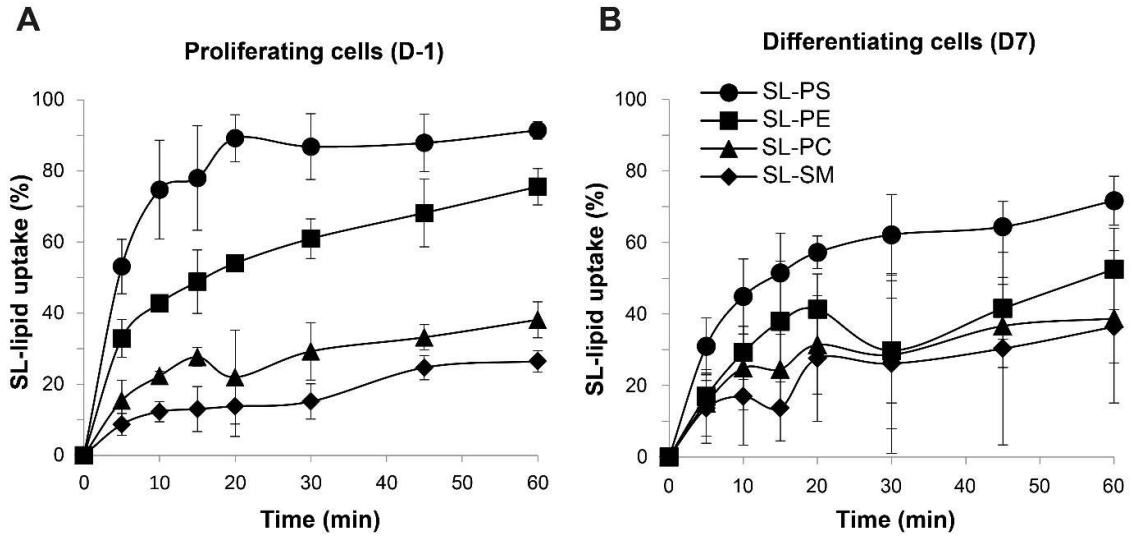

Fig. S1. Internalization of spin-labeled lipids from the plasma membrane of proliferating and differentiating L6 rat skeletal muscle cells. Internalization of the indicated spin-labeled (SL) lipids was analyzed during proliferation at D-1(A) and differentiation at day 7 (B) using back-exchange to albumin and expressed as the percentage of total label added at time zero. Data are mean ± S.D. from at least two (A) and three (B) independent experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

