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Supplemental Information

GAA Deficiency in Pompe Disease Is Alleviated by Exon Inclusion in iPSC-Derived Skeletal Muscle Cells

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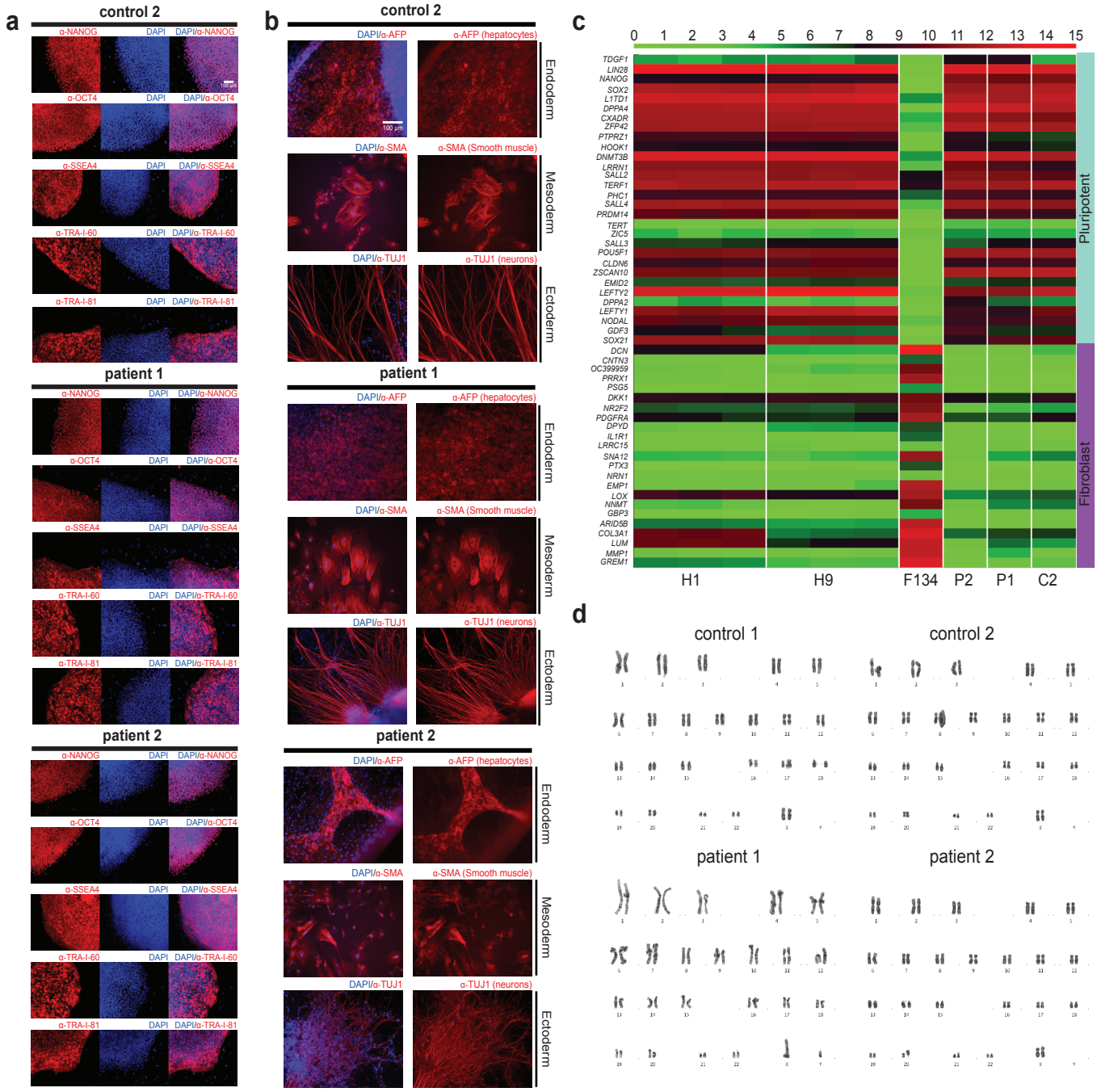
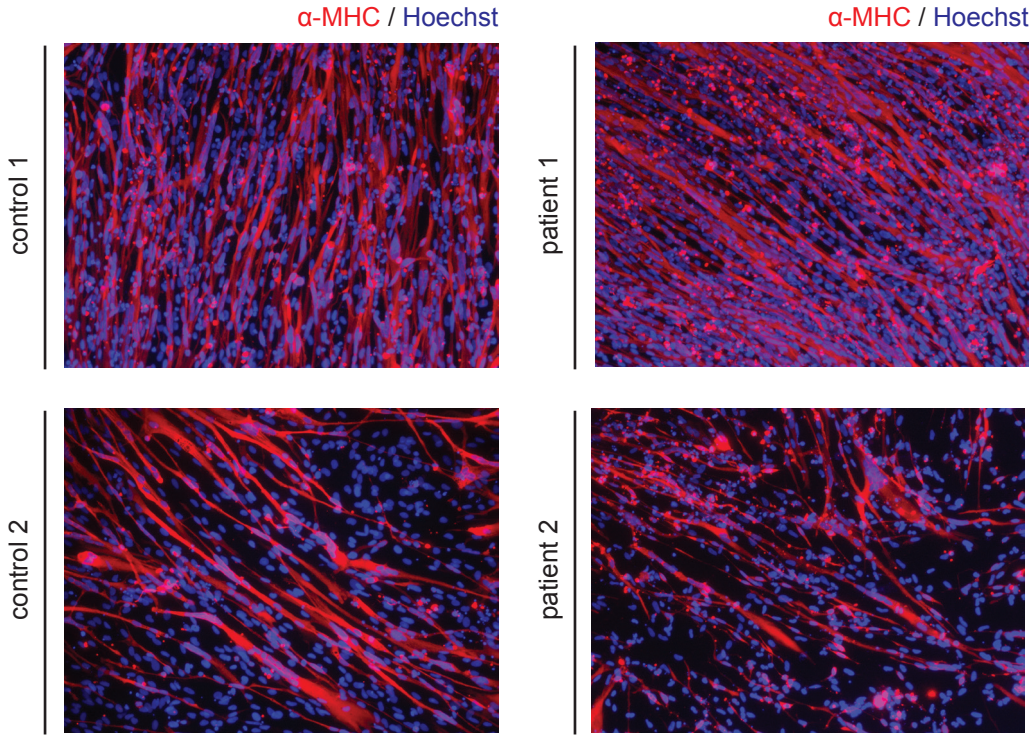


