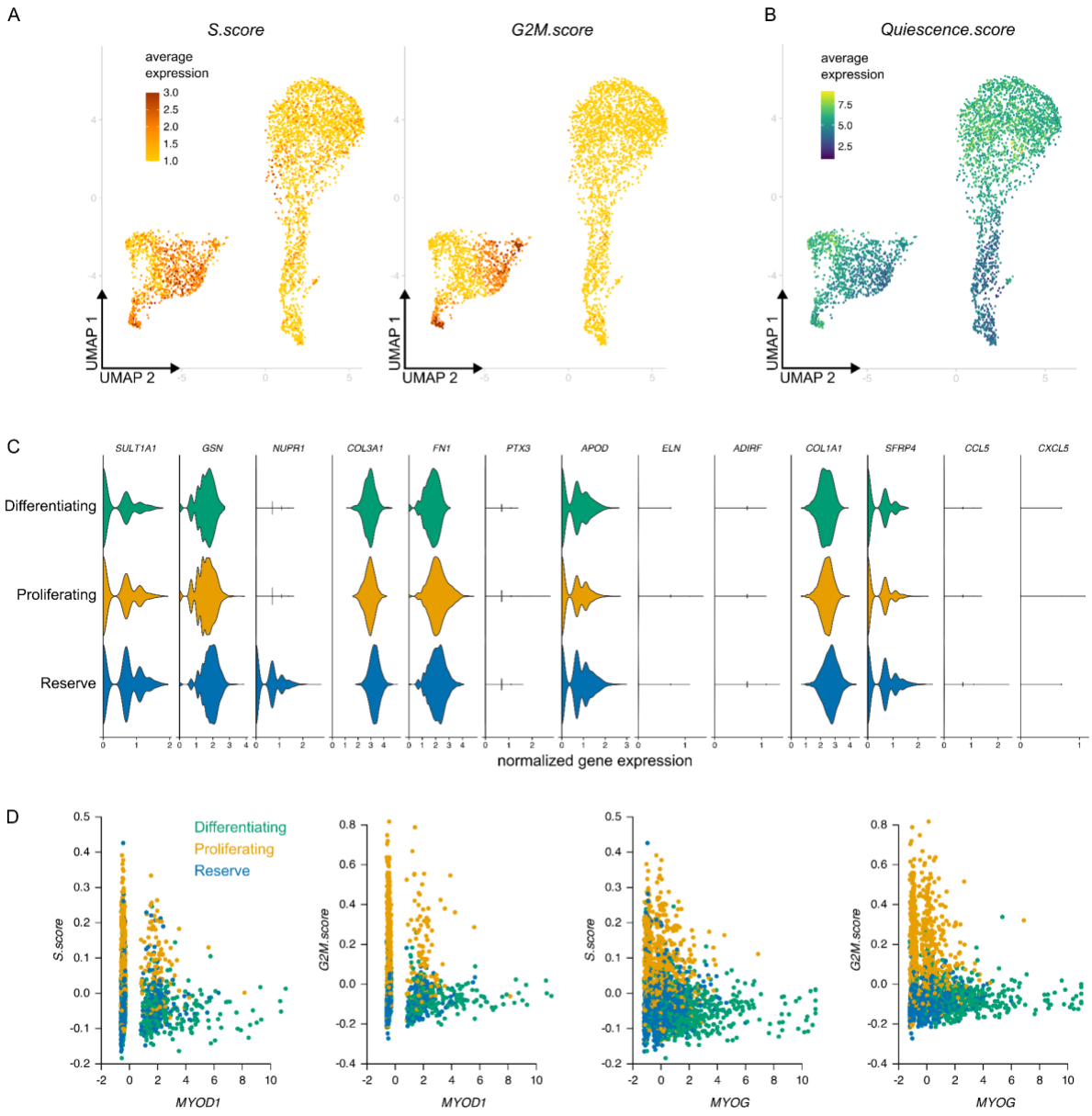
Supplementary Figure 2: Single-nuclei RNA-sequencing quality control (related to Figure 1)

A: UMAP of nuclei from all five time points prior to quality control, where nuclei are coloured according to clusters identified through shared nearest neighbour (SNN) modularity optimisation.
B: Number of single-nucleotide polymorphisms (SNPs) identified per nucleus, which were used to assign genetic background, where nuclei are grouped and coloured by identified clusters.
C: Average number of unique molecular identifiers (UMIs) overlapping a SNP per nucleus for each cluster.
D: Total number of counts (reads) per nucleus for each cluster.
E: Total number of features (genes) per nucleus for each cluster.