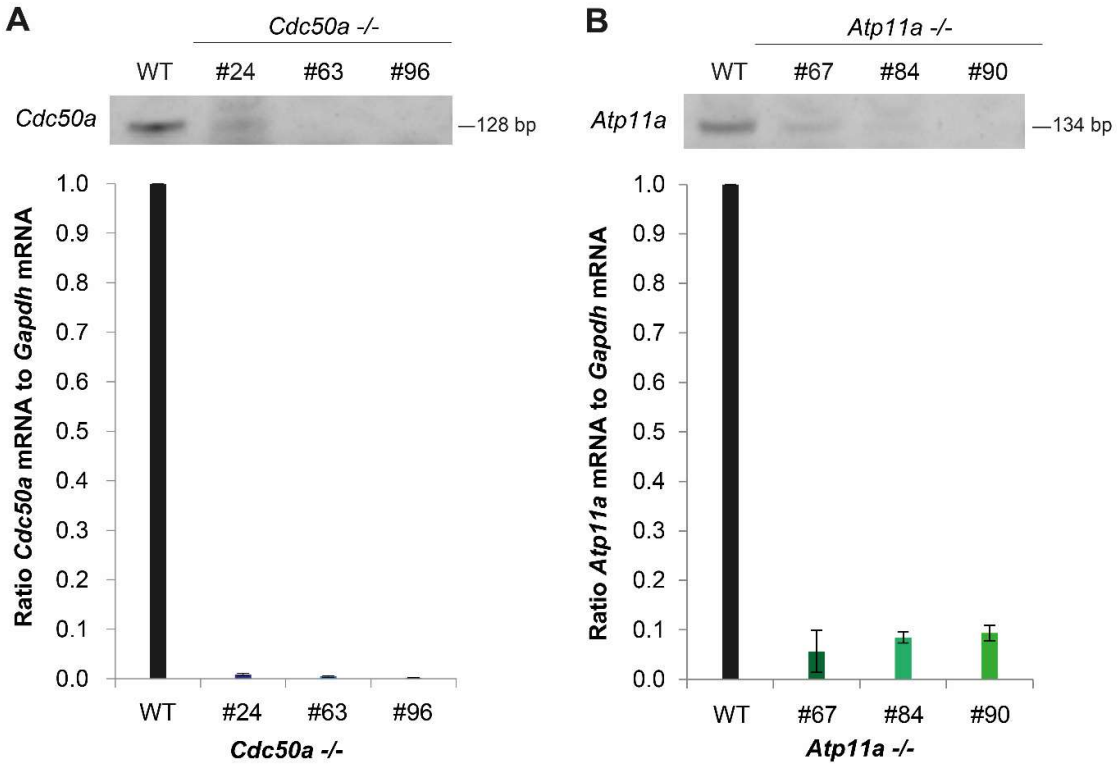

Fig. S2. Validation of C2C12 knockout cell lines. Analysis of the mRNA expression of *Cdc50a* and *Atp11a* transcripts in wild-type (WT) and clonal KO isolates by qRT-PCR. Ct values of genes of interest (GOI) were normalized to the housekeeping gene *Gapdh*. Top panels: agarose gel electrophoresis of the RT-PCR products. The experiment was carried out in duplicates and results shown are the mean ± S.D. of two independent experiments.