Figure S1. Generation and characterization of induced pluripotent stem cells.

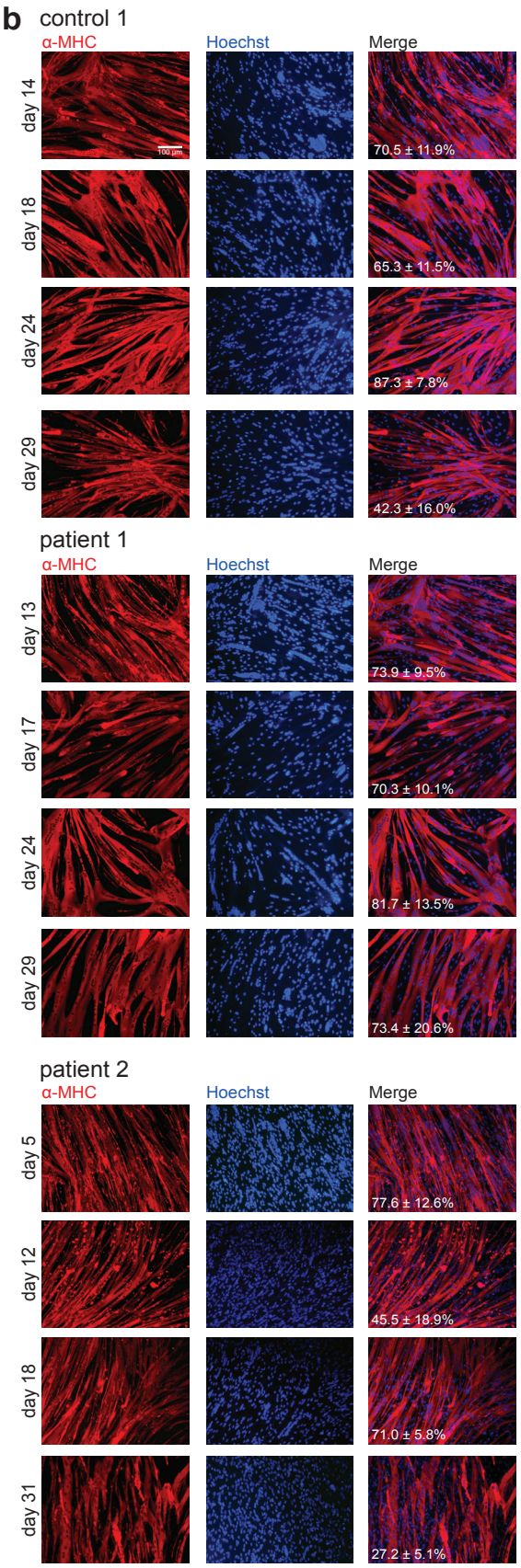
(a) Immunofluorescent analysis of iPSC from control 2 and patient 1 and 2 with antibodies to NANOG, OCT4, SSEA4, TRA-1-60 an TRA-1-81 (red). DAPI was used to stain nuclei (blue). Control 1 iPSC were published previously[1]. **(b)** *In vitro* differentiation of iPSC lines from **(a)** into derivatives of the three germ layers. Stainings for α-Fetoprotein (AFP) show hepatocytes (endoderm; red), stainings for smooth muscle actin (SMA) show smooth muscle cells (mesoderm, red), and neuron-specific class III α-tubulin (TUJ1) stainings show neurons (ectoderm, red). DAPI staining shows nuclei in blue. **(c)** Microarray analysis of mRNA expression of pluripotency and fibroblast genes. iPSCs are marked as P2, P1 and C2 (patients 2 and 1, and control 2, respectively). For comparison, human embryonic stem cell lines H1 and H9 and fibroblast line F134 were also analyzed. **(d)** Karyotype analysis of the four iPSC lines used in this study. All lines have normal karyotypes. Representative karyotypes of 10 nuclei per cell line are shown.