A
Differentiating
Proliferating
Reserve
Expression
-2 -1 0 1 2
B
0 h
24 h
48 h
72 h
96 h
NCAM1
MYMK
MYL1
MYH2
MYMX
TPM1
ACTA1
MYOD1
CHRNG
TNNT1
MYF5
MYOG
CHRNA1
DES
MB
Differentiating
CENPF
VEGFC
CDC20
PLK1
CENPA
BUB1
MCM5
MYBL2
CCNB1
MCM2
MCM4
MCM6
CCND1
MKI67
CENPE
Proliferating
NOTCH2
FTL
RACK1
FTH1
RPS2
RPSA
TPT1
RPS14
RPL11
NEB
PAX7
NOTCH3
DLL1
HEY1
HEY2
Reserve
Expression
-2 -1 0 1 2
C
Differentiating
Proliferating
Reserve
nuclei [%]
100
75
50
25
0
0 h
24 h
48 h
72 h
96 h

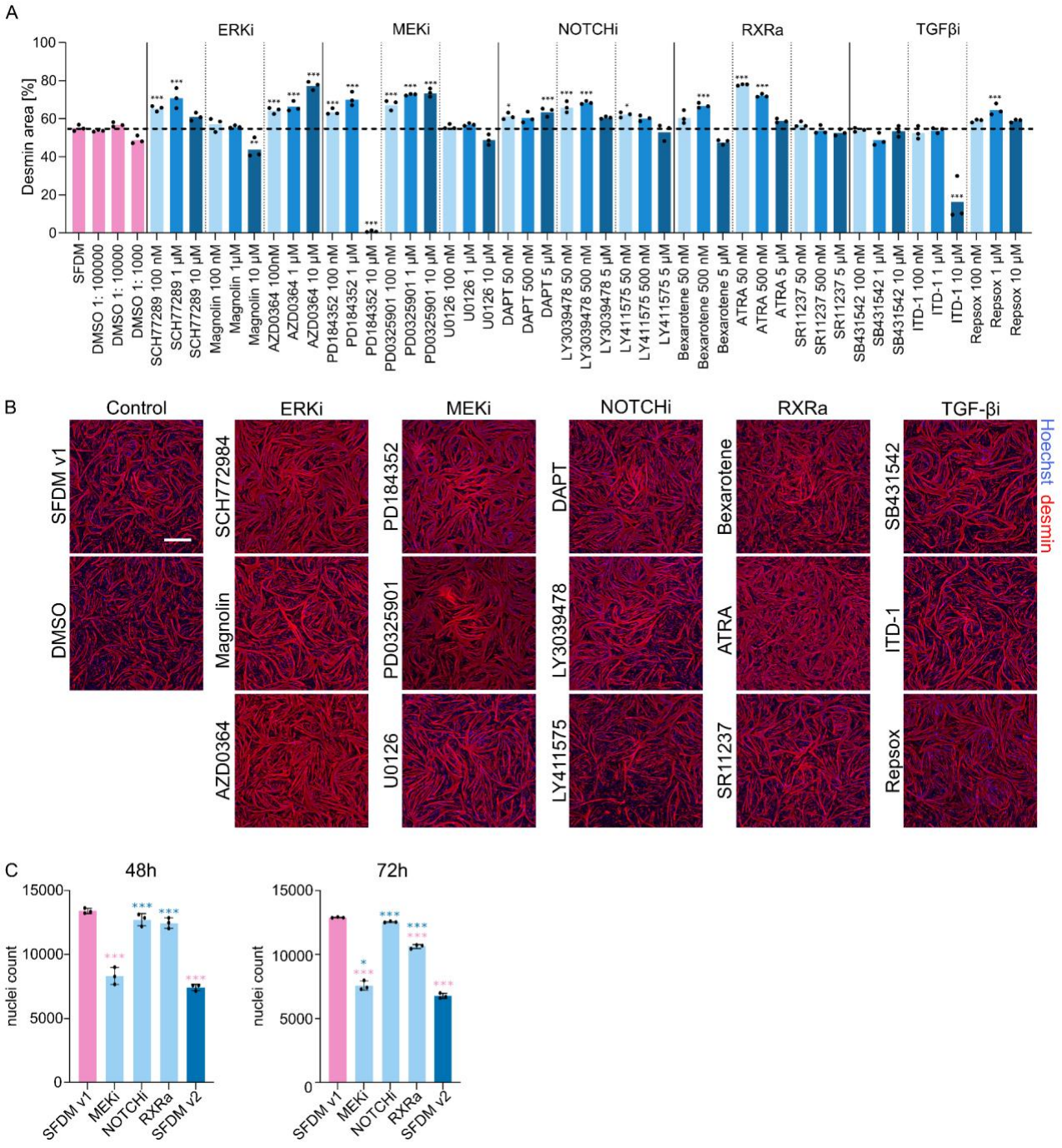
Supplementary Figure 3: Identification and characterisation of SC subpopulations (related to Figure 2)

