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Supplemental Information

Myostatin: a Circulating Biomarker Correlating with Disease in Myotubular Myopathy Mice and Patients

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Supplementary information

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Supplementary tables

Supplementary Table 1. Antisense oligonucleotide sequences used in this study, and targeted region of the *Dnm2* gene

	Sequence	Targeted region	Reference
ASO-Ctrl (control)	GGCCAATACGCCGTCA	No homology to mouse genome	[1]
DYN101 (targeting *Dnm2*)	GGCATAAGGTCACGGA	Exon 17	[1]

Sequences do not target exon 11 harbouring the p.R465W mutation.

Supplementary Table 2. Primers used for quantitative PCR analysis

Gene	**Forward primer**	**Reverse primer**
Rpl27	5'-AAGCCGTCATCGTGAAGAACA-3'	5'-CTTGATCTTGGATCGCTTGGC-3'
Dnm2	5'ACCCCACACTTGCAGAAAAC-3'	5'CGCTTCTCAAAGTCCACTCC-3'
Mstn (Gdf8)	5'-GCACTGGTATTTGGCAGAGTA-3'	5'- CACACTCTCCTGAGCAGTAAT-3'
AcvRIIb	5'- GCTCAGCTCATGAACGACT-3'	5'- CTCTGCCACGACTGCTTGT -3'
Fstn	5'- AAAACCTACCGCAACGAATG-3'	5'- TTCAGAAGAGGAGGGCTCTG -3'
Gdf11	5'- ATCAGCCGGGAGGTAGTGAA-3'	5'- CTGGGCCATGCTTATGACCGT-3'
Pre-miR-27a	5'-GCTTAGCTGCTTGTGAGCAA-3'	5'-GGTCCAGGGGGCGGAA-3'
Pre-miR-206	5'-CCAGGCCACATGCTTCTTTAT-3'	5'-CCAAAACCACACACTTCCTTAC-3'

Supplementary Table 3. Transcriptomics data from *Mtm1*$^{-/y}$ mice. Transcriptomics data generated from RNAseq of tibialis anterior muscles in WT and *Mtm1*$^{-/y}$ mice at 2 or 7 weeks of age, represented as Log2 fold change (LFC). P<0.05 values highlighted in bold.

Gene		LFC wild type versus *Mtm1*$^{-/y}$ mice		LFC Mtm1$^{-/y}$ versus *Mtm1*$^{-/y}$ *Dnm2*$^{-/+}$mice		LFC wild type versus *Mtm1*$^{-/y}$ *Dnm2*$^{-/+}$mice	
Gene name	Accession number	2 weeks	7 weeks	2 weeks	7 weeks	2 weeks	7 weeks
AcvRIIB	NM_007397.3 NM_001313757.1	0.20496	-0.31953	-0.14742	0.61394	0.05112	0.28053
Cebpa	NM_001287523.1	0.87843	1.09515	-0.43850	0.04380	0.43216	1.12936
Cebpb	NM_009883.4	-0.16426	0.36777	-0.17221	0.99464	-0.34000	1.36327
Cebpd	NM_007679.4	-0.06872	0.86015	0.25675	0.10013	0.18327	0.96194
Cebpg	NM_009884.3	**-0.62751**	**-0.92029**	0.38291	0.28650	-0.25269	-0.64234
Fstn	NM_001301373.1 NM_008046.3 NM_001301375.1	**1.06118**	**2.76568**	-0.29028	**-1.42604**	0.76271	**1.32725**
Gdf11	NM_010272.2	0.70512	**1.57504**	-0.18480	**-1.51455**	0.51185	0.05522
miR-27a	NR_029746.1	**2.85030**	**2.61976**	-1.56849	-1.62360	1.27258	*1.00195*
miR-206	NR_029593.1	**2.60111**	**3.92160**	-2.59326	-1.64692	0.01645	*2.23938*
Mstn	NM_010834.3	**-1.02877**	**-1.94102**	0.69297	0.47260	-0.34363	**-1.4777**
Murf1	NM_001039048.2 NM_001369245.1	**1.25695**	**1.51663**	-0.03128	**-0.72095**	**1.21758**	**0.78874**
Myc	NM_001177352.1 NM_001177354.1	0.38964	**1.63689**	0.13340	0.38796	0.51366	**1.24116**
Myogenin	NM_031189.2	**1.59291**	**3.81960**	-0.77485	-0.58433	0.81100	**3.22743**
Smad2	NM_010754.5 NM_001252481.1 NM_001311070.1	0.34558	0.33336	**-0.31109**	-0.47437	0.02696	-0.14888
Smad3	NM_016769.4	-0.20634	**-0.49153**	0.16633	-0.18901	-0.04772	**-0.68908**
Smad9/Smad8	NM_019483.5	-0.63330	**1.39845**	-0.18048	-0.84205	-0.82060	0.55441