Supplementary Figure S2a

a



Supplementary Figure S2b,c



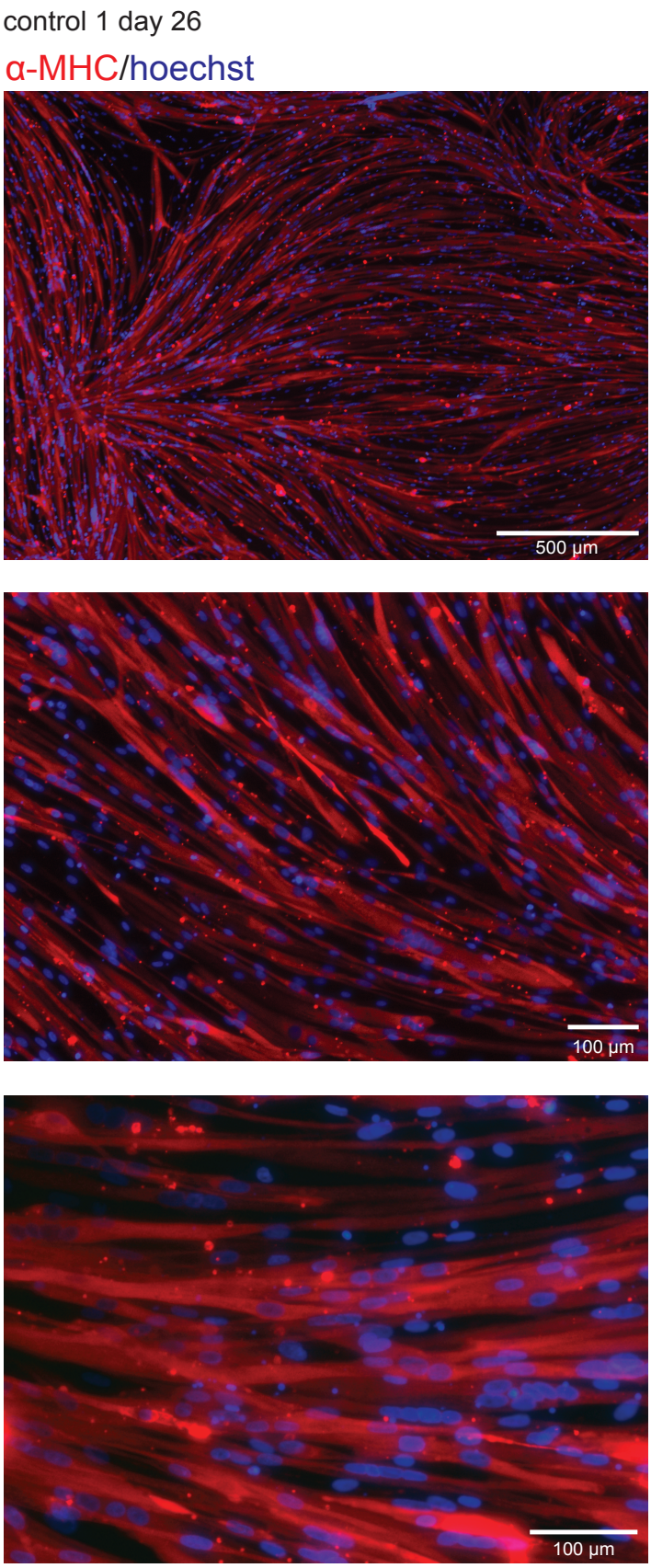
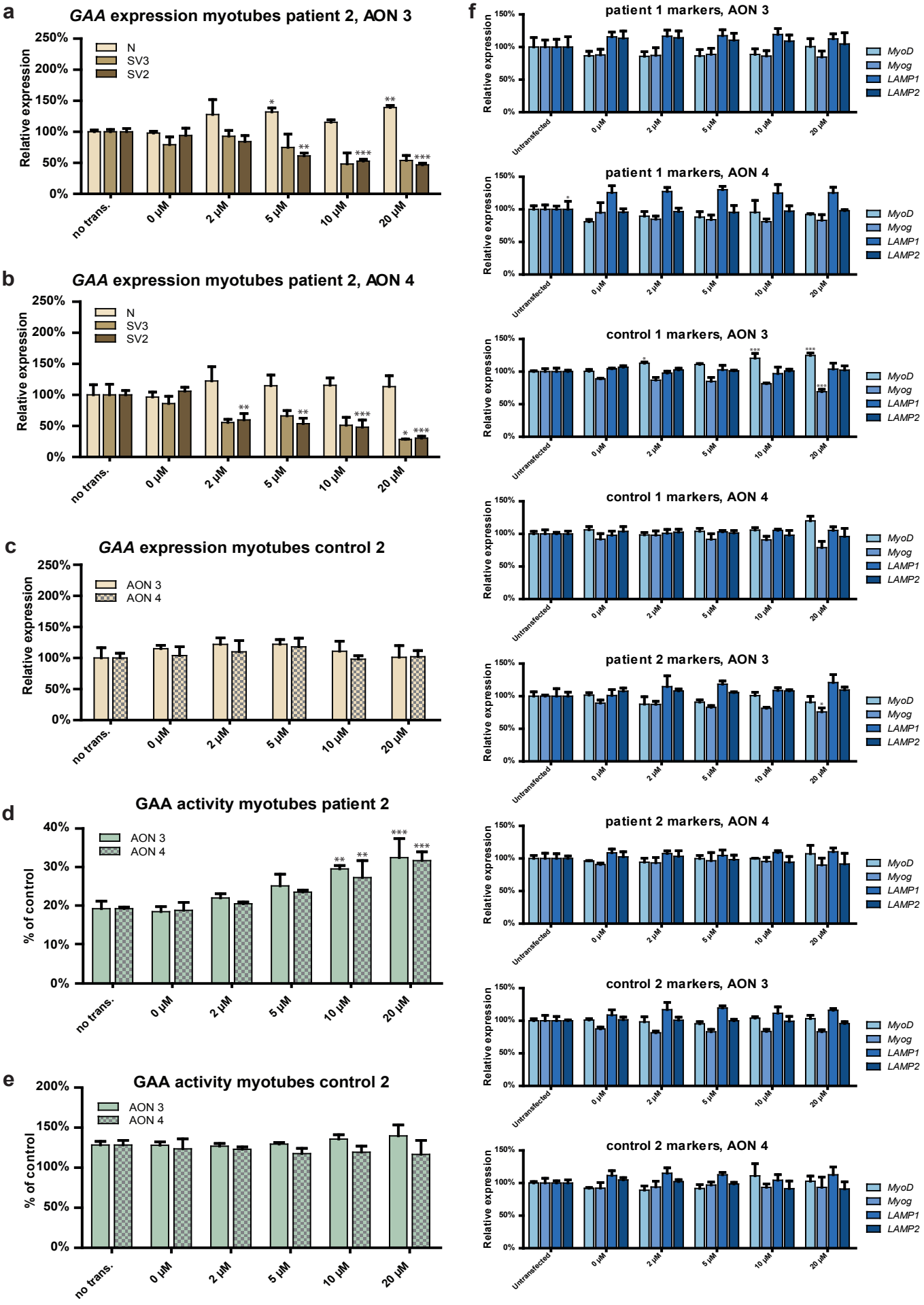