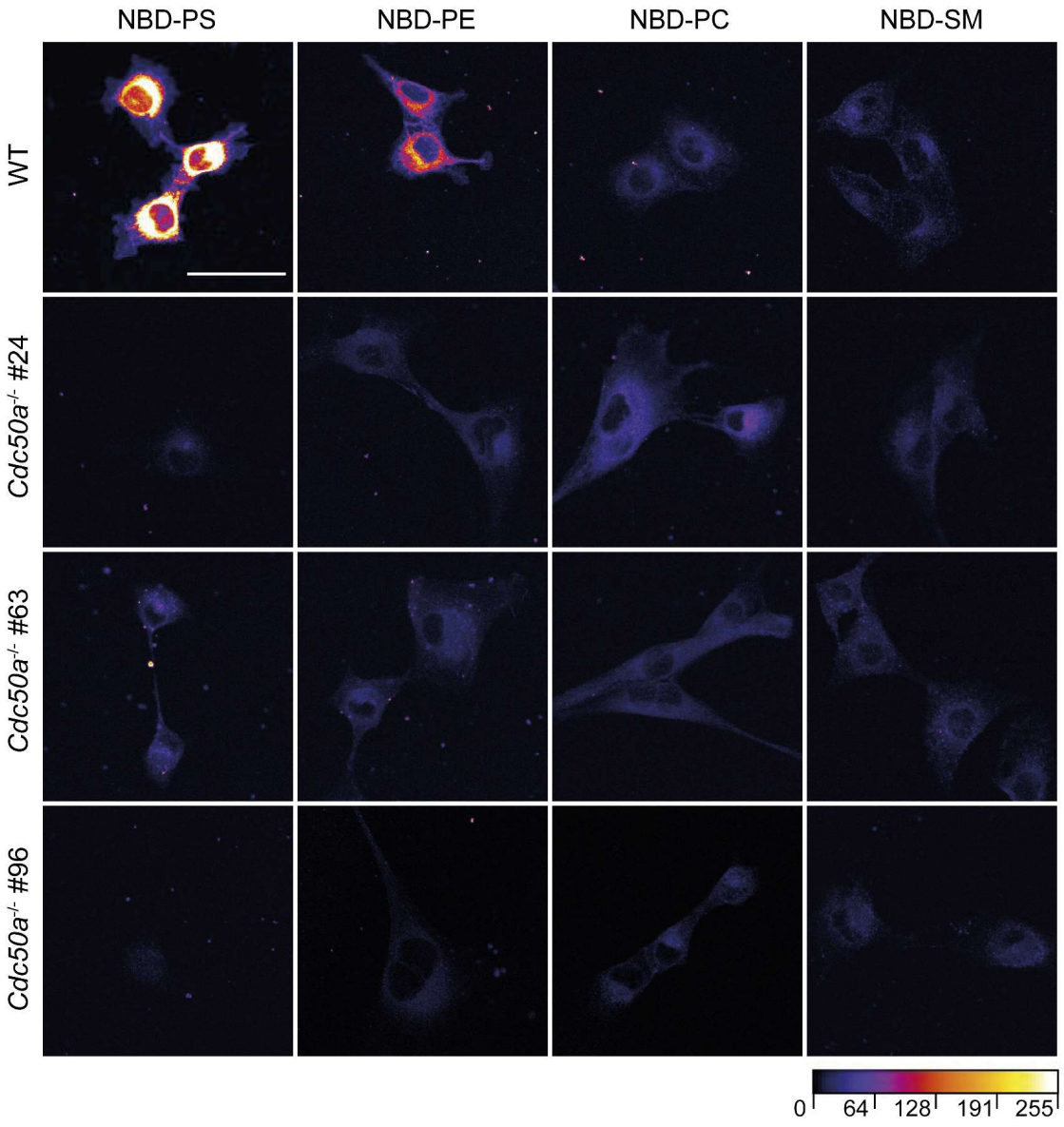

Fig. S3. CDC50A-deficient C2C12 cells are impaired in aminophospholipid uptake. Representative confocal images of proliferating wild-type (WT) and CDC50A-deficient cells (lines #24, #63 and #96) labeled with the indicated NBD-lipids for 60 min and subjected to back-exchange with albumin. Images were color-coded with the FIRE look-up-table to highlight intensity variations. In CDC50A-deficient cells, all NBD-lipids were hardly internalized and predominately detected in intracellular punctate structures. Scale bar, 50 µm. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