Supplementary Table 4. ROC analysis from studies 1,3 and 5 (shown in figure 2G). This table shows for hanging time performance in 10 second intervals, the identified corresponding myostatin value the rate of True Positives (indicating myostatin level correctly predicts hanging test ability at the identified cut-off value) and the corresponding rate of False Positives (where myostatin incorrectly predicts hanging test performance). 37ng/ml was identified for all hanging times to have a true positive rate over 0.8 .

Hanging time	Myostatin value	True Positive Rate	False Positive Rate
10	37ng/ml	0.80851	0.1
20	37ng/ml	0.80851	0.1
30	37ng/ml	0.80851	0.1
40	37ng/ml	0.80851	0.1
50	37ng/ml	0.82609	0.09091
60	37ng/ml	0.82609	0.09091

Supplementary methods

Antisense oligonucleotides (ASO) injections in *Dnm2*$^{RW/+}$ mouse line. The *Dnm*2 R465W heterozygous KI (*Dnm2*$^{RW/+}$) mouse line was generated as previously described [2]. We analyzed male *Dnm*2 R465W heterozygous KI (*Dnm2*$^{RW/+}$) C57BL/6J strain mice. Intraperitoneal injections of 25mg/kg of ASO were performed in *Dnm2*$^{RW/+}$ or wild type mice, weekly from 8-12 weeks of age. Myostatin measurements

were performed from plasma taken at 12 weeks of age, using the protocol described in the main text of Koch et al. Animal experimentation was approved by the institutional ethical committee Com'Eth IGBMC-ICS; APAFIS#14725-2018041809558996.

Transcriptomics analysis. WT, *Mtm1*$^{-/y}$ and *Mtm1*$^{-/y}$*Dnm2*$^{+/-}$ mice were housed and sacrificed as the other cohorts. Animal experimentation was approved by the institutional ethical committee Com'Eth IGBMC-ICS; APAFIS#5453-2016052510176016. TA muscles were extracted at 2w (disease onset in *Mtm1*$^{-/y}$) and 7w (late disease state in *Mtm1*$^{-/y}$) from 4 male mice per group and timepoint. *Mtm1*$^{-/y}$*Dnm2*$^{+/-}$ mice do not develop the *Mtm1*$^{-/y}$ phenotypes [3]. Total RNA was extracted using Trizol reagent (Invitrogen, UK) and 1µg of total RNA was reverse transcribed to cDNA by Superscript IV reverse transcriptase (Thermofischer Scientific). RNA-Seq libraries were generated from polyA mRNA using TruSeq Stranded mRNA Sample Preparation Kit (Illumina, Part Number RS-122-2101). RNAseq was performed on Illumina HiSeq4000 sequencer with single 50 nucleotide read to 50 million read average per samples. Reads were mapped onto mm10 assembly of mouse genome using STAR version 2.5.3a [4]. Quantification of gene expression was performed using HTSeq v0.6.1p1 [5] and gene annotations from Ensembl release 90, on uniquely aligned reads. Comparisons of interest and statistical analyses were performed as described previously [6] implemented in the DESeq2 Bioconductor library (DESeq2 v1.16.1).