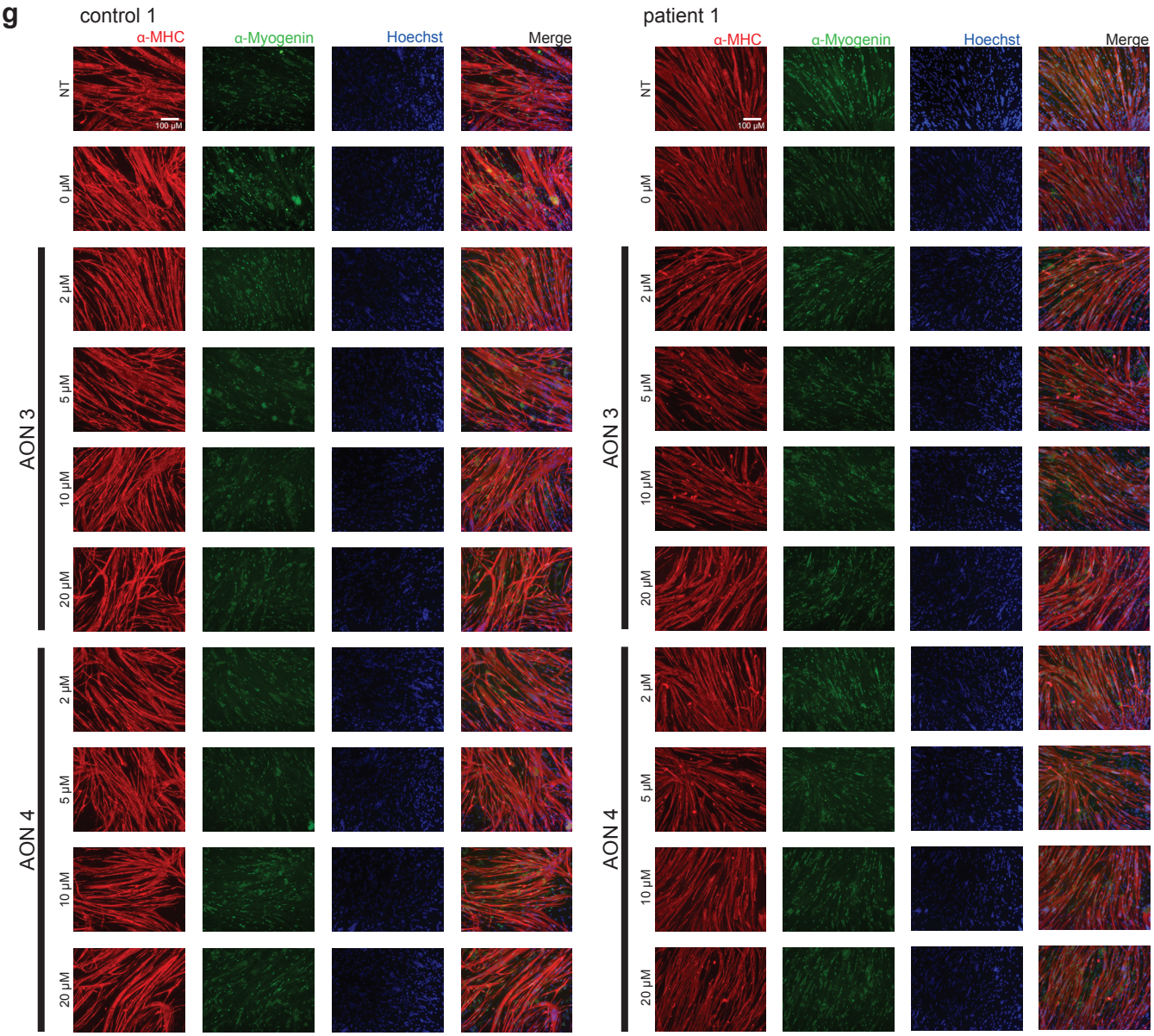
Figure S2. Purification and expansion of iPSC-derived myogenic progenitors

