Muscle-Specific DNM2 Overexpression Improves Charcot–Marie–Tooth Disease In Vivo and Reveals a Narrow Therapeutic Window in Skeletal Muscle

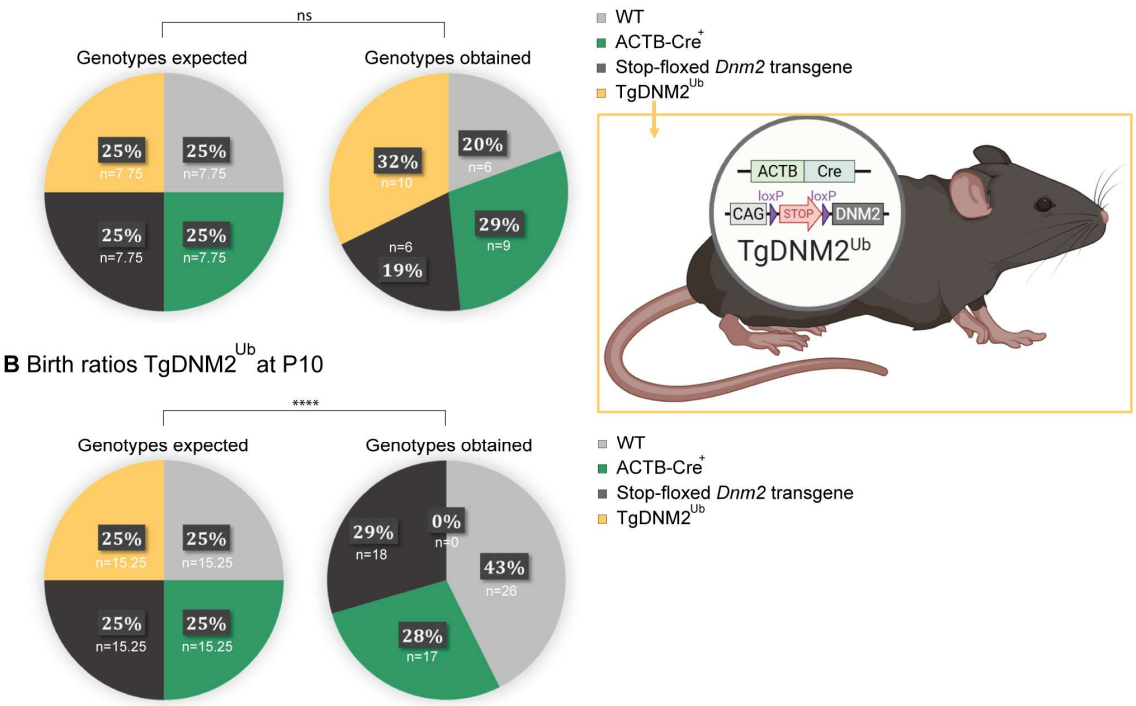
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SUPPLEMENTARY MATERIALS

Supplementary Fig. S1 to S5.