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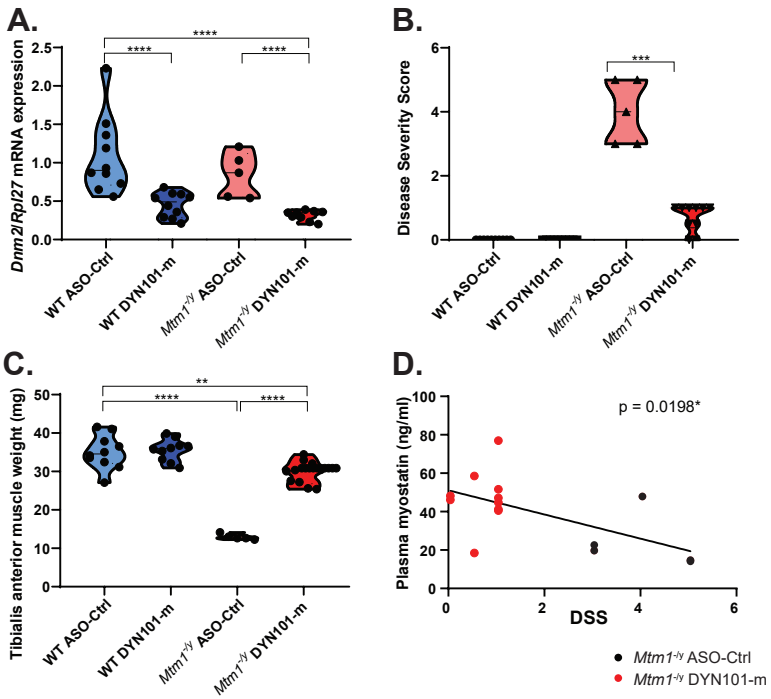
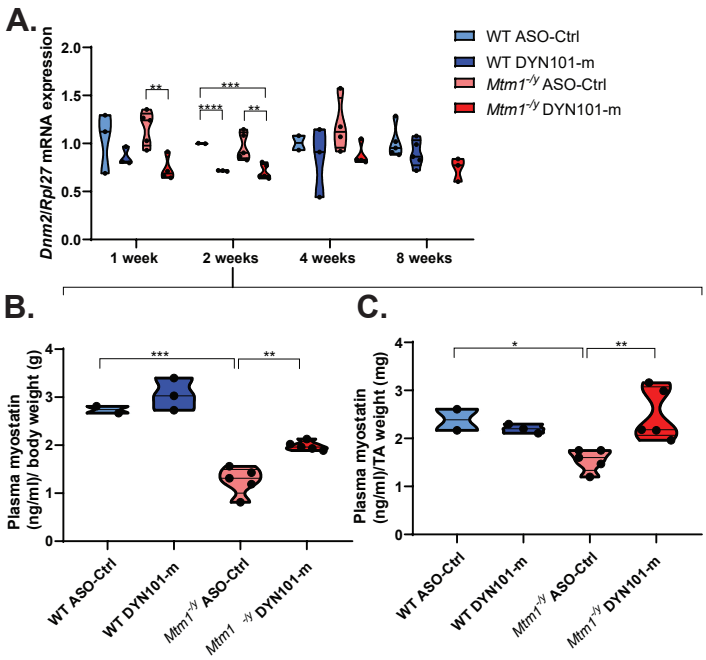
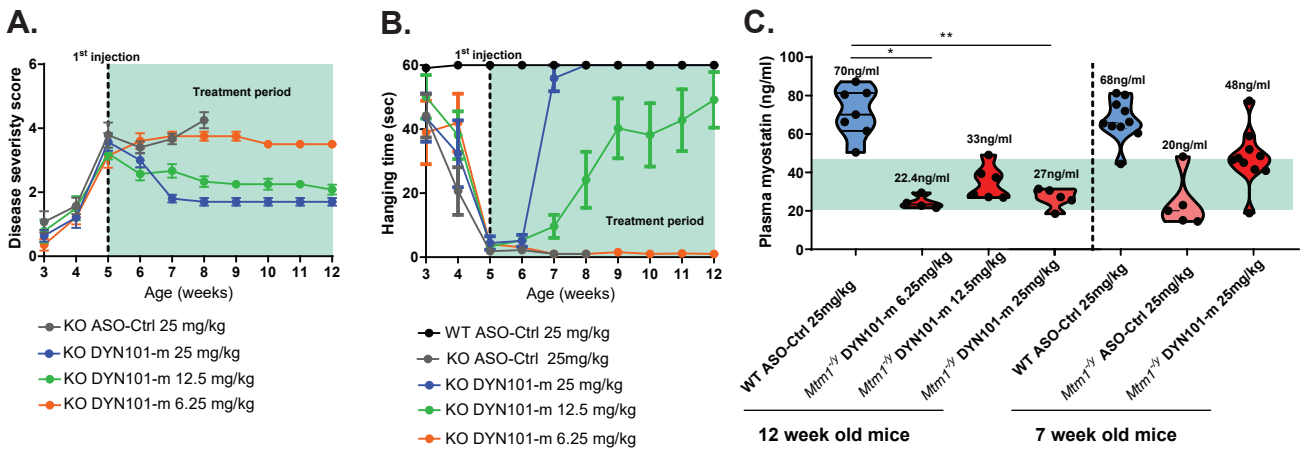