(a) Myogenic progenitors from (Figure 1aI-II) were purified by FACS for HNK-1-/C-MET+ cells, and directly differentiated into myotubes without expansion. Cells were stained with an MF-20 antibody to MHC (red). Nuclei were stained with Hoechst (blue). Attempts to differentiate freshly purified myogenic progenitors using standard differentiation medium (DMEM/F12 1% ITS-X, 1% P/S/G) failed as cells died. Therefore, we first applied a 2-day period in DMEM HG, 2% horse serum, 1% P/S/G, followed by a 6 day period in standard differentiation medium. These results are shown here. **(b)** Capacity to differentiate into multinucleated myotubes during expansion. Myogenic progenitors were expanded and at several time points during expansion, a subculture from the expansion was differentiated for 4 days and stained for MHC expression (anti-MF20, red). Nuclei were stained with Hoechst (blue). The fusion index of each differentiation is shown at the bottom. Data are means +/- SDs of five technical replicates. We noted that, upon differentiation, myogenic progenitors from patient 2 (generated from iPSC clone 1 and used in the experiments shown in Figure 1 and 4 and Supplementary Figure S3b) showed shorter myotubes and in some cases abnormal fusion into large round MHC-positive multinucleated structures. To investigate this further, we generated independent myogenic progenitors from this iPSC clone, but these also showed similar properties. We then generated myogenic progenitors from an independent iPSC clone 2 from patient 2 (which expressed pluripotency markers and showed *in vitro* differentiation into derivatives of the three germ layers, data not shown). These showed formation of multinucleated myotubes that were similar to those formed from patient 1 and the healthy controls, and we used these cells in Supplementary Figure S2b. Myogenic progenitors from patient 2 clones 1 (Figure 1c) and 2 (data not shown) showed highly similar proliferation curves upon expansion of at least 10^{12} in 31 days. **(c)** Examples of myogenic differentiation after expansion of myogenic progenitors shown at different magnifications. Staining was as in (I). Multiple aligned myonuclei were seen in extended myotubes.