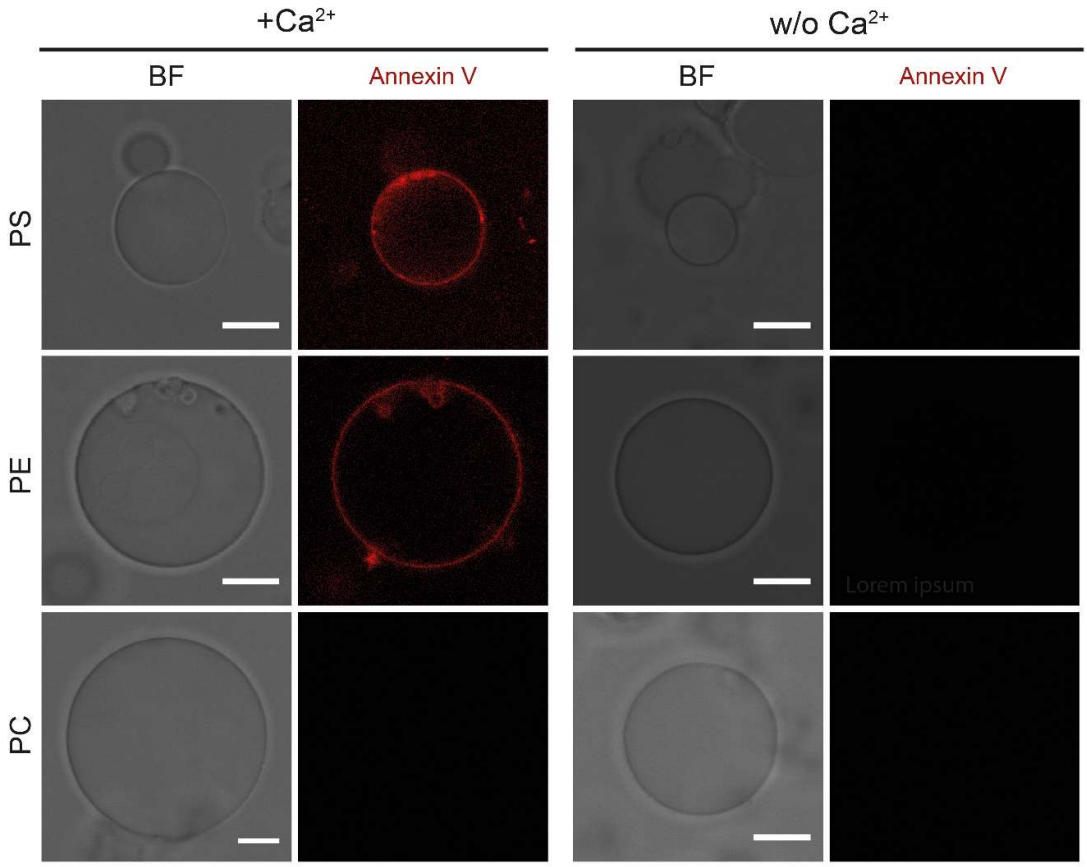

Fig. S4. Annexin V-Alexa 568 binds PS and PE. Giant unilamelar vesicles were prepared from different lipids and incubated with Annexin V-Alexa 568 (0.5 µL/mL) in the presence or absence of Ca^{2+}. Vesicles were analyzed by bright field (BF) and fluorescence microscopy. PC: PC only; PE: PC/PE, (9/1, mol/mol); PS: PC/PS, (9/1, mol/mol). Scale bar, 10 µm.