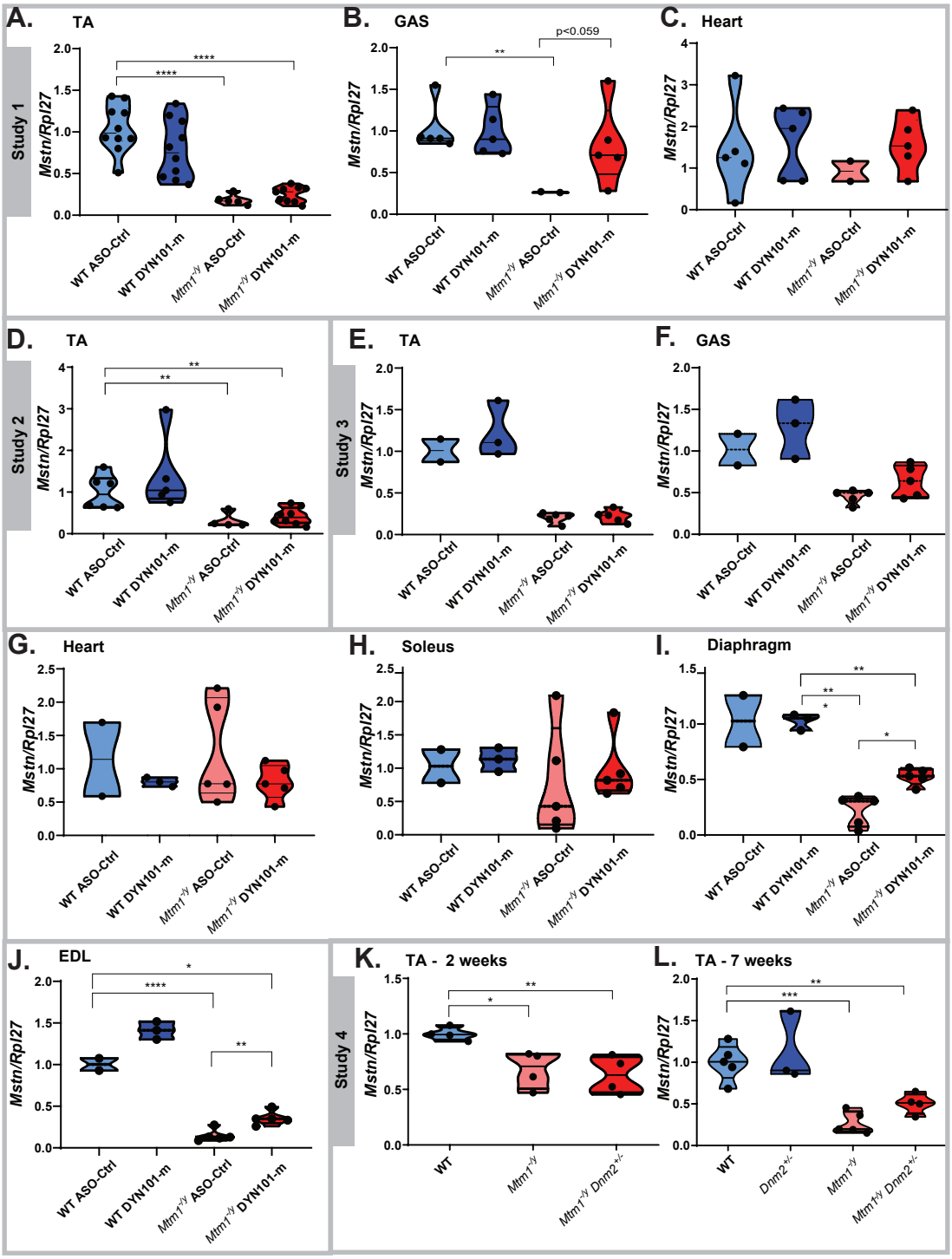
Supplementary figure 1: Circulating myostatin is reduced in *Mtm1*$^{-/y}$ mice, and improved in response to antisense oligonucleotide mediated reduction of *Dnm2*. (A) *Dnm2* mRNA expression quantified from tibialis anterior muscles by qRT-PCR analysis, relative to *Rpl27* expression, in wild type (WT) and *Mtm1*$^{-/y}$ mice, treated with ASO targeting *Dnm2* (DYN101-m) or ASO-control (ASO-Ctrl). (B) Disease severity score (DSS) from wild type and *Mtm1*$^{-/y}$ mice, treated with DYN101-m targeting *Dnm2* mRNA or ASO control (ASO-Ctrl), at 7 weeks of age. (C) Tibialis anterior (TA) muscle mass from WT and *Mtm1*$^{-/y}$ mice, treated with ASO targeting *Dnm2* (DYN101-m) or ASO-control (ASO-Ctrl). (D)
Linear regression analysis was performed between plasma myostatin levels (figure 1E) and disease severity score (B) in *Mtm1*$^{-/y}$ mice following ASO-Ctrl (black dots) or DYN101-m administration (red dots). Line of best fit shown, slope=-6.21+/-2.34, 95% confidence interval (CI) -11.25 to -1.1590, p value displayed. Pearson correlation analysis was also performed (r=-0.5931, p=0.0198*). Each point represents one mouse, minimum 5 mice per group. Results in (A)-(C) presented as violin plots, one dot per mouse. *p<0.05, **p<0.01, ***p<0.001. All results from study 1 (see table 1 for details).



