

Supplementary materials

Secretoglobin 3A1 in activated muscle satellite cells contributes to myosin heavy chain IIX and IIB fiber differentiation

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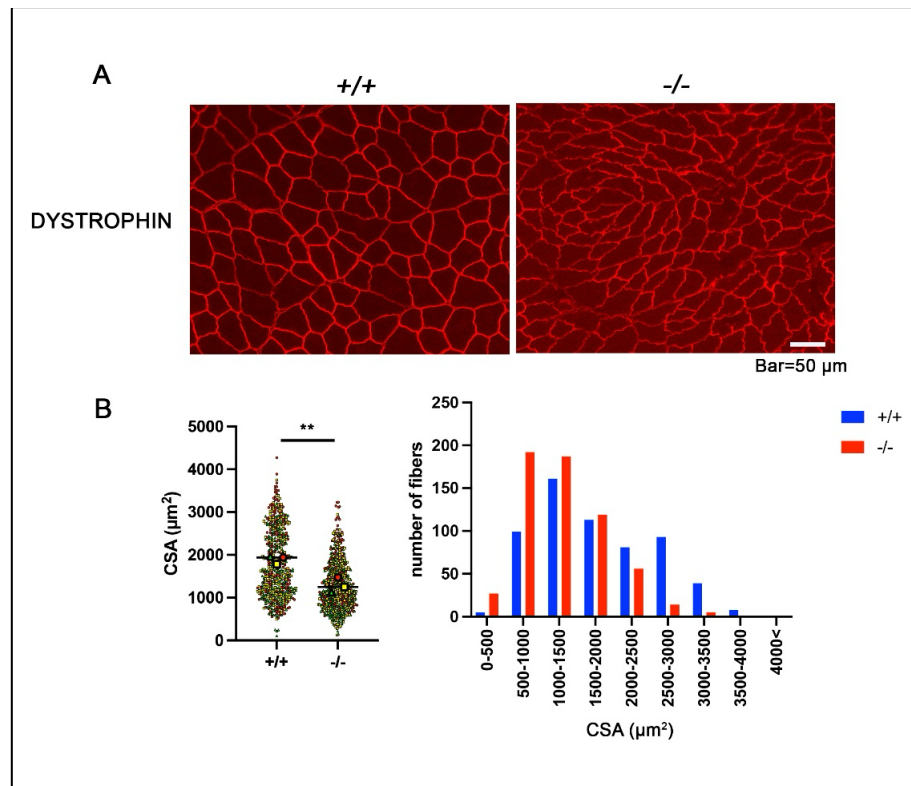


Fig. S1 Immunostaining of DYSTROPHIN in 18-week-old *Scgb3a1*^{+/+} and *Scgb3a1*^{-/-} TA muscle.

(A) Representative images of immunofluorescent staining of TA muscle of 18-week-old *Scgb3a1*^{+/+} (+/+) or *Scgb3a1*^{-/-} (-/-) mice for the DYSTROPHIN expressions. Bar= 50 μm.

(B) Details of muscle fiber size distribution between +/+ and -/- mice. Right Figure depicts the distribution of fiber size (n=3 mice, each 100 fibers per mouse). ***p*<0.01 by Student's *t*-test.

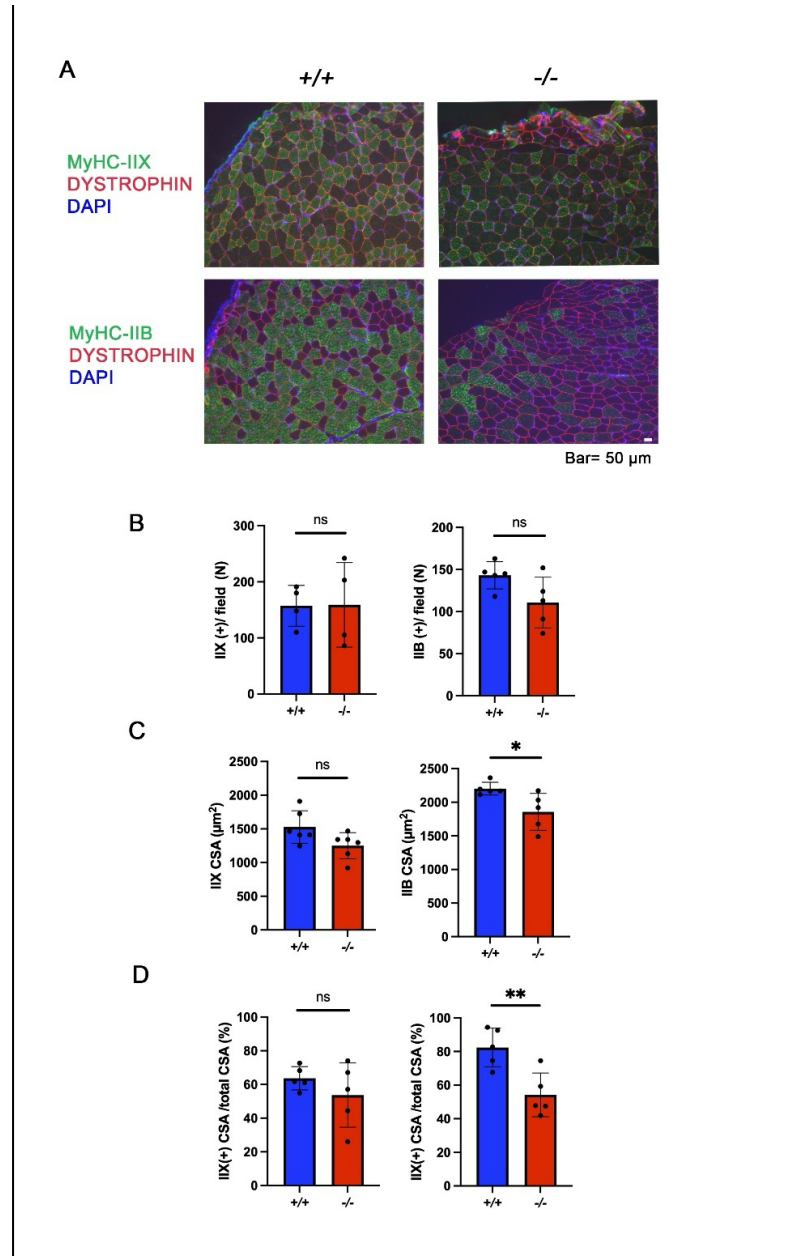


Fig. S2 Immunostaining of MyHC-IIX and MyHC-IIB in 4-week-old *Scgb3a1*^{+/+} and *Scgb3a1*^{-/-} TA muscle.