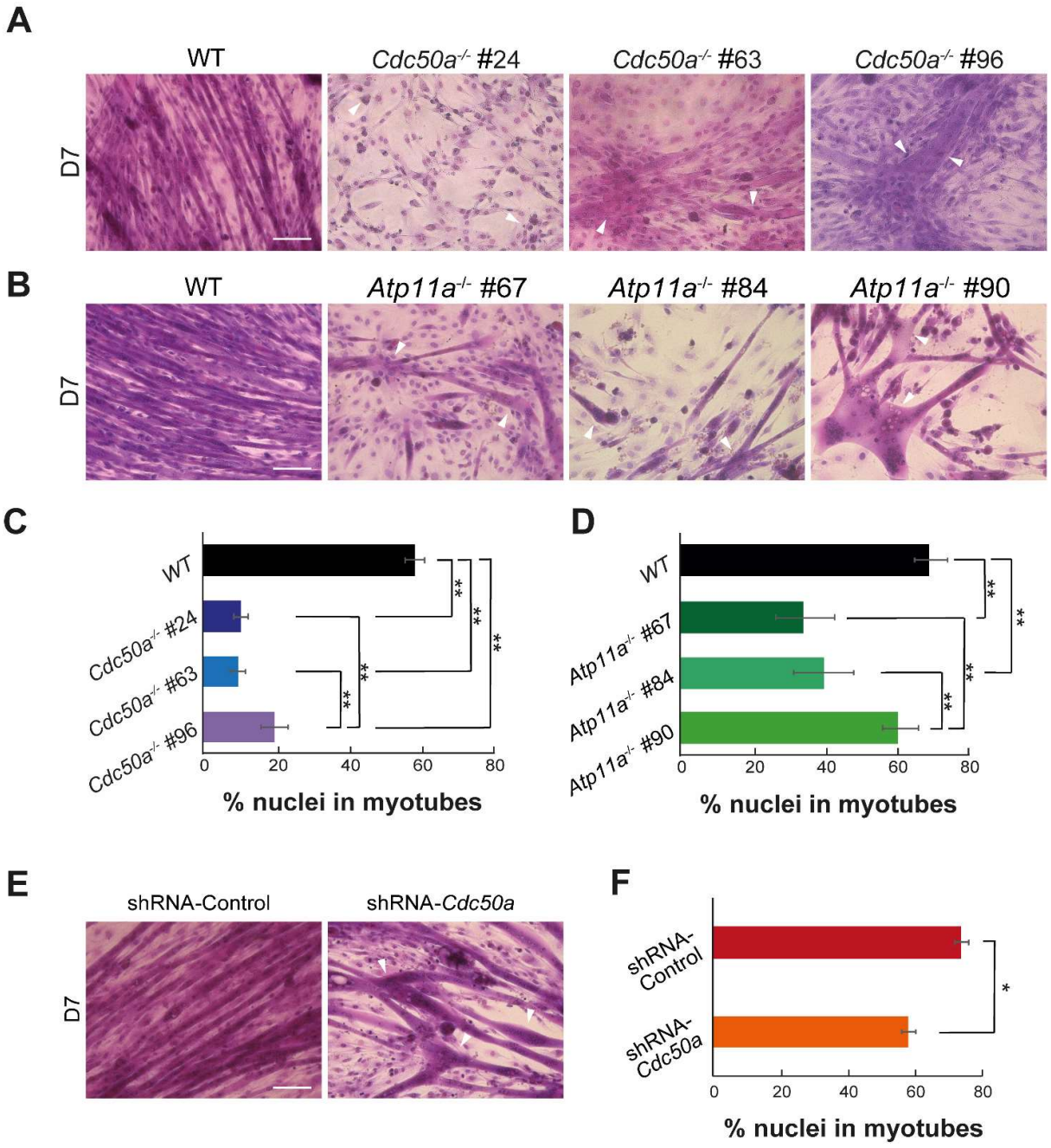

Fig. S5. High passage C2C12 cells lacking CDC50A or ATP11A are affected in myotobe formation. A, B, E) Representative microscopy images of wild-type (WT), three independently isolated CDC50A-deficient lines, three independently isolated ATP11A-deficient cell lines, and CDC50A-knockdown cells during differentiation at day 7 (D7). For knockdown, cells were stably transduced with scrambled shRNA control lentivirus or shRNA lentivirus targeted against CDC50A. Scale bars, 100 µm. Arrowheads indicate myotubes with abnormal morphology. C, D, F) Fusion index calculated at day 7 of differentiation. Data represents the mean ± S.D. of three experiments (ANOVA and TukeyHSD, **P<0.01).