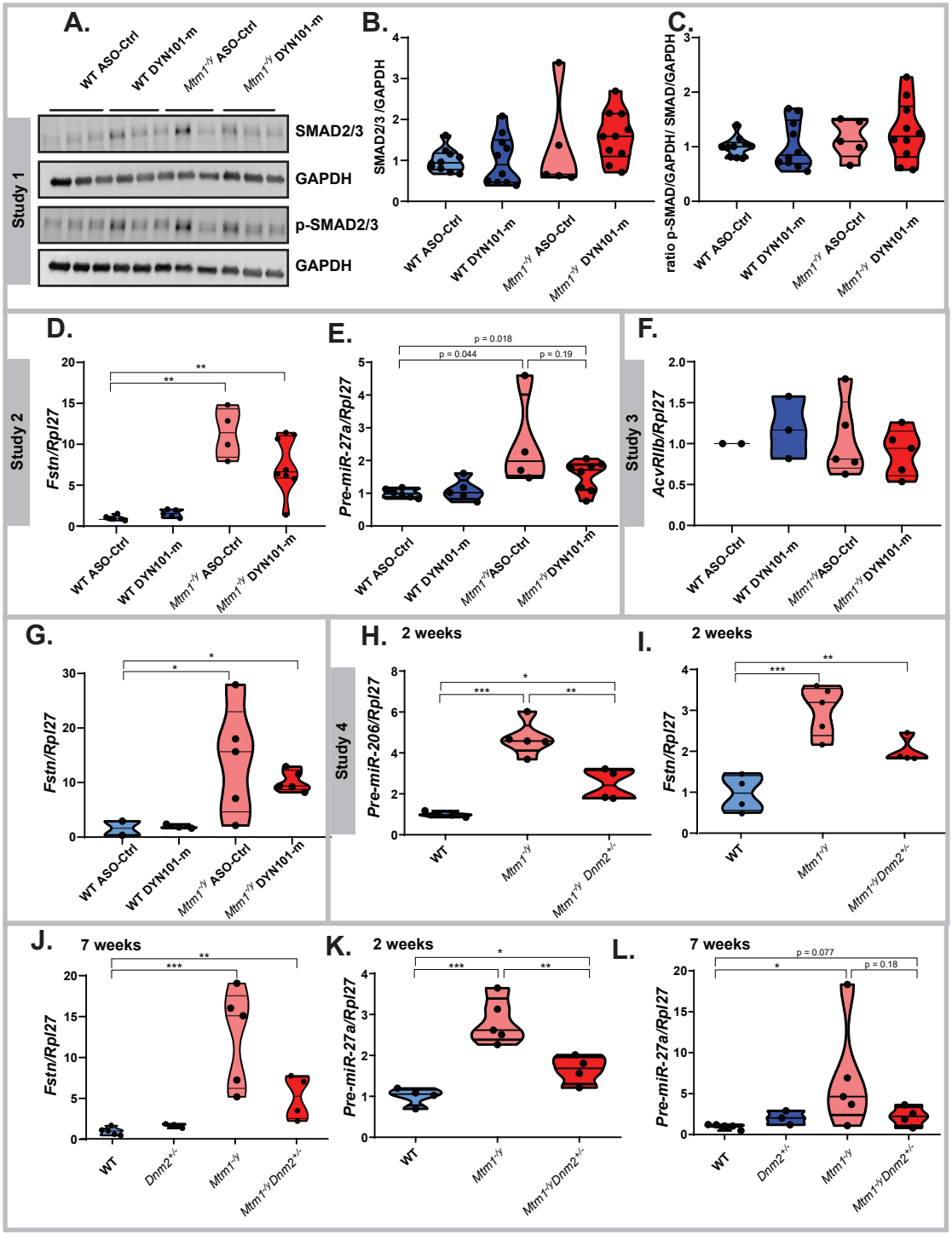
Supplementary figure 2. Circulating myostatin levels respond to *Dnm2* reduction in a time-dependent manner, correlating with reduced disease severity in *Mtm1*$^{-/y}$ mice. (A) *Dnm2* mRNA expression quantified by qRT-PCR analysis, relative to *Rpl27* expression from tibialis anterior muscles, in wild type (WT) and *Mtm1*$^{-/y}$ mice, treated with DYN101-m targeting *Dnm2,* or ASO control (ASO-Ctrl), 1, 2, 4 and 8 weeks post single injection. Myostatin plasma protein relative to body weight (B) or tibialis anterior muscle weight (C), 2 weeks post single injection. Results presented as violin plots, one dot per mouse. *p<0.0125, **p<0.01, ***p<0.001, ****p<0.0001. All results from study 3.