(A) Representative images of immunofluorescent double staining of TA muscle of 10-month-old *Scgb3a1*^{+/+} (+/+) or *Scgb3a1*^{-/-} (-/-) mice for the MyHC-IIX and DYSTROPHIN or MyHC-IIB and DYSTROPHIN expressions in intact TA muscle. Bar= 50 μ m. (n=3). (B) Each MyHC fiber numbers per independent field (n=5). (C) Average CSA of MyHC-IIX and -IIB between +/+ and -/- mice (n=5 independent fields). (D) Average each MyHC (+) CSA per total muscle CSA between +/+ and -/- mice (n=5 independent fields). Total fiber number; IIX>600, IIB>500.

* p <0.05, ** p <0.01 by Student's t -test. ns, not significant.

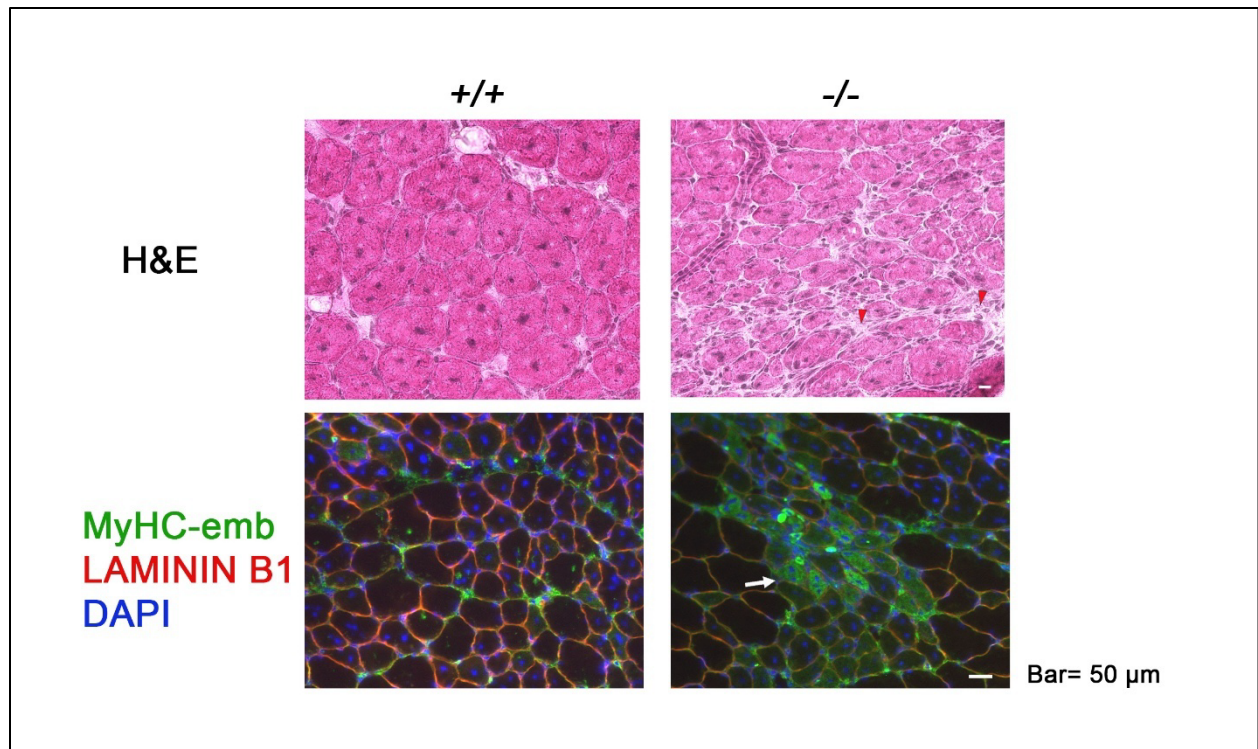


Fig. S3 TA muscle of *Scgb3a1*^{+/+} and *Scgb3a1*^{-/-} 1 week after BaCl₂ injection. **(Upper)** Representative image of HE staining of TA muscle after 1 week of BaCl₂ injection. Red arrowheads indicate large interstitial region. **(Bottom)** Immunostaining of Embryonic MyHC (MyHC-emb) and LAMININ B1 after 1 week of BaCl₂ injection. White arrow shows expression of MyHC-emb. Bar=50 μm. Nuclei were counterstained with DAPI.