Supplementary Figure S3a-f



Supplementary Figure S3g



Supplementary Figure S3h

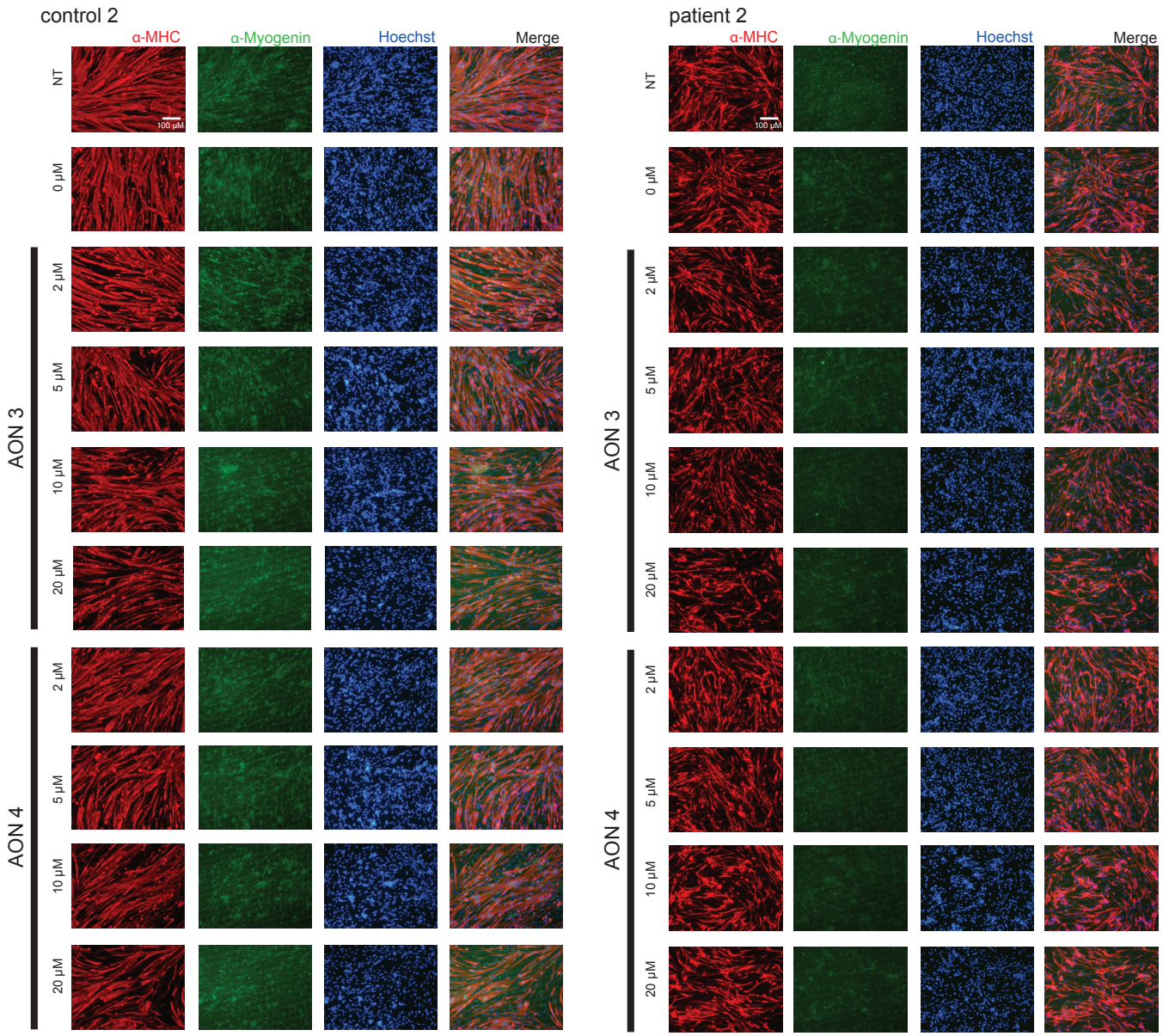
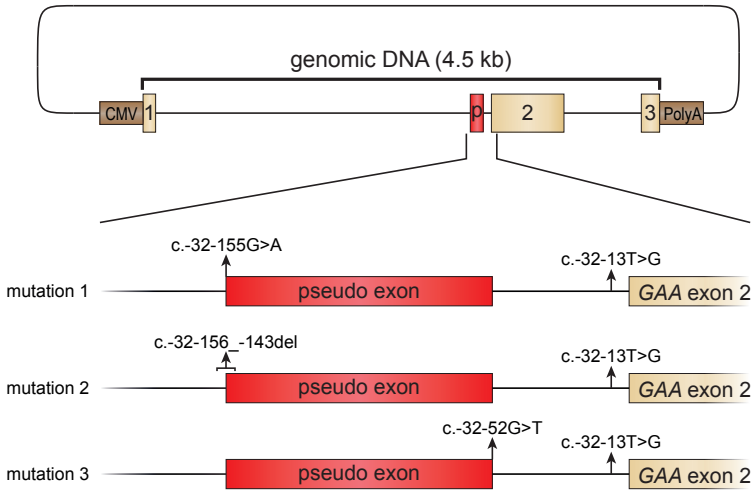


Figure S3. Promotion of exon inclusion in patient-derived myotubes.