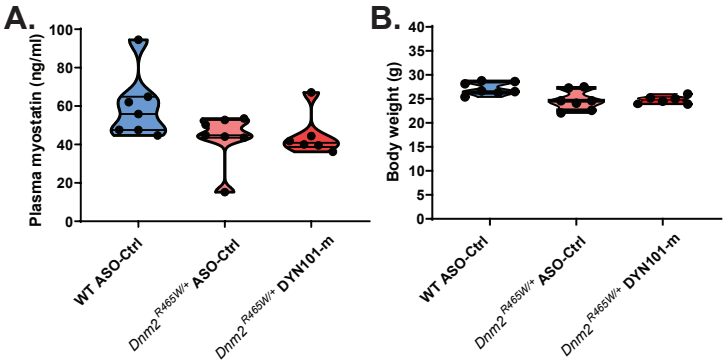
Supplementary figure 3. Circulating myostatin is reduced in *Mtm1*$^{-/y}$ mice, and improved in response to antisense oligonucleotide mediated reduction of *Dnm2*. (A) Disease severity score (DSS) from wild type and *Mtm1*$^{-/y}$ mice, injected with DYN101-m targeting *Dnm2* mRNA (6.25, 12.5 or 25mg/kg) or ASO control (ASO-Ctrl, 25mg/kg), weekly from 5-12 weeks of age. (B) Hanging test performed from 3-12 weeks of age, maximum time 60 secs. For (A-B) green shading highlights treatment period, graphs represent mean+s.e.m. (C) Myostatin protein levels in plasma (ng/ml) in 12 week old mice. Results presented as violin plot, one dot per mouse, median values listed for each group. For comparison results from figure 1E from 7 week old mice are shown on right hand side. Green shading highlights range between minimum and maximum values from 7 week old *Mtm1*$^{-/y}$ mice. *<0.05, **<0.01, ***<0.001. N=7 mice per group. All results from study 5.