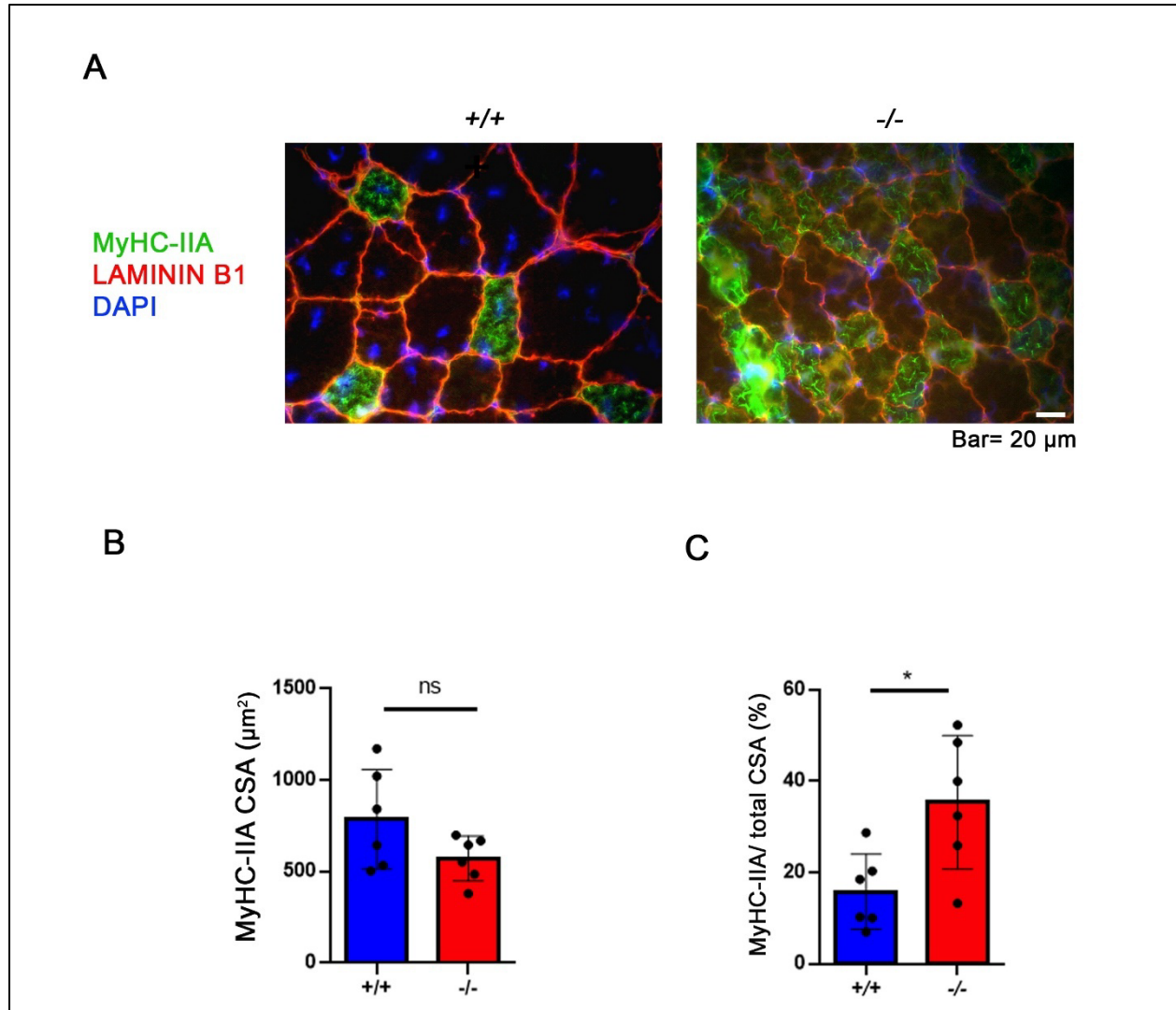


Fig. S4 MyHC IIA immunostaining in *Scgb3al*^{+/+} and *Scgb3al*^{-/-} tissues after BaCl₂ injection. (A) Representative images of immunofluorescent double staining of TA muscle of 1-month-old *Scgb3al*^{+/+} ($+/+$) or *Scgb3al*^{-/-} ($-/-$) mice for the MyHC-IIA and LAMININ B1 expressions after BaCl₂ injection. Bar= 20 μ m. (n=3). (B) MyHC-IIA (+) fiber size CSA between *Scgb3al*^{+/+} ($+/+$) or *Scgb3al*^{-/-} ($-/-$) mice after BaCl₂ injection (n=6 independent fields/3 mice). (C) Percentage of MyHC-IIA+ area per total muscle area between *Scgb3al*^{+/+} ($+/+$) or *Scgb3al*^{-/-} ($-/-$) mice after BaCl₂ injection (n=6 independent fields/3 mice). Total IIA fibers>200. * p <0.05 by Student's t -test. ns, not significant.

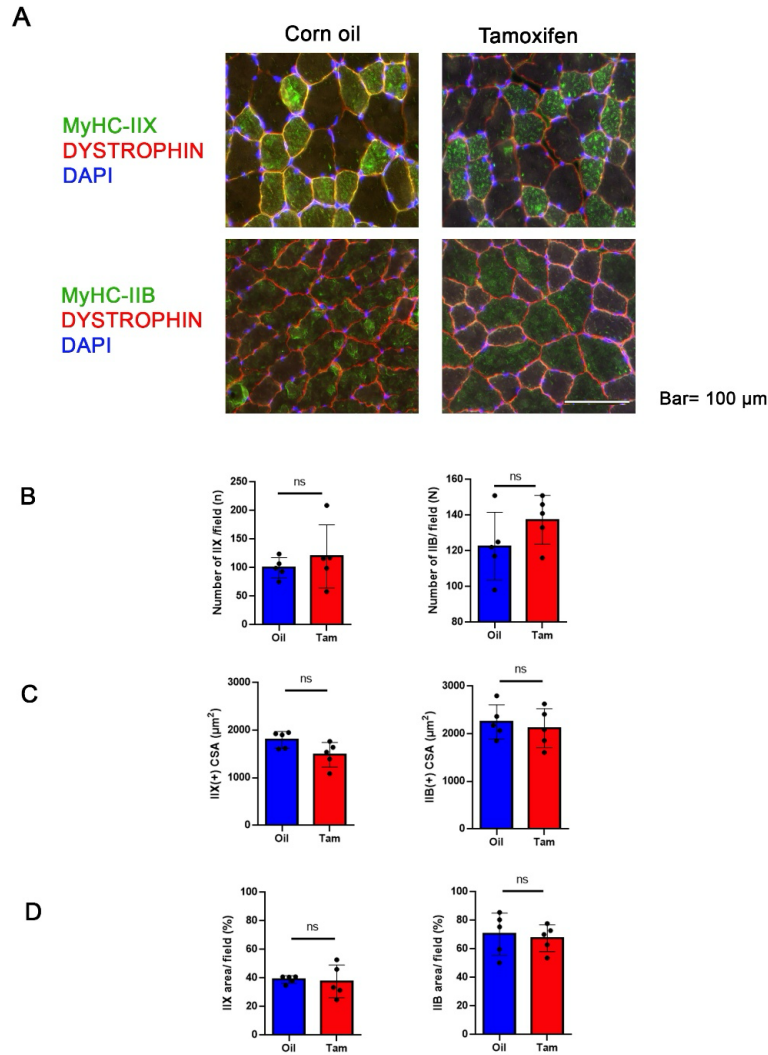


Fig. S5 Immunofluorescent images of MyHC-IIX and MyHC-IIB in *Pax7^{CreERT2};Scgb3a^{If/f}* mice treated with or without tamoxifen.

(A) Representative image of MyHC-IIX or MyHC-IIB and DYSTROPHIN in intact TA muscle of 4 weeks after administration of corn oil (Control) or Tamoxifen (CKO). (B) Each MyHC fiber numbers per independent field (n=5). (C) Average CSA of MyHC-IIX and -IIB between +/+ and -/- mice (n=5 independent fields). (D) Average each MyHC (+) CSA per total muscle CSA between +/+ and -/- mice (n=5 independent fields). Total fiber number>500. ns, not significant by Student's *t*-test.