A: Heatmap showing normalised expression of 50 most significantly upregulated genes per SC subpopulation.
B: Heatmap showing average normalised expression of 15 most differentially expressed genes between timepoints within each subpopulation, sorted by UPGMA clustering.
C: Quantification of subpopulation sizes over all five timepoints of the snRNA-seq dataset.



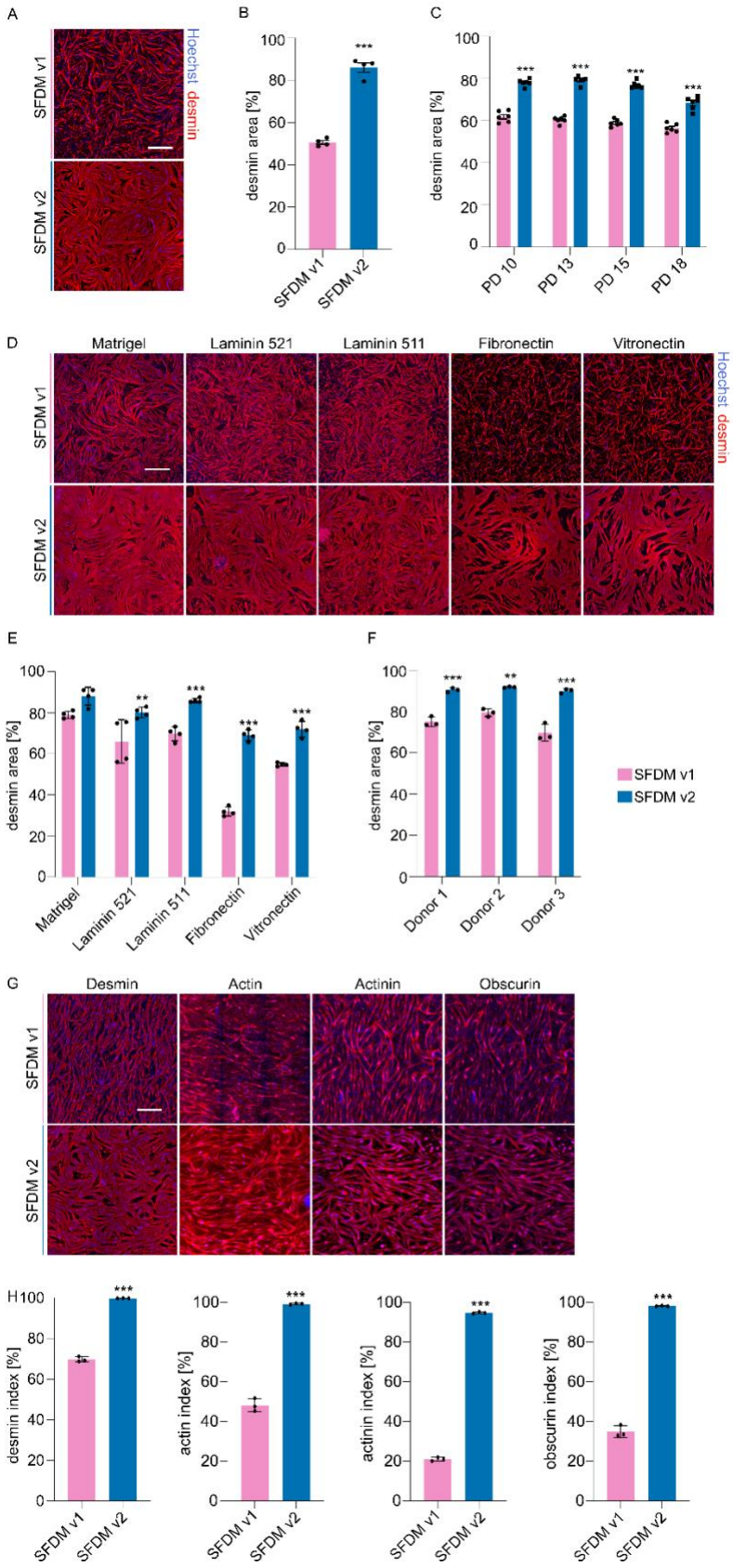
Supplementary Figure 4: Analysis of cell cycle and quiescence in SC subpopulations (related to Figure 2)