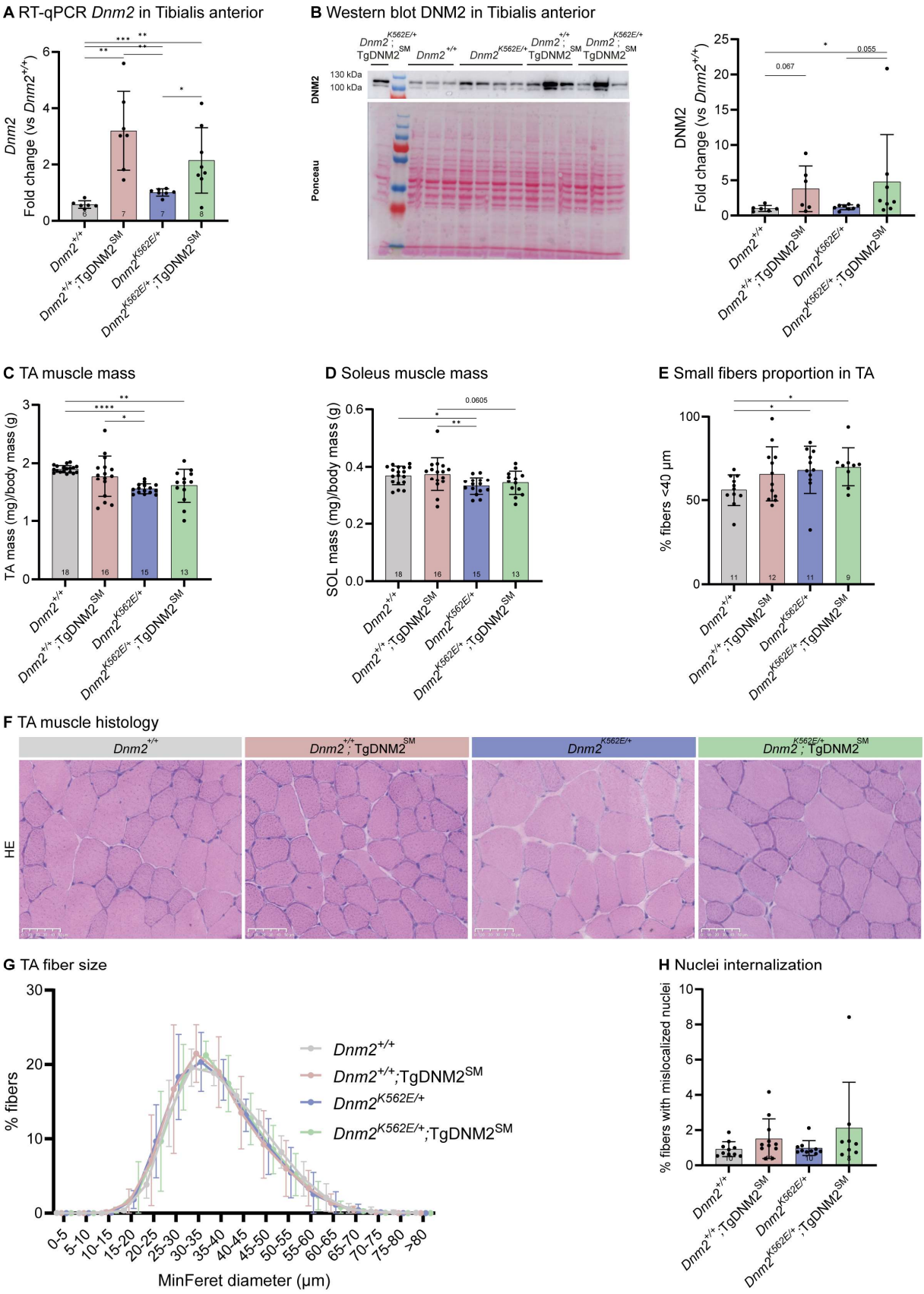
Supplementary Table S1. Reagents used. Antibodies used for immunofluorescence and western blots. Primers used for PCR and qRT-PCR.

Supplementary Table S2. Number of mice used per test and statistical analysis performed. For each test, number total of mice and sex-repartition as well as statistical analysis.