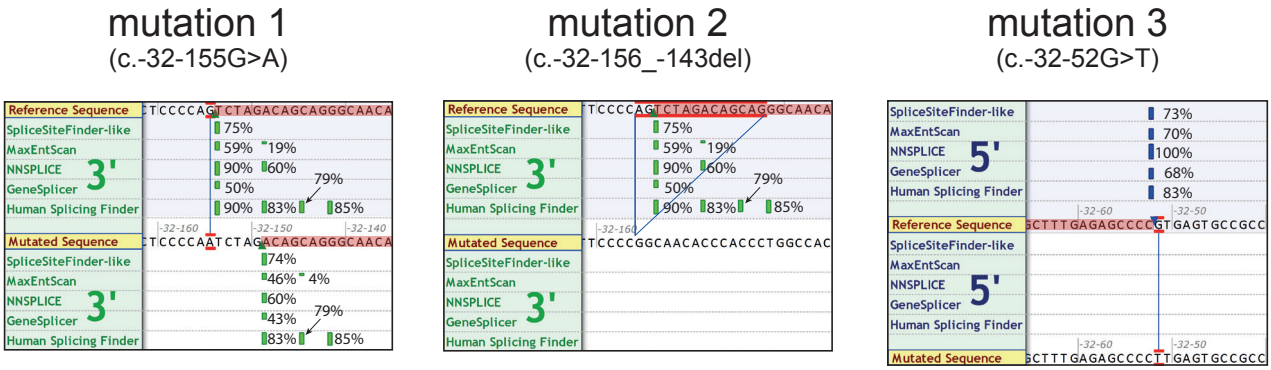
(a) Effect of AON 3 on *GAA* pre-mRNA splicing in myotubes from patient 2, measured with RT-qPCR analysis of individual splicing products. Data were normalized using a panel of reference genes (*MyoD*, *Myogenin*, *LAMP1*, *LAMP2*) that did not change during treatment (see Supplementary Figure S3f). **(b)** As **(a)**, but using AON 4. **(c)** Effects of AON 3 and 4 on expression of the N form of *GAA* mRNA in myotubes from control 2. Normalization was as in (a). (d) Effects of AON 3 and 4 on GAA enzymatic activity in myotubes from patient 2. (e) Effects of AON 3 and 4 on GAA enzymatic activity in myotubes from control 2. (f) Effects of AON 3 and 4 on expression of reference genes (*MyoD*, *Myog*, *LAMP1*, *LAMP2*) in myotubes from patients and controls. These genes were used for normalization of all RT-qPCR data from myotubes, unless otherwise stated. (g) Morphology of differentiated myotubes, obtained from purified myogenic progenitors from control 1 and patient 1, with and without AON treatment. Cells were stained with antibodies against Myosin Heavy Chain (MHC) (red), and Myogenin (green). Nuclei were visualized with Hoechst (blue). (h) Same as (g), but for control 2 and patient 2. In all experiments, data represent means +/- SD of three biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplementary Figure S4a-c