A: UMAP of nuclei from all five timepoints, coloured by average expression of cell cycle genes related to S (left) or G2M phase (right), as assigned by the CellCycleScoring() function in Seurat.
B: UMAP as in A, coloured by expression of a 'quiescence signature' score calculated by averaging over all 597 significantly upregulated genes in a quiescent subpopulation of proliferating SCs (Messmer *et al*., 2023).
C: Violin plots showing normalised expression of 15 most upregulated genes in quiescent SCs (Messmer *et al*., 2023), separated and coloured by subpopulation.
D: Scatter plots comparing expression of *MYOD1*/*MYOG* with cell cycle genes in individual nuclei, separated and coloured by subpopulation.



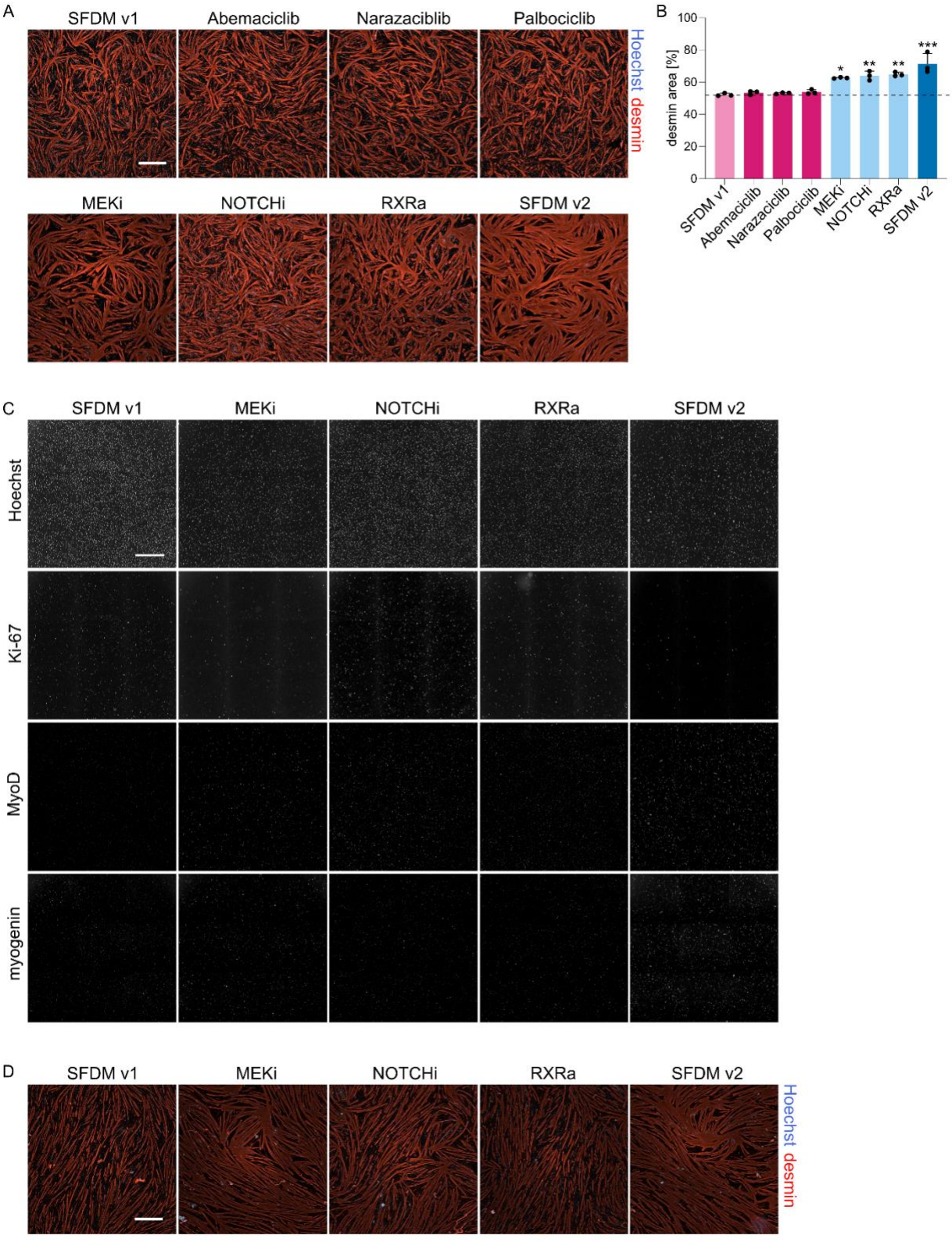
Supplementary Figure 5: Differentiation compound screening (related to Figure 3)

A: Mean desmin areas after 72 h differentiation with indicated compounds (targeting MEK/ERK, RXR, NOTCH and TGF-β pathways) tested at the indicated range of concentrations, n = 3.
B: Representative fluorescence images for compound screening shown in Fig. 3a. Red, desmin; blue, Hoechst. Scale bar, 500 µm.
C: Nuclei counts for the images in Fig. 3C. Error bars show s.d., n = 3.
*p<0.01, **p<0.001, ***p<0.0001.


A
SFDM v1
SFDM v2
Hoechst desmin
B
desmin area [%]
SFDM v1
SFDM v2
C
desmin area [%]
PD 10
PD 13
PD 15
PD 18
D
Matrigel
Laminin 521
Laminin 511
Fibronectin
Vitronectin
Hoechst desmin
E
desmin area [%]
Matrigel
Laminin 521
Laminin 511
Fibronectin
Vitronectin
F
desmin area [%]
Donor 1
Donor 2
Donor 3
SFDM v1
SFDM v2
G
Desmin
Actin
Actinin
Obscurin
H
desmin index [%]
actin index [%]
actinin index [%]
obscurin index [%]