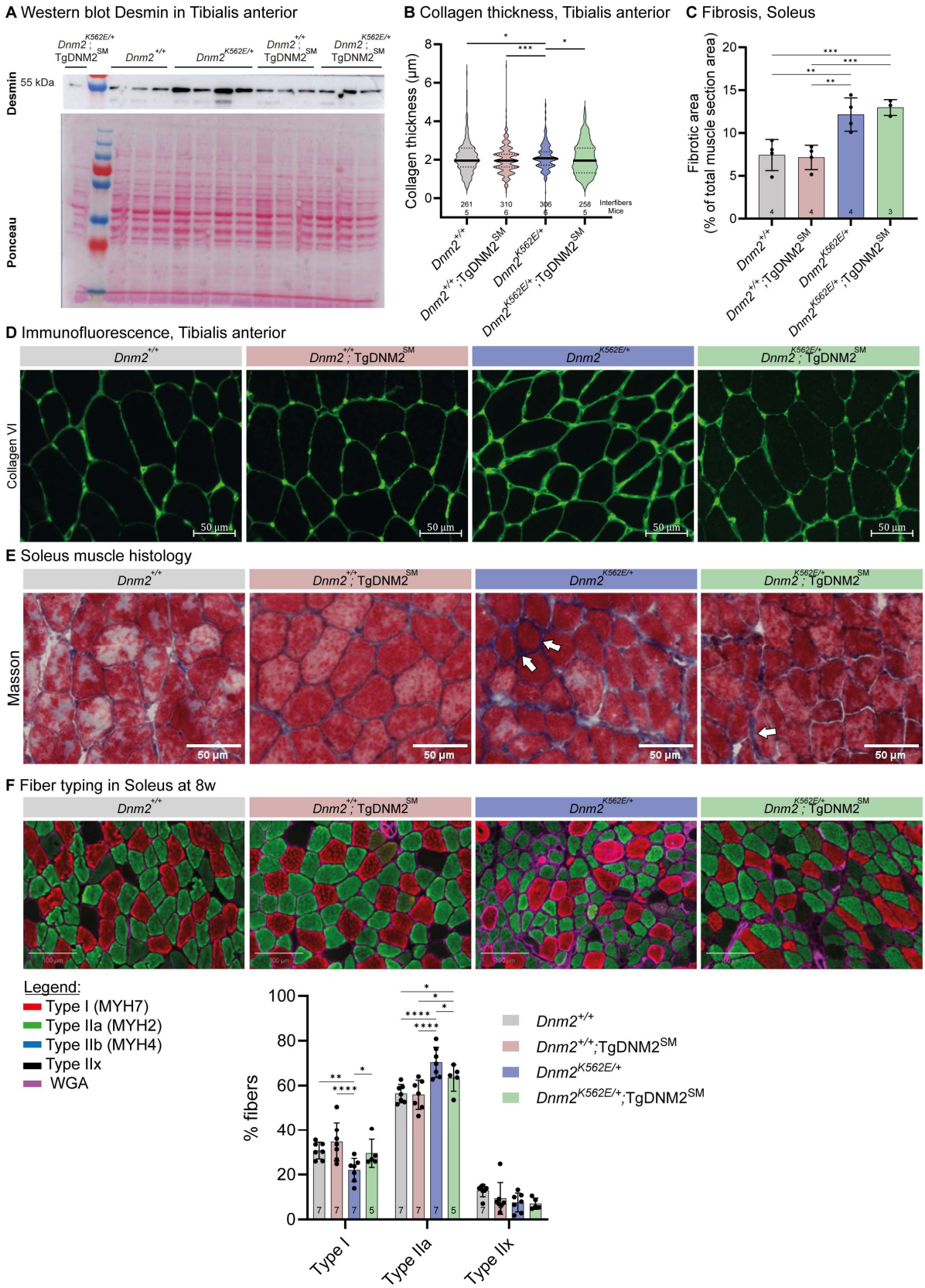


Group Name	Description
WT	No Cre, no *Dnm2* transgene
ACTB-Cre⁺	Cre only
Stop-floxed *Dnm2* transgene	CAG-LoxP-STOP-LoxP-*Dnm2*
TgDNM2^{Ub}	Cre excises STOP → DNM2 overexpression