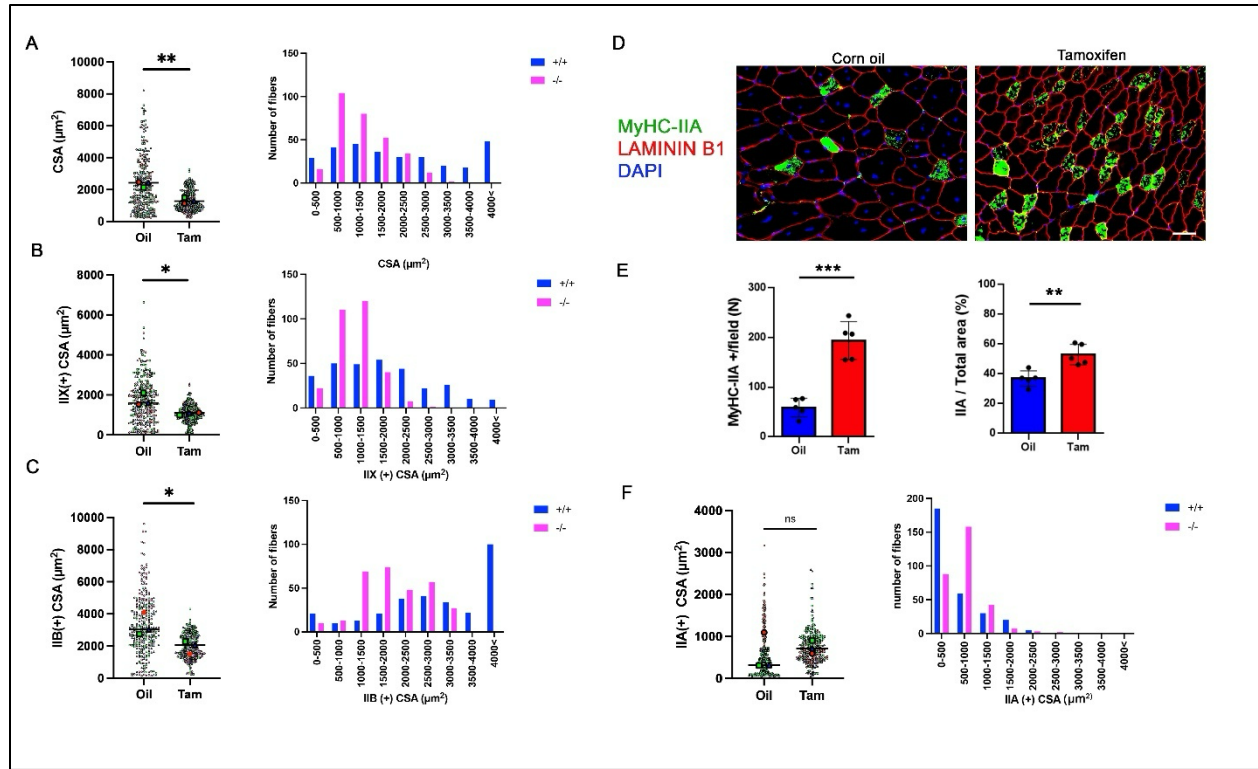


Fig. S6 *Pax7^{CreERT2};Scgb3a^{Iff}* mice treated with Tam show severe MyHC-IIIX, IIB differentiation defects.

(A) Distribution of CSA of HE stained TA muscle of Oil (control) or Tam treated followed by BaCl₂ injection. n=3 mice, each 100 fibers per mouse. (B) Distribution of CSA of MyHC-IIIX (+) fibers determined by immunofluorescent analysis. n=3 mice, each 100 fibers per mouse. (C) Distribution of CSA of MyHC-IIB (+) fibers determined by immunofluorescent analysis. n=3 mice, each 100 fibers per mouse. (D) Double immunostaining of MyHC-IIA and LAMININ B1 of control or Tamoxifen treated TA muscle followed by BaCl₂ injection. Nuclei were counterstained with DAPI. Bar=50 μm. (E) (Left) MyHC-IIA+ fiber numbers per fields (n=5, total fiber numbers>1000). (Right) Percentage of MyHC-IIA area per total area using data shown on left graph (n=5 independent fields). (F) Distribution of CSA of MyHC-IIA (+) fibers determined by immunofluorescent analysis. n=3 mice, each 100 fibers per mouse. **p*<0.05, ***p*<0.01, ****p*<0.005, ns; not significant by Student's *t*-test.

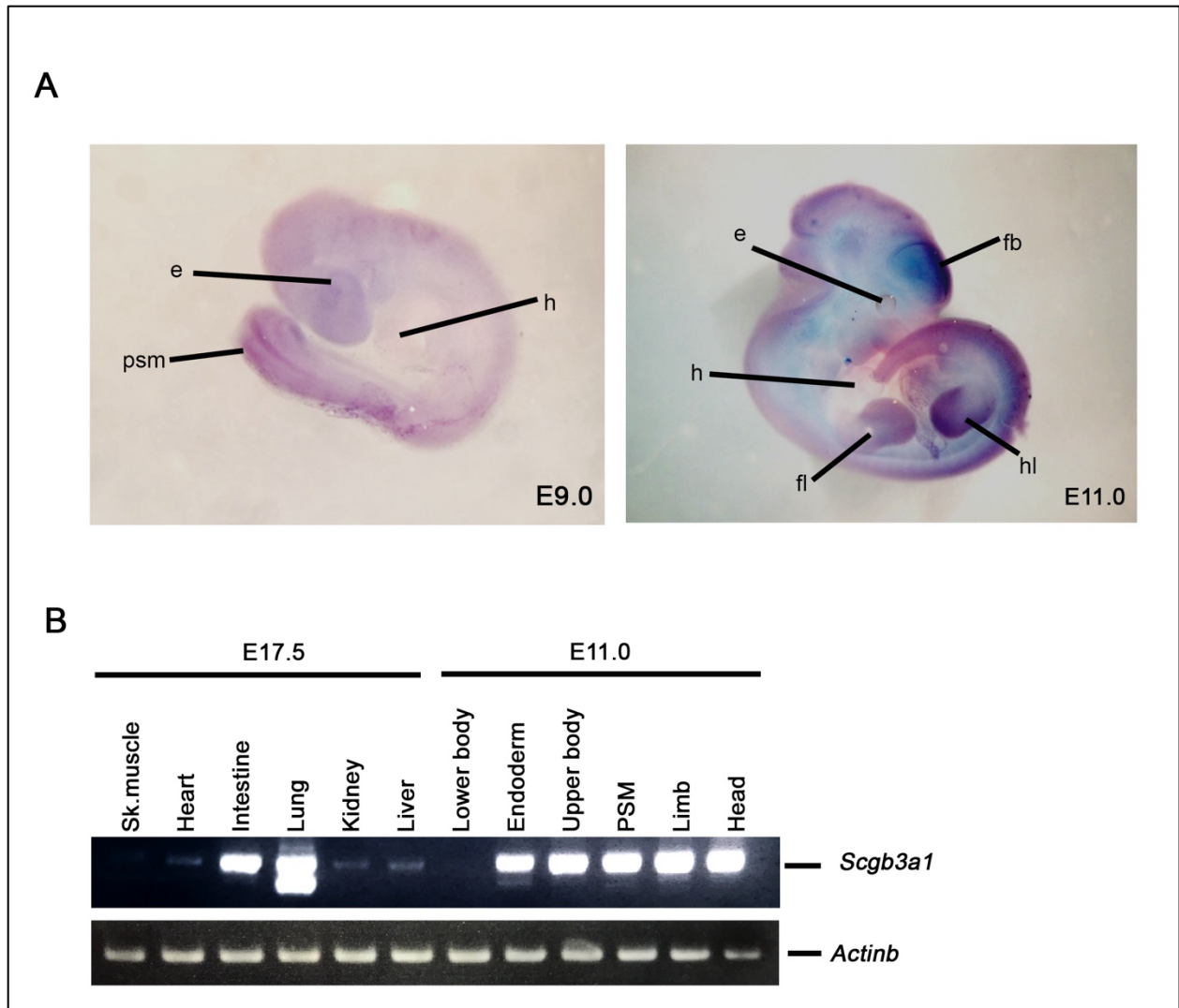


Fig. S7 Expression of *Scgb3a1* mRNA in mouse embryos. (A) Whole-mount in situ hybridization was carried out using mouse E9.0 (left) or E11.0 (right) embryos with DIG-*Scgb3a1* antisense RNA probe. n=3. e, eye; h, heart; psm, presomitic mesoderm; fb, forebrain; fl, forelimb; hl, hindlimb. (B) PCR results for *Scgb3a1* mRNA in E17.5 and E11.0 tissues. E11.0 endoderm is a mixture of heart and liver. PSM, presomitic mesoderm.