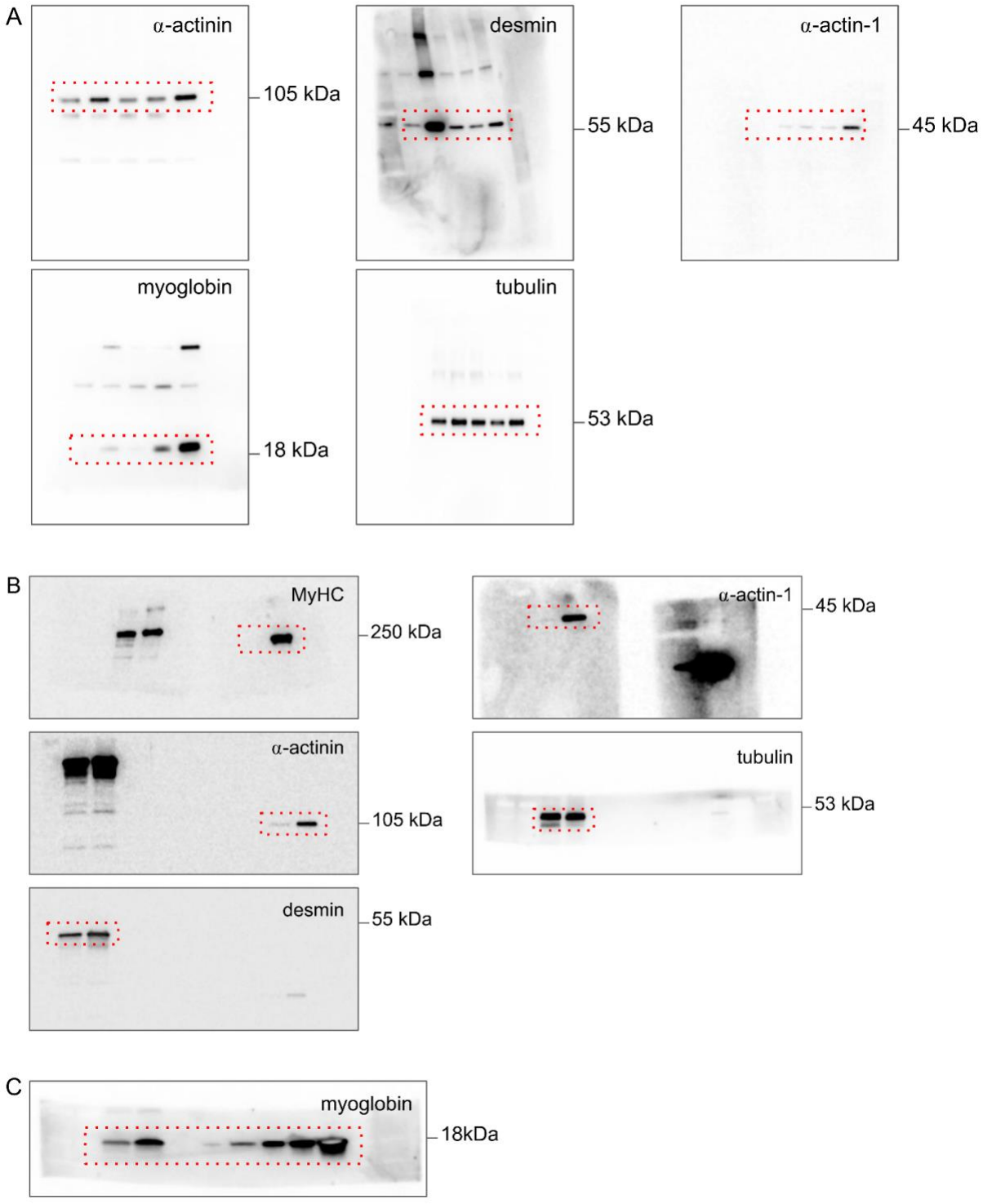
Supplementary Figure 6: Analysis of SFDM v2 for cultured meat bioprocesses (related to Figure 3)

A: Representative fluorescence images after 72 h myogenic differentiation after proliferation in a 40 L stirred-tank bioreactor. Blue, Hoechst; red, desmin. Scale bar, 500 µm.
B: Mean desmin areas for images in A. Error bars indicate s.d., n = 4.
C: Mean desmin areas after 72 h myogenic differentiation for SCs at different PDs. Error bars show s.d., n = 6.
D: Representative fluorescence images after 72 h of myogenic differentiation on indicated extracellular matrix (ECM) protein coatings. Scale bar, 500 µm.
E: Mean desmin areas for images in D. Error bars show s.d., n = 4.
F: Mean desmin areas after 72 h myogenic differentiation for SCs from three different donor cattle. Error bars show s.d., n = 3.
G: Representative fluorescence images stained for non-desmin muscle-related proteins after 72 h of myogenic differentiation.
H: Quantification of the images in G. Error bars show s.d., n = 3.
*p<0.01, **p<0.001, ***p<0.0001.