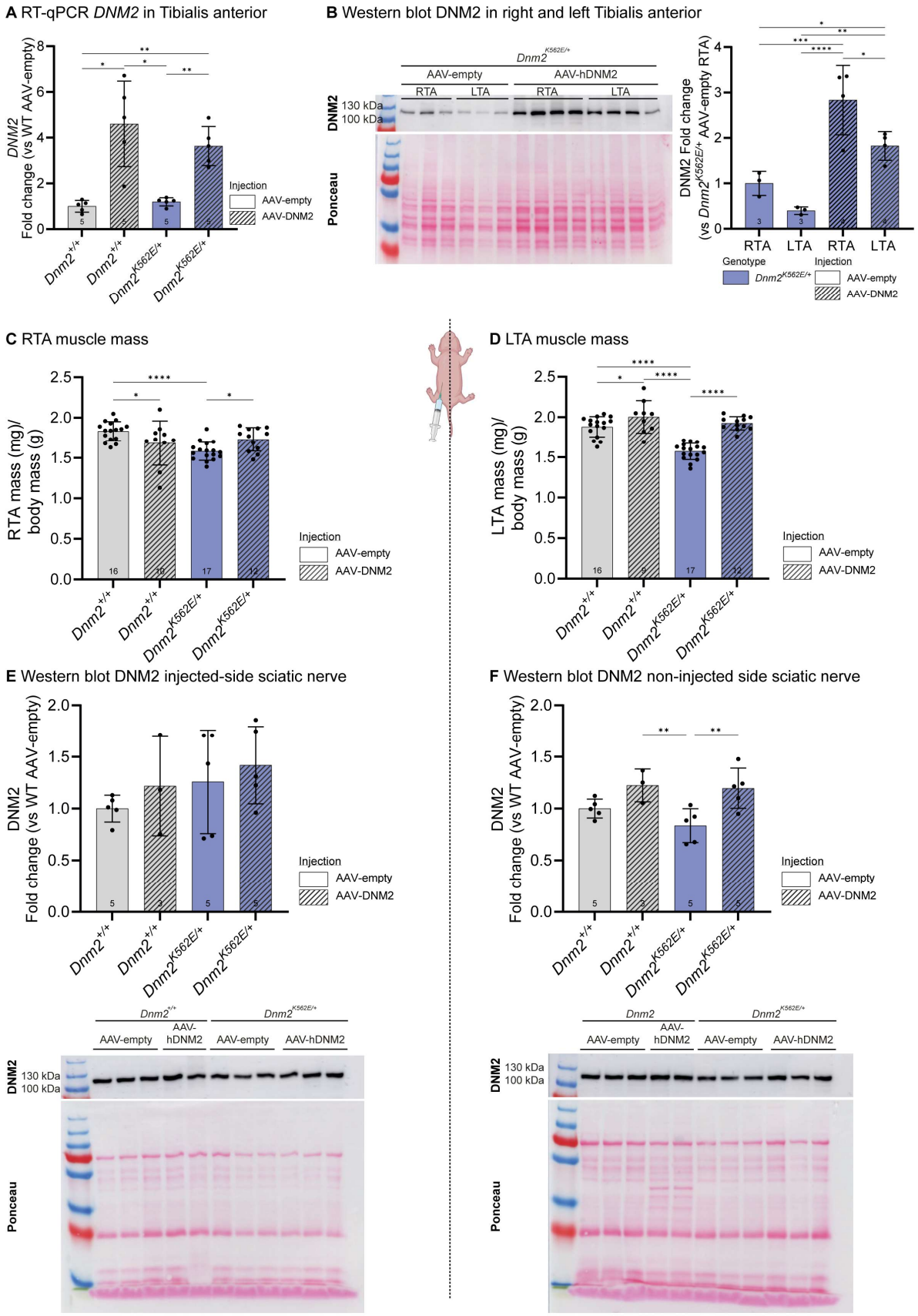
Supplementary Fig S1. The TgDNM2^{Ub} mouse line shows perinatal lethality. (A) Birth ratio expected and obtained at E18.5 from four different litters, in percentage and n number (*n*=31). **(B)** Birth ratio expected and obtained at P10 for the four groups in percentage and n number (*n*=61). ****p<0.0001. (A-B) Chi-squared test.



Supplementary Fig S2. Muscle-specific DNM2 overexpression does not improve *Dnm2*-CMT muscle atrophy. (A) RT-qPCR analysis of *Dnm2* expression in TA at 8w (6≤*n*≤8). **B)** Representative western blot and quantification of DNM2 protein in TA, normalized to Ponceau S staining (6≤*n*≤8). **(C-D)** Muscle mass of **(C)** TA and **(D)** Soleus normalized to body mass at 8w (13≤*n*≤18). **(E)** Proportion of small fibers (MinFeret<40 µm) in TA sections (9≤*n*≤12). **(F)** TA transversal sections stained with hematoxylin-eosin (HE). Scale bar= 50 µm. **(G)** TA fibers distribution based on their MinFeret diameter (9≤*n*≤12). **(H)** Proportion of fibers with internalized nuclei (8≤*n*≤11). Each dot represents a mouse. Values are represented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (A-E) ANOVA test. (H) Kruskal-Wallis test.