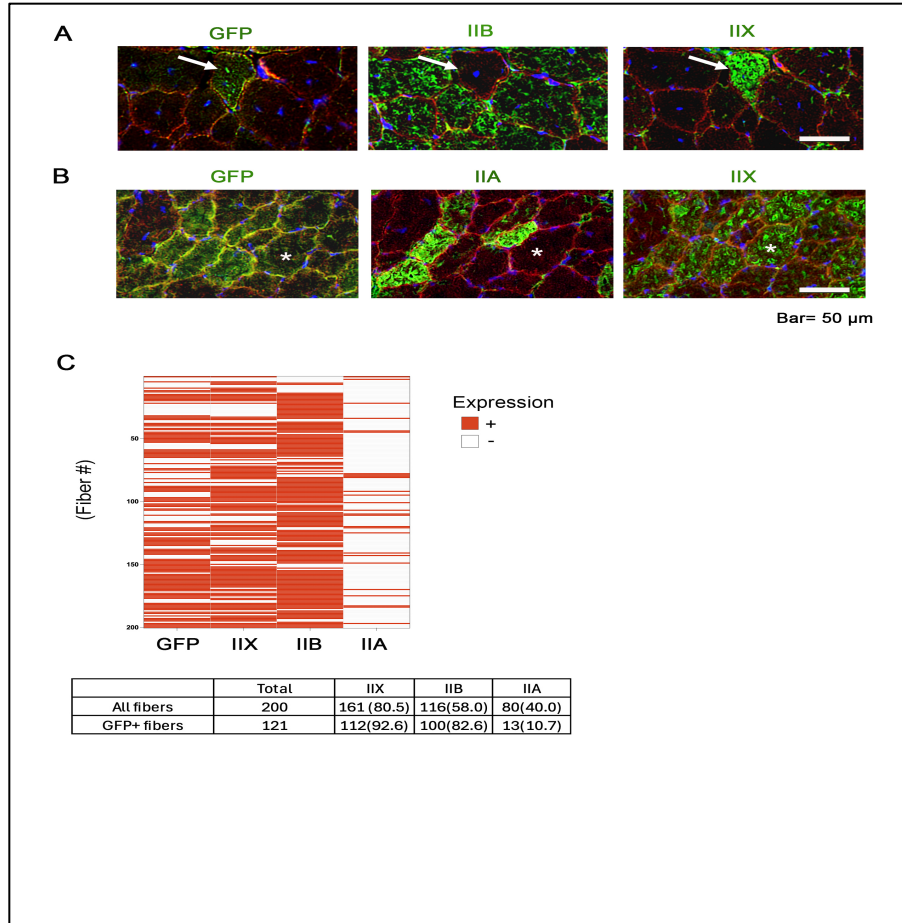


Fig. S8 GFP (+) fibers are composed of MyHC-IIIX (+) fibers.

(A) Double immunofluorescent staining of GFP (green) and tdTomato (red) (left), MyHC-IIB (green) and DYSTROPHIN (red) (center), and MyHC-IIIX (green) and DYSTROPHIN (red) (right) in serial sections of TA muscle of *SCGB3A1^{CreERT2};mT/mG* knock-in mice after 3 weeks of BaCl₂ injection. Nuclei were counterstained with DAPI (blue). Arrows depict the same fiber. Bar= 50 μ m. (B) Double immunofluorescent staining of GFP and tdTomato (left), MyHC-IIA and DYSTROPHIN (center), and MyHC-IIIX and DYSTROPHIN (right) in serial sections of TA muscle of *SCGB3A1^{CreERT2};mT/mG* knock-in mice after 3 weeks of BaCl₂ injection. Nuclei were counterstained with DAPI (blue). Asterisks depict the same fiber. Bar= 50 μ m. (C) Quantification of co-expression of GFP with MyHC-IIIX, MyHC-IIB, and MyHC-IIA in 200 fibers. Red bars indicate positive expressions in each fiber. Bottom: Total number of fibers of each fast-type MyHC coinciding with GFP expression or with GFP+ fibers. Numbers in parentheses indicate the percentage relative to the GFP expression pattern or to GFP+ fibers.