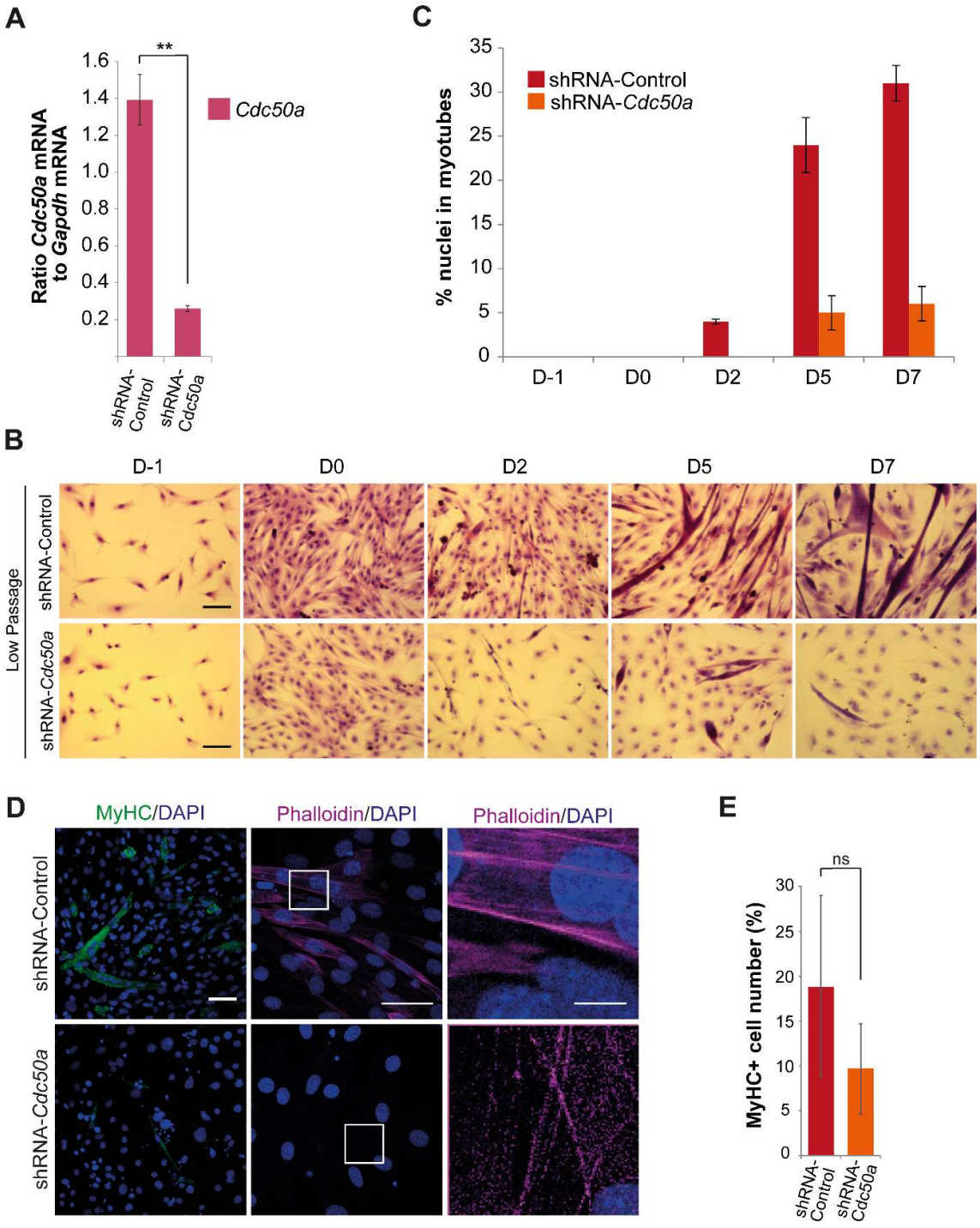

Fig. S6. Cdc50a knockdown in C2C12 cells impairs myotube formation. C2C12 cells were transduced with lentivirus carrying shRNA targeting CDC50A (shRNA-CDC50A) or the same amount of lentivirus carrying shRNA-Control. A) qRT-PCR was used to detect the efficiency of shRNA-*Cdc50a* mediated knockdown in proliferating C2C12 cells. The expression level of *Cdc50a* in the knockdown cells

was reduced to ~20% of the level in shRNA-Control. Data are presented as mean ± S.D. of three experiments (ANOVA and TukeyHSD, **P < 0.01). B) Representative microscopy images of low passage control and CDC50A-knockdown cells during proliferation (D-1) and differentiation at days 0 (D0), 2 (D2), 5 (D5) and 7 (D7). Scale bar, 100 µm. C) Calculation of fusion indices showed that shRNA-Cdc50a cells were severely deficient in myotube formation. Data represents the mean ± S.D. of two distinct fields of one experiment. D) Confocal imaging of control and CDC50A-knockdown cells immunostained at day 2 of differentiation for MyHC (left panel, green) and F-actin (middle panel, phalloidin, red). Nuclei (DAPI) are in blue. Right panels show zoomed-in images of the white boxes, adjusted with different tonal values, brightness and contrast, in order to enhance the low signal intensities. Scale bars: 50 µm (left and middle panels), 10 µm (right panel). E) Percentage MyHC-positive cells at day 2 of differentiation. Bars indicate S.D. from the mean of two independent experiments (ANOVA and TukeyHSD; ns, not significant).

Table S1. Cell media and material

Product	Company	Catalogue number
Dulbeccos's Modified Eagle's Medium - high glucose	Sigma-Aldrich	D6046-500ML
Dulbeccos's Modified Eagle's Medium - low glucose	Sigma-Aldrich	D6046-500ML
Fetal Bovine Serum	Capricorn Scientific	FBS-12A
Horse serum	Sigma-Aldrich	F9665-100ML
Penicilin/Streptomycin	Sigma-Aldrich	P0781-100ML
Trypsin	Sigma-Aldrich	T4549-100ML
Glutamin	Sigma-Aldrich	G7513-100ML
T-25 flasks	Sarstedt	83.3910
T-75 flasks	Sarstedt	83.3911
35 mm Petri dishes	Ibidi	81156
Hanks' Balanced Salt Solution	Sigma-Aldrich	H6648-500ML
Duclbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537-500ML
Goat serum	SouthernBiotech	0060-01
DAPI	Sigma-Aldrich	D9524-5MG
Bovine Serum Albumin	Sigma-Aldrich	A7030-50G
Triton X-100	Roth	3051.3

Table S2. Primers used for PCR

Target	Type	Primer sequence	Amplicon size
Cdc50a WT.A	Fwd	GACCCTAGTGCTTTGCTTGTAAGT	590 bp
	Rv	CACTATGGAAAGCTCAACAGTCAG	
Cdc50a HDR.A	Fwd	GTTGCTACTTAACCTCAGTAC	463 bp
	Rv	CTACTGCACTTATATACGGTTC	
Cdc50a WT.C	Fwd	CCTATGCCTTCTCTGTCAGC	306 bp
	Rv	CTTTTAGACAAGAAGGGATATTGATC	
Cdc50a HDR.C	Fwd	CTTCCATTTCTTGTGCTTAG	387 bp
	Rv	GTGAAGGAGAGATGCGAG	
Atp11a WT.A	Fwd	GTCTCAACCTGACGGTGTTG	504 bp
	Rv	GACTCCATCTCCACATCCTG	
Atp11a HDR.A	Fwd	GTCTCAACCTGACGGTGTTG	387 bp
	Rv	GCACTTATATACGGTTCTCCC	
Atp11a WT.B	Fwd	GAAACATAAGGCAGCCTAAAC	308 bp
	Rv	GGCAAACCTGCTTTGAAAAG	
Atp11a HDR.B	Fwd	GAAACATAAGGCAGCCTAAAC	434 bp
	Rv	GCGAAAAAGAACGTTCACG	
Atp11a WT.C	Fwd	GTTGCTAGGTGTTTCCAGG	408 bp
	Rv	GCCCTTAACTGTCAGACTACAC	
Atp11a HDR.C	Fwd	GTTGCTAGGTGTTTCCAGG	416 bp
	Rv	GTGAAGGAGAGATGCGAGC	