Supplementary Fig S3. Additional data on muscle-specific DNM2 overexpression from embryogenesis: key muscle protein expression, extracellular matrix and fiber type. (A) Representative western blot of Desmin protein level in TA at 8w and relative Ponceau S staining. **(B)** Collagen thickness (258≤*n*=interfibers≤310, 5≤*n*=mice≤6). **(C)** Fibrotic area in Soleus muscle (blue area on Masson's trichrome/total area) (3≤*n*≤4). **(D)** Immunolabelling of collagen VI in transversal TA sections. Scale bar= 50 µm. **(E)** Soleus transversal sections stained with Masson Trichrome; arrows indicate fibrosis. Scale bar= 50µm. **(F)** Immunolabelling of fiber types in transversal Soleus sections and quantification. Scale bar= 100 µm. (B) Individual interfiber spaces are plotted. (C, F) Each dot represents a mouse. Values are represented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001. (B) Kruskal-Wallis test. (C, F) ANOVA test.



Supplementary Fig S4. Additional data on postnatal DM2 delivery for muscle and nerves. (A) RT-qPCR analysis of *Dnm2* expression in TA (random sides) at 8w (*n*=5). **(B)** Representative western blot and quantification of DNM2 protein level in right (RTA, IP-injected side) and left TA (LTA, contralateral side) in *Dnm2*$^{K562E/+}$ mice at 8w, normalized to Ponceau S staining (3≤*n*≤4). **(C-D)** TA muscle mass normalized to body mass at 8w: **(C)** RTA and **(D)** LTA (9≤*n*≤17). **(E-F)** Representative western blot and quantification of DNM2 protein level in sciatic nerve at 8w, normalized to Ponceau S staining in **(E)** IP-injected side (3≤*n*≤5) and **(F)** contralateral side (3≤*n*≤5). Each dot represents a mouse. Values are represented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (A-F) ANOVA test.

A Part I : Muscle-specific DNM2 overexpression (TgDNM2^{SM}) : summarized phenotypes and therapeutic effect

	In vivo tests	*Dnm2*$^{+/+}$	*Dnm2*$^{K562E/+}$ ♂	*Dnm2*$^{K562E/+}$ ♀
8w	Body weight			
	Hanging time			
	Body stretch			
	Body length			
	Stride			
	Paw angle			
	Stance phase			

	Muscular tissue analyses	*Dnm2*$^{+/+}$	*Dnm2*$^{K562E/+}$
TA	DNM2 level	3.8x ↗	4.1x ↗
	TA muscle mass		
	Fiber size		
	Nuclei internalization		
	Desmin level		
	Desmin IF		
	Integrin IF		
	Collagen fibrosis		
Soleus	Soleus muscle mass		
	CytC level		
	OXPHOS complex V	↗	
	OXPHOS complex III		
	OXPHOS complex II		
	OXPHOS complex I		
	NADH intensity		
	NADH internalization		
	mtDNA content		
	WGA defects IF		
	Masson staining		
	Fiber type		

Legend

No initial phenotype
DNM2 overexpression provided:
Phenotype worsening
No rescue
Tendency to rescue
Partial rescue
Total rescue

B Part II : Postnatal DNM2 overexpression (AAV P3 IP) : summarized phenotypes and therapeutic effect

General phenotype	*Dnm2*$^{+/+}$		*Dnm2*$^{K562E/+}$ ♂	*Dnm2*$^{K562E/+}$ ♀
Body weight				
Body length after death				
Hanging time				
Notched bar				
Muscular tissue analyses	Right side 💉	Left side	Right side 💉	Left side
DNM2 level	5.7X ↗	3.8X ↗	5.3X ↗	3.7X ↗
TA mass		*Increased*		
Fiber size				
Internalized nuclei				
Internalized SDH				
Desmin localization				
β1-integrin localization				
Neurological tissue analyses	Injected side	Non-inj. side	Injected side	Non-inj. side
DNM2 level				

Supplementary Fig. S5. Overview of the improvements provided by DNM2 overexpression in *Dnm2*$^{K562E/+}$ mice. (A) Summary of the therapeutic effects from transgenic overexpression of murine DNM2 in striated muscles of *Dnm2*$^{+/+}$ and *Dnm2*$^{K562E/+}$ mice from embryogenesis, based on in vivo tests and muscle tissue analyses at 8w. **(B)** Summary outcomes following intraperitoneal (IP) injection of human DNM2 at postnatal day 3 (P3) in control and *Dnm2*$^{K562E/+}$ mice, with assessments at 8 weeks. TA= Tibialis anterior. Tendency to rescue= no significant difference between treated mutant mice and either untreated mutants or controls. Partial rescue= treated mutants differ from both untreated mutants and controls. Total rescue= treated mutants differ from untreated mutants but are similar to controls.