A
SFDM v1
Abemaciclib
Narazaciblib
Palbociclib
Hoechst desmin
MEKi
NOTCHi
RXRa
SFDM v2
B
desmin area [%]
100
80
60
40
20
0
*
**
**
***
SFDM v1
Abemaciclib
Narazaciclib
Palbociclib
MEKi
NOTCHi
RXRa
SFDM v2
C
SFDM v1
MEKi
NOTCHi
RXRa
SFDM v2
Hoechst
Ki-67
MyoD
myogenin
D
SFDM v1
MEKi
NOTCHi
RXRa
SFDM v2
Hoechst desmin

Supplementary Figure 7: Mechanistic understanding of SFDM v2 components (related to Figure 4)

A: Representative fluorescence images after 72 h myogenic differentiation with the indicated treatments. Blue, Hoechst; red, desmin. Scale bar, 500 µm.
B: Mean desmin areas for images in A. Error bars indicate s.d., n = 3.
C: Representative fluorescence images corresponding to Fig. 4d. Target proteins are indicated next to their respective panels. Scale bar, 500 µm.
D: Representative fluorescence images corresponding to Fig. 4e. Blue, Hoechst; red, desmin. Scale bar, 500 µm.
*p<0.01, **p<0.001, **p<0.0001.



Supplementary Figure 8: Uncropped western blots (related to Figures 3 & 5)

A: For Fig. 3d.
B: For Fig. 5f.
C: For Fig. 5g.

Supplementary Table 1: Medium formulations used in this study

Compound	Supplier	Concentration
Serum free growth medium (SFGM)		
DMEM/F-12	P04-041262B, PAN Biotech	
α-linolenic acid	L2376, Sigma Aldrich	1.0 µg ml^{-1}
bFGF-2	100-18B, Peprotech	10 ng ml^{-1}
Bovine Serum Albumin (BSA)	A9418, Sigma Aldrich	5.0 mg ml^{-1}
bHGF	100-39H, Peprotech	50 ng ml^{-1}
Hydrocortisone	H0888, Sigma Aldrich	0.1 mM
Insulin, Transferrin, Selenium, Ethanolamine (ITSE)	00-101, biogems	1%
GlutaMax	35050061, ThermoFisher	2 mM
D-glucose	G7021, Sigma Aldrich	17.7 mM
L-ascorbic acid 2-phosphate (Vitamin C)	A8960, Sigma Aldrich	155 µM
Penicillin/Streptomycin/Amphotericin (PSA)	17-745E, Lonza	1%
T3	T6397, Sigma Aldrich	20 ng ml^{-1}
Serum free differentiation medium version 1 (SFDM v1)		
DMEM	A14430-01, Gibco	
EGF-1	AF-100-15, Peprotech	10 ng ml^{-1}
D-glucose	G7021, Sigma	5.5 mM
GlutaMax	35050061, ThermoFisher	2 mM
Human Serum Albumin	Rc HA NW20, Richcore Lifesciences	0.5 mg ml^{-1}
ITSE	00-101, biogems	2%
L-ascorbic acid 2-phosphate (Vitamin C)	A8960, Sigma Aldrich	40 µM
Lysophosphatidic acid (LPA)	L7260, Sigma Aldrich	1 µM
MEM Amino Acids Solution	11130-051, ThermoFisher	0.50%
$NaHCO_3$	P2256, Sigma Aldrich	6.5 mM
PSA	17-745E, Lonza	1%
Soy hydrolysates	58903C, Merck	1%
Sodium l-lactate	71718, Sigma	10 mM
Sodium pyruvate	P2256, Sigma Aldrich	0.5 mM
Serum free differentiation medium version 2 (SFDM v2)		
SFDM v1 +		
ATRA (trans-retinoic acid)	554720, Millipore	500 nM
DAPT, gamma-Secretase inhibitor	ab120622, Abcam	5 µM
PD0325901	S1036, Selleckchem	1 µM