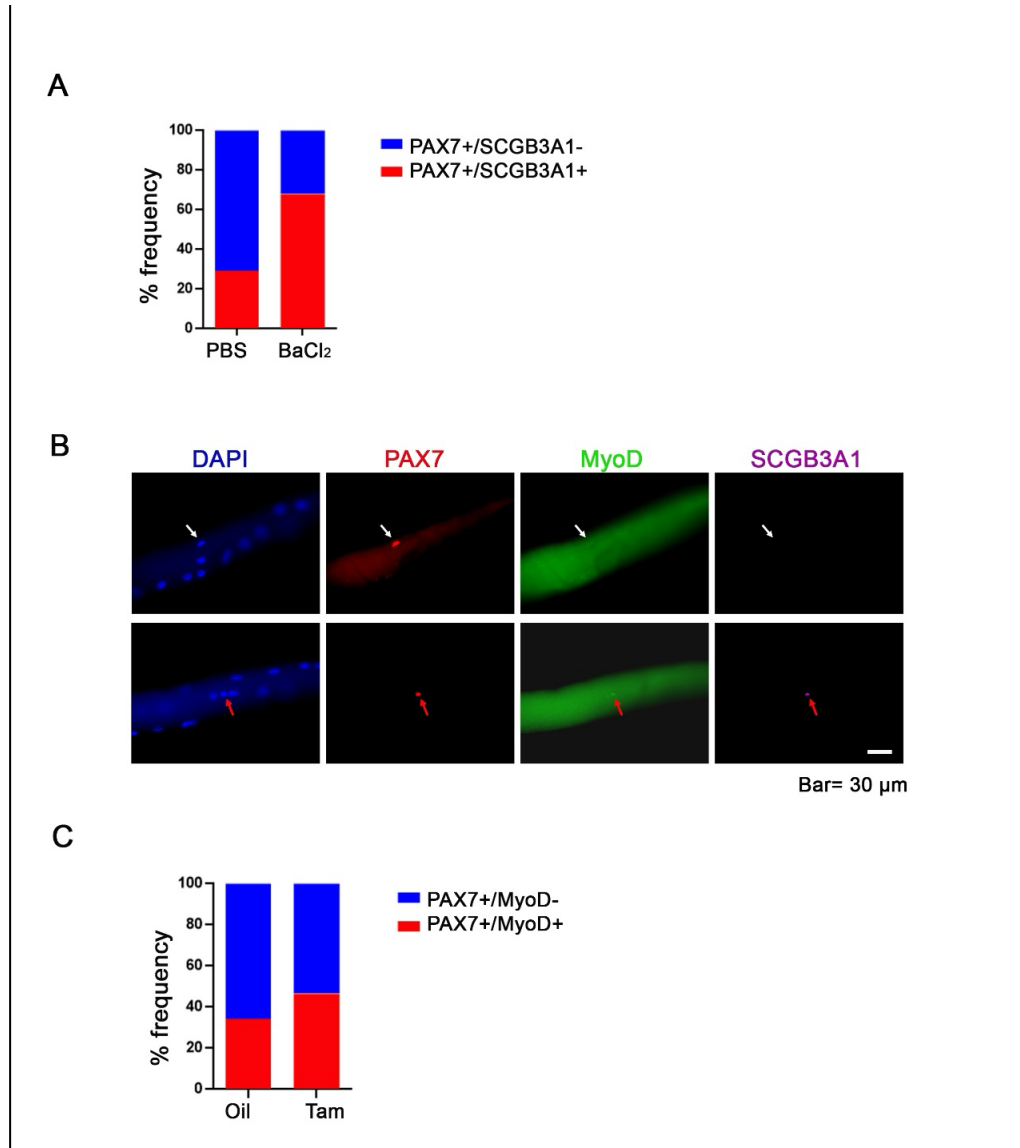


Fig. S9 SCGB3A1 is colocalized with PAX7+/MyoD+ activated satellite cells. (A) Percentage of PAX7+/SCGB3A1- or PAX7+/SCGB3A1+ satellite cells in control (PBS) or BaCl₂ treated EDL muscle examined 1 week after BaCl₂ treatment. n=3 mice. Total number of PAX7(+) cells= PBS; 17, BaCl₂; 50. (B) Immunostaining of EDL muscle with BaCl₂ injection carried out at 4 weeks of age. Muscles were collected 1 week after BaCl₂ injection. Individually separated single muscle fibers were stained with anti-PAX7, anti-MyoD and anti-SCGB3A1 antibodies. Representative image of PAX7(+)/MyoD(-)/SCGB3A1(-) (white Arrows) and PAX7(+)/MyoD(+)/SCGB3A1(+) cells (red arrows) after BaCl₂ injection. Nuclei were counterstained with DAPI. Bar=30 μ m. (C) Percentage of quiescent (PAX7+/MyoD-) or activated (PAX7+/MyoD+) satellite cells in control (oil) or CKO (Tam) EDL muscle of 26-week-old mice. n=3 mice. Total number of PAX7(+) cells = Oil; 61, Tam; 86.

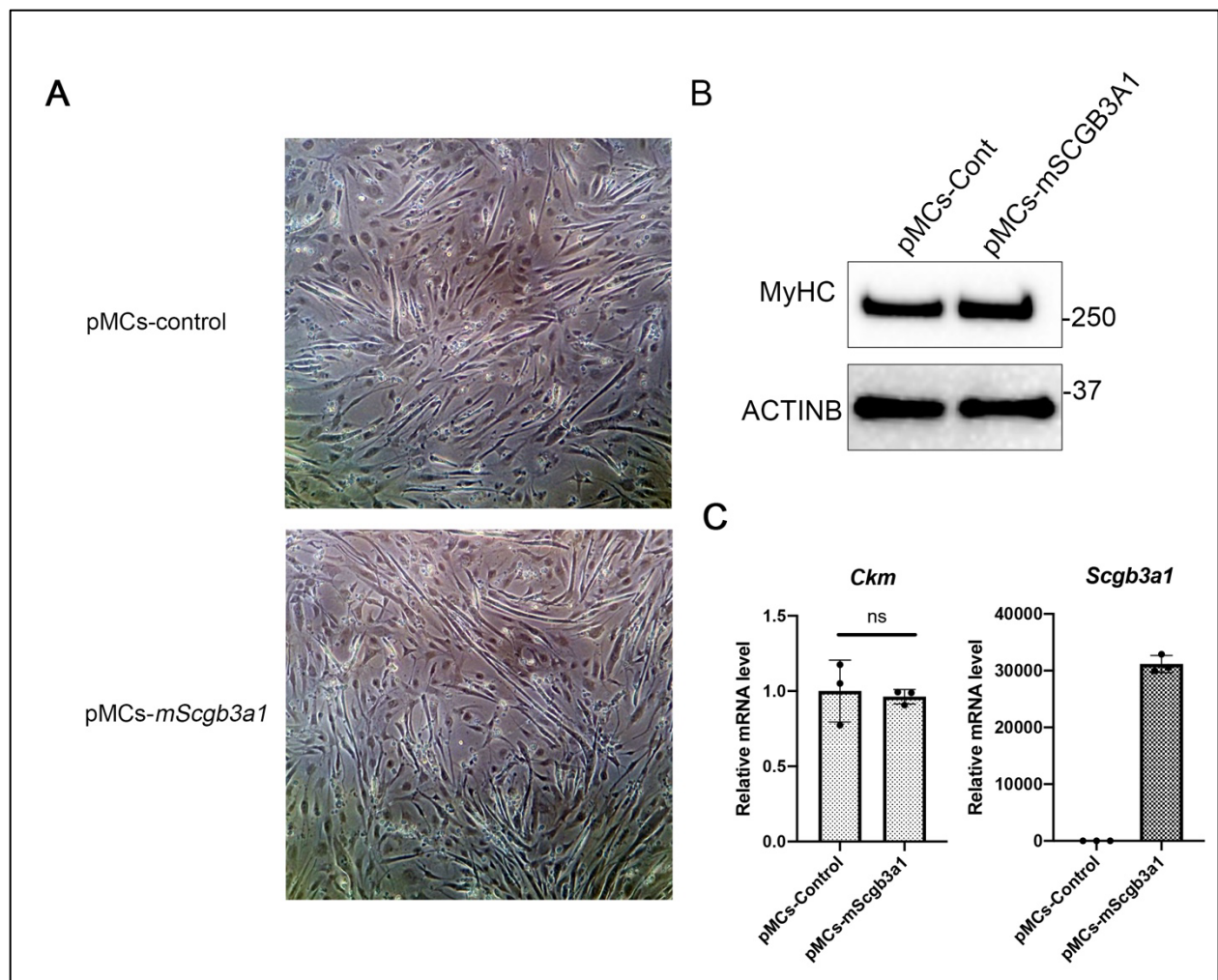


Fig. S10 Constitutive *Scgb3a1* expression in C2C12 does not promote terminal differentiation. (A) Overall morphology of cell culture of C2C12-pMCs-*mScgb3a1* or equivariant control vector clone for 7 days in DM (differentiation medium). (B) Immunoblotting results of culture of (A) for anti-MyHC antibody. Anti-ACTINB antibody was used for internal loading control. (C) qPCR results of culture of (A) for *Creatin muscle kinase* (*Ckm*) and *Scgb3a1*. All data were normalized to *Gapdh* gene. n=3. qPCR was carried out in triplicate per sample. ns: not significant by Student's *t*-test.