Table S3. Primers used for RT-PCR

Target	Type	Primer sequence	Amplicon size
Atp8a1	Fwd	TGAGAGATCGTGCAGAAAGGAG	155 bp
	Rv	GGGCTTTCAGTGGGAACCAAAAC	
Atp8a2	Fwd	GCGTACTTCTGGTTGGGATTG	166 bp
	Rv	CGTTACTGTCTCGAAGCATCG	
Atp8b1	Fwd	CAATTTTCGGCAGCATCGCACTG	137 bp
	Rv	CAGCCAGATGTATGGCTGTCTC	
Atp8b2	Fwd	GACACAATCCTGCTAGACAGGC	150 bp
	Rv	GGCCCACTCCTCATAGTACTCTTC	
Atp8b3	Fwd	ACTACGAGCCACTGGACATGC	162 bp
	Rv	CGTCTGAATCATAGATGCAGCC	
Atp8b4	Fwd	GGCCACATCATTGGTCATTGTGG	140 bp
	Rv	AGACACCATCACTGTGCATGGC	
Atp8b5	Fwd	CTAGTGCAGACGACTCTGATTGG	166 bp
	Rv	AGATGCTGGGGTACCTGAGACAC	
Atp9a	Fwd	GGCCACTGTCCTACAAGACGTTC	160 bp
	Rv	GCCACCATCAGTAGCTCAGTGAG	
Atp9b	Fwd	ACCCTGTTACGCCAGCATACC	169 bp
	Rv	TGCCGATGTGTCTGAACTGTG	
Atp10a	Fwd	GTGGATGCTGCCTTTCAAAGCC	138 bp
	Rv	AATACCCAGGTGCAGCAGGAAG	
Atp10b	Fwd	CAGTACCAATGTCTGTCCTTCCC	146 bp
	Rv	CACCTCCCCACAGATCTTTGTCG	
Atp10d	Fwd	CCTCACCTTCTGGATCACCTTG	161 bp
	Rv	CAATCACCAGGTGGAGGAGAATG	

Atp11a	Fwd	GCTGACCTTTGATTCTGTGCGG	142 bp
	Rv	TCTAGACCGGACCTGGTCTAC	
Atp11b	Fwd	GGACCAGCTTATCTCTTGCACTCAG	168 bp
	Rv	CATCACCAACAGCCAGTGTTATCGG	
Atp11c	Fwd	GAAACTGCTATTGGTTCATGGA	143 bp
	Rv	CATAGAGTGGCTGTTGTGAG	
Cdc50a	Fwd	GCCAGTAAACTGGCATAAGGCAG	159 bp
	Rv	GGGTGTAAATCATCTCTCCGCTC	
Cdc50b	Fwd	CTACCACGTCAAGTTCCGCAAC	160 bp
	Rv	CATCCACACCACGAAGTCCTG	
Cdc50c	Fwd	CCTTCCGGAGAAAATGGAGGG	173 bp
	Rv	TGATGGGAGTGTCATTGTGGG	
Gapdh	Fwd	CATCACTGCCACCCAGAAGACTG	153 bp
	Rv	ATGCCAGTGAGCTTCCCGTTCAG	

Knockout analysis by RT-PCR			
Cdc50a mRNA	Fwd	GGCAGTATATGAGCTAGACCCTGA	128 bp
	Rv	CTCTCCGCTCTATGAGACGATAC	
Atp11a mRNA	Fwd	CATATCCATGCTGTCCAGTGGG	134 bp
	Rv	CATCGGCCAATGCTTTGAGTC	
Housekeeping gene			
Gapdh	Fwd	CATCACTGCCACCCAGAAGACTG	153 bp
	Rv	ATGCCAGTGAGCTTCCCGTTCAG	