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Muscle immunofluorescence antibodies (dilution)		
Integrin β1, rat monoclonal (1:250)	Sigma-Aldrich	MAB1997
↳ GARat Alexa 488, goat polyclonal (1:250)	Thermo Scientific	A-11006
Desmin, rabbit polyclonal (1:250)	Abcam	AB15200
↳ GAR Alexa 555, goat polyclonal (1:250)	Invitrogen	A21430
Collagen VI, rabbit polyclonal (1:250)	Novus Biologicals	NB 120-6588
↳ GAR Alexa 555, goat polyclonal (1:250)	Invitrogen	A21430
Anti-type I fibers: MYH7: mouse IG2b (1:50)	DSHB	BA-D5
↳ GAM IgG2b Cy3 (1:100)	Jackson Immunoresearch	115-165-207
Anti-type IIa fibers: MYH2: mouse IgG1 (1:50)	DSHB	SC-71
↳ GAM IgG1 Alexa 488 (1:100)	Jackson Immunoresearch	115-545-205
Anti-type IIb fibers: MYH4: mouse IgM (1:50)	DSHB	BF-F3
↳ GAM IgM DyLight 405 (1:100)	Jackson Immunoresearch	115-475-075
WGA, Wheat Germ Agglutinin, Alexa 647 (1:200)	Thermo Scientific	W32466
DAPI (1:1000)	/	/
Western blot antibodies (dilution)		
DNM2, rabbit polyclonal (1:1000)	Homemade (2865)	N/A
DNM2, rabbit polyclonal (1:1000)	ThermoFisher	PA5-19800
Desmin, rabbit polyclonal (1:1000)	Abcam	15200
OXPHOS, mouse monoclonal antibody cocktail (1: 1000)	ThermoFisher	45-8199
cytC, rabbit polyclonal (1:1000)	Cell Signaling	4272
GAR perox, goat polyclonal (1:10000)	Jackson Immunoresearch	111-036-045
GAM perox, goat polyclonal (1:10000)	Jackson Immunoresearch	115-036-068

Genotyping PCR oligos	5'-sequence-3'
6115 Er KE	TACACTGTCTGCACTGTCGAGCCCTG
6116 Ef KE	GCCATCTTCAACACAGAGCAGAGGTG
Cre 160	GAACCTGATGGACATGTTCAGG
Cre 161	AGTGCGTTCGAACGCTAGAGCCTGT
Sf 10692	GGCCCACCATTATCCGCCC
Wr 4035	CCTTTAAGCCTGCCCAGAAG
AAV titration qPCR oligos	
hDNM2 for	ATCAGGTGGACACTCTGGAGC
hDNM2 rev	GCATAGCTGATCTCCCGTCG
CMVe-CAG For5	TACGGTAAACTGCCCACTTG
CMVe-CAG Rev8	AGGAAAGTCCCATAAGGTCA
qPCR oligos	5'-sequence-3'
Rps11 for	CGCGTGGTGAATAAGGAAGC
Rps11 rev	GTAAGCACGCTCCGTCTGAA
Rpl27 for	AAGCCGTCATCGTGAAGAACA
Rpl27 rev	CTTGATCTTGGATCGCTTGGC
Dnm2 ex6	ACCCCACACTTGCAGAAAAC
Dnm2 ex8	CGCTTCTCAAAGTCCACTCC
Dnm2 ex10 m+h	GTCAAGCTGAAAGAGCCCTG
Dnm2 ex13 m+h	CTTCTTGTTCAGCTGCGTGC
Nd1 for	AAGTTGATCGTAACGGAAGC
Nd1 rev	CCCATTCGCGTTATTCTT

Supplementary Table S1. Reagents used. Antibodies used for immunofluorescence and western blots. Primers used for PCR and qRT-PCR.