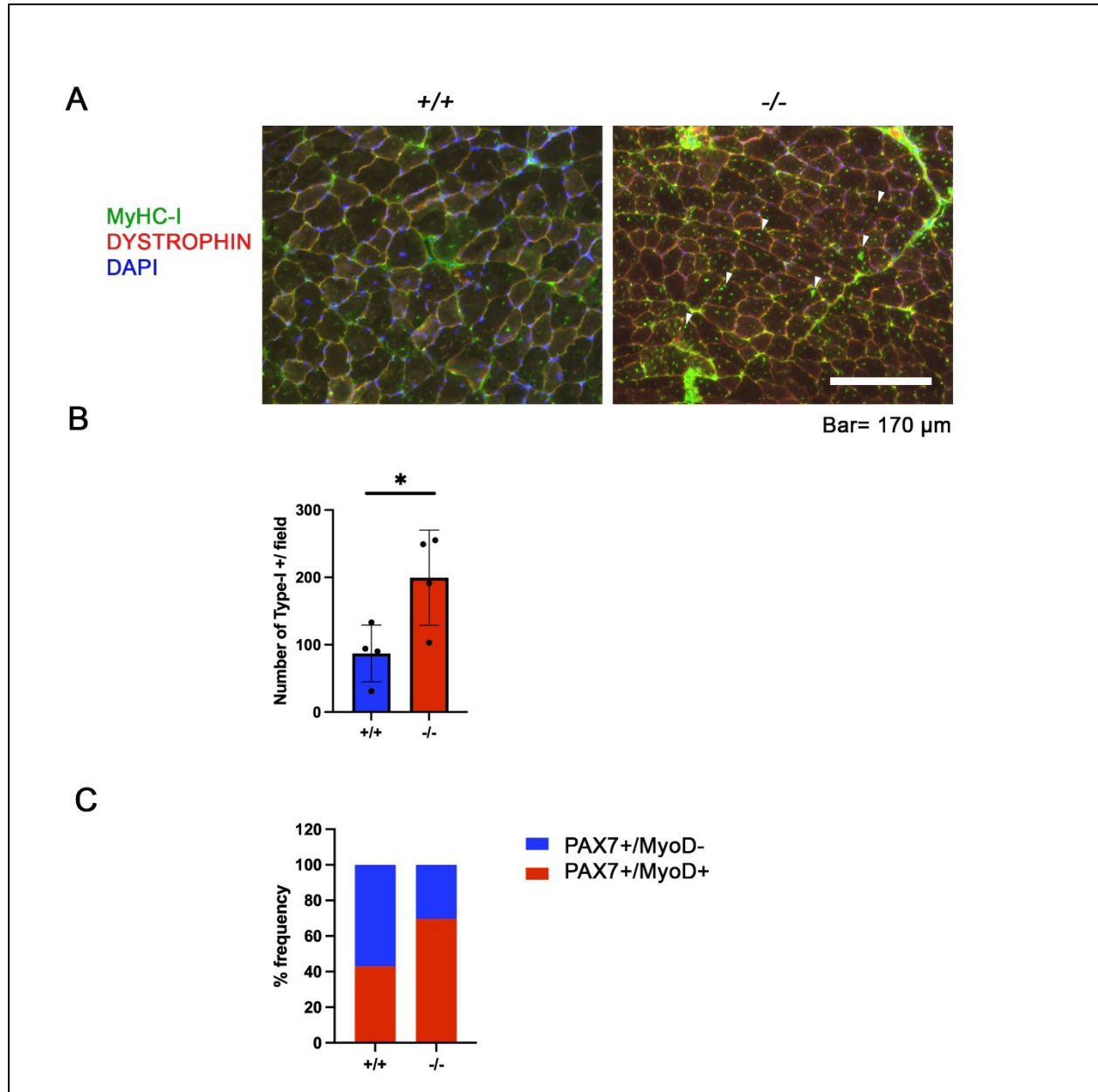


Fig. S11 Aged *Scgb3a1*^{-/-} mice exhibit MyHC-I in intact TA muscles.

(A) Representative images of immunofluorescent double staining of TA muscle of 22-month-old *Scgb3a1*^{+/+} (+/+) or *Scgb3a1*^{-/-} (-/-) mice for the MyHC-I and DYSTROPHIN expressions. Arrowheads indicate the emergence of scattered MyHC-I expressions in *Scgb3a1*^{-/-} TA muscles. Bar= 170 μ m. (n=3). Nuclei were counter stained with DAPI. (B) MyHC-I+ fiber numbers per fields (n=4, total fiber numbers>500). (C) Percentage of quiescent (PAX7+/MyoD-) or activated (PAX7+/MyoD+) satellite cells in aged *Scgb3a1*^{+/+} (+/+) or *Scgb3a1*^{-/-} (-/-) EDL muscle of 84-week-old mice. n=3 mice. **p*<0.05 by Student's *t*-test.