Supplementary Table 2: Antibodies used in this study

Target	Colour	Supplier	Reference	Dilution	Application
α-actin-1	-	Abcam	ab184705	1:5,000	Western Blot
α-actinin	-	Sigma-Aldrich	A7811	1:1,600 (IF); 1:2,500 (WB)	IF Western Blot
f-actin	Atto550	Sigma-Aldrich	19083	1:300	IF
desmin (rabbit)	-	Abcam	ab227651	1:400	IF
desmin (goat)	-	Abcam	ab80503	1:100	IF
Ki-67	-	Miltenyi Biotech	130-108-060	1:100	IF
MyoD	-	LifeSpan BioSciences	LS-C88010-100	1:100	IF
myogenin	-	Abcam	ab219998	1:100	IF
myoglobin	-	Abcam	ab231725	1:2,500	Western Blot
myosin (MyHC)	-	Abcam	ab51263	1:2,000	IF
myosin (MyHC)	-	Abcam	ab11083	1:5,000	Western Blot
obscurin	-	biotechne	NBP3-05247	1:800	IF
Pax7	-	Developmental Studies Hybridoma Bank	Cat# PAX7	1:100	IF
tubulin	-	Abcam	ab4074	1:10,000	Western Blot
goat	AF546	Invitrogen	A-11056	1:300	IF
human	DyLight488	Invitrogen	SA5-10126	1:300	IF
mouse	AF647	Invitrogen	A32787	1:300	IF
mouse	AF488	Invitrogen	A-11001	1:1,000	IF
mouse	AF633	Thermo Fisher Scientific	A21050	1:500	IF
mouse	DyLight650	biotechne	NBP1-75147C	1:300	IF
mouse	HRP	DAKO	P0447	1:2,000	Western Blot
rabbit	AF488	Thermo Fisher Scientific	A21206	1:300	IF
rabbit	DyLight550	Thermo Fisher Scientific	SA5-10033	1:250	IF
rabbit	AF594	Thermo Fisher Scientific	R37119	1:300	IF
rabbit	HRP	Abcam	ab6721	1:10,000	Western Blot

Supplementary Table 3: Small molecule compound screening

Compound	Supplier	Concentration	Pathway
Abemaciclib	MedChemExpress	100 nM	CDKi
Narazaciclib	MedChemExpress	100 nM	CDKi
Palbociclib	MedChemExpress	100 nM	CDKi
SCH772984	Selleckchem	1 μM	ERKi
Magnolin	Selleckchem	1 μM	ERKi
AZD0364	Selleckchem	1 μM	ERKi
PD184352	Selleckchem	1 μM	MEKi
PD0325901	Selleckchem	1 μM	MEKi
U0126-EtOH	Selleckchem	1 μM	MEKi
DAPT	Abcam	5 μM	NOTCHi
LY3039478	Selleckchem	5 μM	NOTCHi
LY411575	Miltenyi Biotec	5 μM	NOTCHi
Bexarotene	Selleckchem	500 nM	RXRa
All-trans-retinoic acid (ATRA)	Millipore	500 nM	RXRa
SR11237	Sigma Aldrich	500 nM	RXRa
SB431542	Selleckchem	1 μM	TGF-βi
ITD-1	MedChemExpress	1 μM	TGF-βi
Repsox	Selleckchem	1 μM	TGF-βi

Supplementary Video 1: Live imaging of differentiating SCs (related to Figure 3)

Representative phase holographic microscopy videos of 2D myogenic differentiation over 96 h with indicated treatments. Scale bar, 500 µm.