Supplementary figure 4. *Mstn* mRNA analysis across cohorts. *Mstn* mRNA analysis relative to *Rpl27* expression from various cohorts. (A-C) Repeated injections from 2-7 weeks of age of antisense oligonucleotides targeting *Dnm2* (DYN101-m) or control (ASO-Ctrl) in wildtype (WT) and *Mtm1*$^{-/y}$ mice (analyzed in figure 1). Analysis of tibialis anterior (TA)(A), gastronemius (GAS)(B) and cardiac (C) muscles shown. (D) Analysis of *Mstn/Rpl27* mRNA from a second independent cohort following repeat ASO injections. (E-J) Single injection cohort (analyzed in figure 2), analysis from the TA (E), GAS (F), cardiac (G), soleus (H), diaphragm (I) and extensor digitorum longus (EDL)(J) muscles, 2 weeks post single injection, age at analysis: 5 weeks. (K-L) Tibialis anterior analysis of WT, *Mtm1*$^{-/y}$ mice, and *Mtm1*$^{-/y}$*Dnm2*$^{+/-}$ mice at 2 weeks (K) or 7 weeks (L) of age. *Dnm2*$^{+/-}$ mice are also shown at 7 weeks of age. Values also represented in table 2 in the main text of this manuscript. Results presented as violin plots, one dot per mouse. Kruskal-Wallis and Mann-Whitney multiple tests were performed. *p<0.0125, **p<0.01, ***p<0.001, ****p<0.0001. Detailed study design listed in table 1.



Supplementary figure 5. Analysis of the myostatin pathway in *Mtm1*$^{-/y}$ mice. (A) Immunoblot for protein expression of total or phosphorylated SMAD2/3 (p-SMAD2/3) and GAPDH (protein loading control) in tibialis anterior muscles. Total SMAD2/3 protein expression (B), or phosphorylated relative to total SMAD2/3 (C) quantified relative to GAPDH loading control in tibialis anterior. *Fstn* expression quantified by qRT-PCR analysis, relative to *Rpl27* levels for study 2 (D), study 3 (G), and study 4 and 2 weeks (I) or 7 weeks (J) of age. *Pre-miR-27a* mRNA expression quantified by qRT-PCR analysis, relative to *Rpl27* expression from study 2 (E), and study 4 and 2 weeks (K) or 7 weeks (L) of age. (F) *AcvRIIb* expression analyzed relative to *Rpl27* expression from study 3. (H) mRNA analysis of *Pre-miR-206* relative to *Rpl27* expression from tibialis anterior skeletal muscles from study 4 at 2 weeks of age. Results presented as violin plots, one dot per mouse, *p<0.0125, **p<0.01, ***p<0.001, ****p<0.0001. (A)-(C) Study 1. (D)-(E) Study 2. (F)-(G) Study 3 (2 weeks post single injection). (H)-(L) Study 4. See table 1 for study details. Tibialis antieror muscles analyzed.



C.

Genotype	Minimum ferrets diameter
WT + ASO-Ctrl	40.3±2.7µm
Dnm2$^{R465W/+}$ + ASO-Ctrl	34.5±2.4µm **
Dnm2$^{R465W/+}$ + DYN101-m	36.8±1.6µm

Supplementary figure 6. Myostatin levels in an autosomal form of Centronuclear myopathy. (A) Circulating myostatin protein levels in plasma (ng/ml) from wildtype (WT) and *Dnm2* R465W knock-in mice (*Dnm2*$^{R465W/+}$), injected with DYN101-m targeting *Dnm2,* or ASO control (ASO-Ctrl), weekly from 8-12 weeks of age, analysis performed at 12 weeks of age. (B) Body weight from same cohort of mice, represented in grams (g). (C) Minimum ferrets diameter, represented as mean+/-SD. A minimal but significant reduction in fiber size was observed in *Dnm2*$^{R465W/+}$ mice compared to WT (14%). N=6-7 mice per group, from study 6. Results presented as violin plot, one dot per mouse. **p<0.01 versus WT mice.