Table S1

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Myosin heavy chain (MyHC) (clone MF20)	DSHB	#MF20, RRID:AB_2147781
mouse anti-MyHC Type I (BA-D5)	DSHB	#BA-D5, RRID:AB_2235587
mouse anti-MyHC IIA (sc-71)	DSHB	#sc-71, RRID:AB_2147165
mouse anti-MyHC IIX (6H1)	DSHB	#6H1, RRID:AB_1157897
mouse anti-MyHC IIB (BF-F3)	DSHB	#BF-F3, RRID:AB_2266724
mouse anti-MyHC-neonatal (N3.36)	DSHB	#N3.36, RRID:AB_528380
mouse anti-Pax7 (DSHB)	DSHB	#PAX7, RRID:AB_2299243
rat anti-HIN1/SCGB3A1	R&D	#MAB2954, RRID:AB_2157009
goat anti-HIN1/SCGB3A1	R&D	#AF2954, RRID:AB_2157011
rabbit anti-Notch3	Proteintech	#55114-1-AP, RRID:AB_10858393
rabbit anti-MyoD	Santa Cruz Biotechnology	#sc-760, RRID:AB_2148870
rat anti-Laminin beta-1 (LT3)	Thermo Fisher Scientific	#MA5-14567, RRID:AB_10981503
mouse anti-Gapdh	Proteintech	#60004-1-Ig, RRID:AB_2107436
rabbit anti-Caspase 3	Cell Signaling Technology	#D3R6Y, RRID:AB_2798429
rabbit anti-Dystrophin	Thermo Fisher Scientific	#PA1-21011, RRID:AB_2091358
rabbit anti-Ki67	Abcam	#ab16667, RRID:AB_302459
mouse anti-Actin (a-Sarcomeric) clone 5C5	SIGMA	#A2172, RRID:AB_476695
rabbit anti-b-actin	Cell Signaling Technology	(#4967, RRID:AB_330288
Bacterial and virus strains		
Biological samples		
Chemicals, peptides, and recombinant proteins		
Tamoxifen		
BaCl ₂	Sigma	# 202738
PBS	Fisher Scientific	#10-010-072

DMEM (Dulbecco's modified Eagle's medium; high glucose, L-glutamine with 110 mg/ml sodium pyruvate)	ThermoFisher Scientific	#11995073
collagenase type I	SIGMA	#SCR103
FBS		
Chicken Embryo Extract	USBiological	#C3999
<i>penicillin - streptomycin</i>	Gibco	#15140122
fibroblast growth factor-2 (FGF-basic)	Peptotech	#450-33
Collagen I	Corning	#CB-40236
horse serum (HS)	ATCC	#30-2040
recombinant mouse HIN-1/SCGB3A1	R&D Systems	#2954-HN-050
Formalin		
MeOH	SIGMA	# 1424109
bovine serum albumin (BSA)	Caibiochem	#2960
4',6-diamidino-2-phenylindole (DAPI)		
tragacanth gum	SIGMA	# G1128
isopentane (methylbutane)	SIGMA	#277258
RIPA buffer	ThermoFisher Scientific	#89900
PMSF	SIGMA	#41216525
4-20 % Criterion Tris HCL precast gel	Bio Rad	#3450104
PVDF	GE Healthcare	# RPN303F
Supersignal West dura	ThermoFisher Scientific	#34075
TRIzol®	Life Technologies	#15596026
SuperScript III reverse transcriptase	ThermoFisher Scientific	# 18080044
puromycin		
Critical commercial assays		
Pierce BCA protein assay kit	ThermoFisher Scientific	#23225
X-treme Gene™ HP DNA transfection reagent	Roche	#6366244001
Deposited data		
Experimental models: Cell lines		
C2C12	ATCC	N/A

Experimental models: Organisms/strains		
Mouse:Scgb3a1-KO	This study	
Mouse:Pax7-CreERT2	The Jackson Laboratory	
Mouse: Scgb3a1 f/f	This study	
Oligonucleotides		
Primers for qRT-PCR, genomic PCR, see Table S1.	This study	
Recombinant DNA		
pGEM-T-mScgb3a1	This study	
pMCs-mScgb3a1-v1	This study	
pMCs-mScgb3a1-v2	This study	
Software and algorithms		
GraphPad Prism (version 7)	GraphPad Software,	https://www.graphpad.com
Zen Black (version 3.1)	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite
Adobe Photoshop CC	Adobe	https://www.adobe.com/uk/products/photoshop
imageJ	imageJ	https://imagej.nih.gov/ij/index.html
Other		

Table S2. List of primers used in this study, related to Methods

Primers used in this study:

Amplicon	F primer (5'-->3')	R primer (5'-->3')	Used for	Reference
<i>Scgb3a1</i> KO	GGTGTTCGCTTTCTTCATGGAC	AGCTCGGTGACACACTTCCT	detection KO allele	This study
<i>Scgb3a1</i> EX2	TGGCCACCTTTCCTAACTTC	AAGGGGAAACTGGGTGACAT	wild-type <i>Scgb3a1</i> exon2	This study
<i>mScgb3a1</i>	CTGAGGCTGTAGGAGCTGTG	ATCTCGTCAGGCATCTCGTC	qPCR	This study
<i>mMyh1</i>	CCAGGACCTTGTGGACAAAC	ATTTGGCCAGGTTGACATTG	qPCR	This study
<i>mMyh4</i>	GCAGGACTTGGTGGACAAAC	ACTTGGCCAGGTTGACATTG	qPCR	This study
<i>mNotch3</i>	CAATGCAGTGGATGAGCTTG	GGCTCCATTTTTCAGCAGAG	qPCR	This study
<i>mHes1</i>	CTACCCAGCCAGTGTCAAC	CGCCTCTTCTCCATGATAGG	qPCR	This study
<i>mHey1</i>	CACCTGAAAAATGCTGCACAC	ACCCCAAACCTCCGATAGTCC	qPCR	This study
<i>mHeyL</i>	TTTCTGAATTGCGACGATTG	ACGGTCATCTGCAAGACCTC	qPCR	This study
<i>mGapdh</i>	CCTTCCGTGTTCCCTACCCC	CCTGCTTCACCACCTTCTTG	qPCR	This study
<i>mCkm</i>	AGGAGATTCTCACTCGCCTTC	GTTGGAGATGTCGAACACG	qPCR	This study
<i>mScgb3a1</i> -cDNA	CCCACTAGTCTGATGACATCTTC	ATCTCGTCAGGCATCTCGTC	ISH probe	This study
<i>Scgb3a1</i> -P2ACreERT2	CAGGTGTTGCTTTCTTCATGG	GTAAACCTCAAGCTGTGGCAG	detection P2ACreERT2 allele	This study
<i>Scgb3a1</i> -GFP	CAGGTGTTGCTTTCTTCATGG	AGACAATCTCCAGAGTCTGG	detection GFP allele	This study

Primers used for preparation of *Scgb3a1* transgenic mice:

Candidate guide RNA	Target site (PAM in bold)
1533	CAATCCTCGGTAGCAGTGAAGGG
1534	AGTGCCCTTCACTGCTACCGAGG
1535	GTCCTGACAATGTTTCGGTTGAGG
1536	TCCTGACAATGTTTCGGTTGAGGG
1537*	CCTGACAATGTTTCGGTTGAGGGG
1538	ATCTCGTCAGGCATCTCGTCAGG
To generate ssDNA	
<i>Scgb3a1</i> -ssDNA-F	CCTAGCCTACCATTAAGCCA
<i>Scgb3a1</i> -ssDNA-R	/5Phos/CAAACAGGTCATCTATATGTGTT

*Candidate guide RNA used for mouse generation

PAM: protospacer adjacent motif