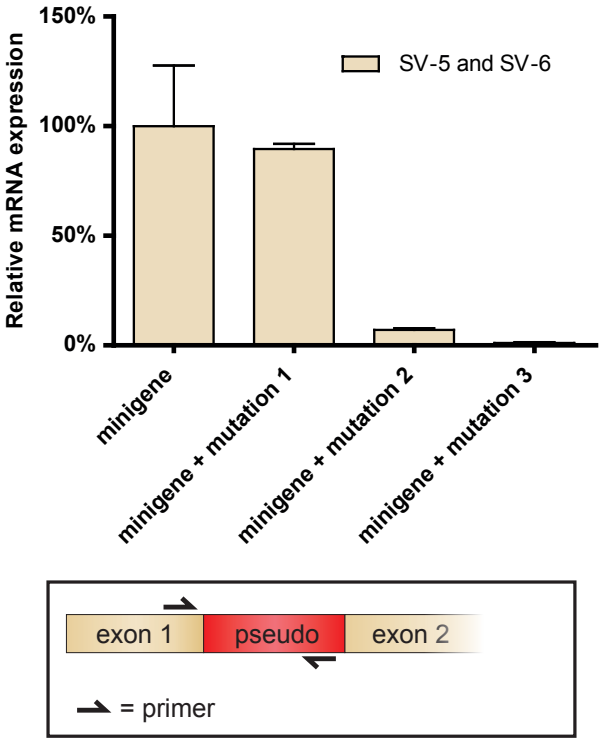
a



b



c



Supplementary Figure S4d,e

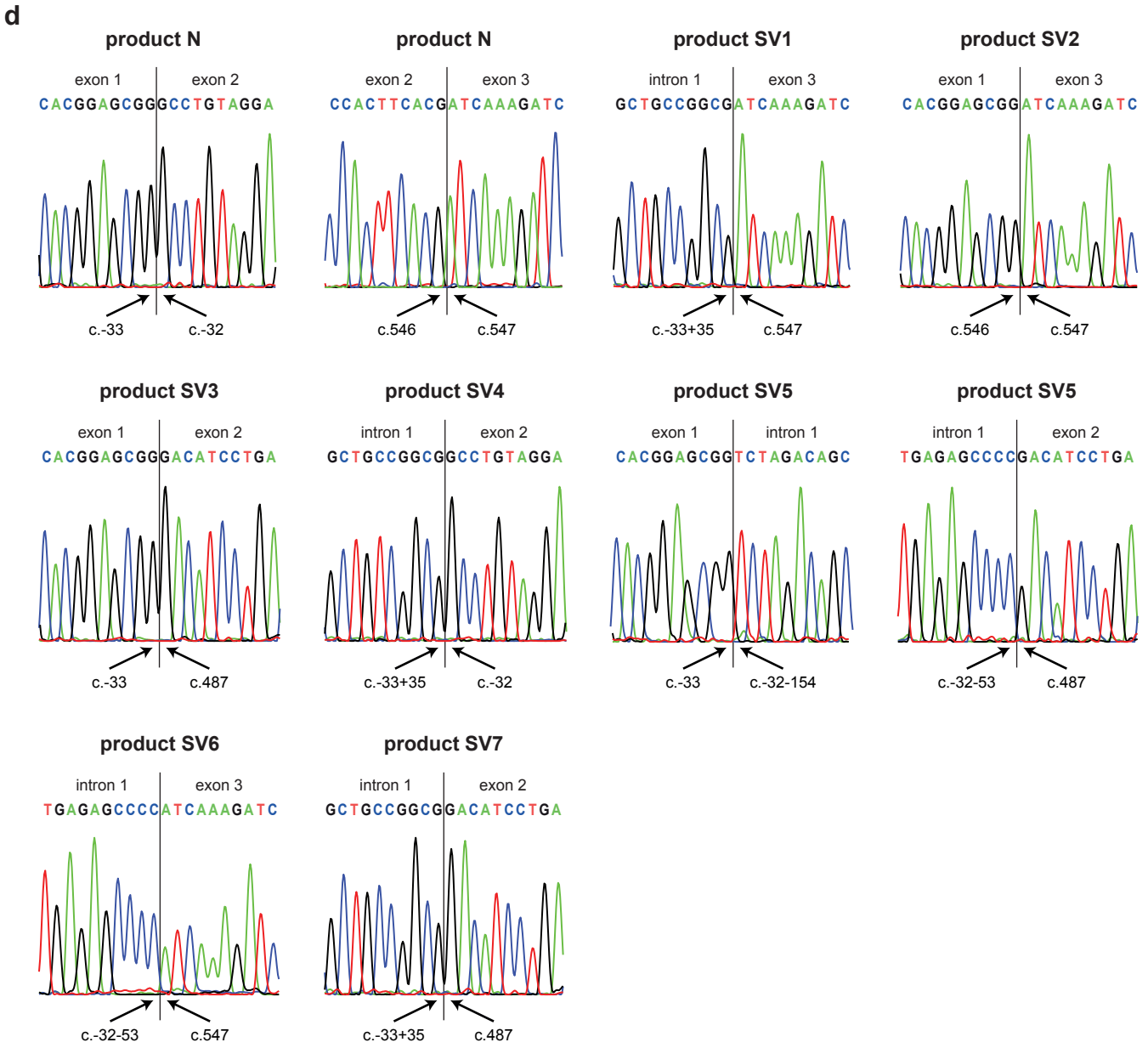


Figure S4. Identification of a natural pseudo exon that competes with *GAA* exon inclusion.

(a) Cartoon of the minigene comprising a 4.5 kb genomic *GAA* sequence including exons 1-3. This sequence was obtained by PCR and cloned into pcDNA3.1. The natural pseudo exon is indicated along with the natural cryptic splice sites that were mutated by site directed mutagenesis. **(b)** Splicing prediction of the effect of the mutations shown in **(a)**. Mutation 1 generated a new predicted 3' splice site 5 nt downstream, whereas mutations 2 and 3 completely abol-ished predicted 3' and 5' splice site, respectively. **(c)** Wild type and mutated minigenes were transfected in HEK293T cells, and expression of *GAA* splice variants containing the natural pseudo exon was quantified by RT-qPCR analysis using the primers indicated. **(d)** Sequence analysis of splicing products from Table 2. **(e)** AON treatment does not change expression of reference genes in myotubes. The experiment of Fig 5B and D was analyzed by RT-qPCR for expression of the reference genes shown. Equal amounts of total RNA were used. Data represent means +/- SD of three biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

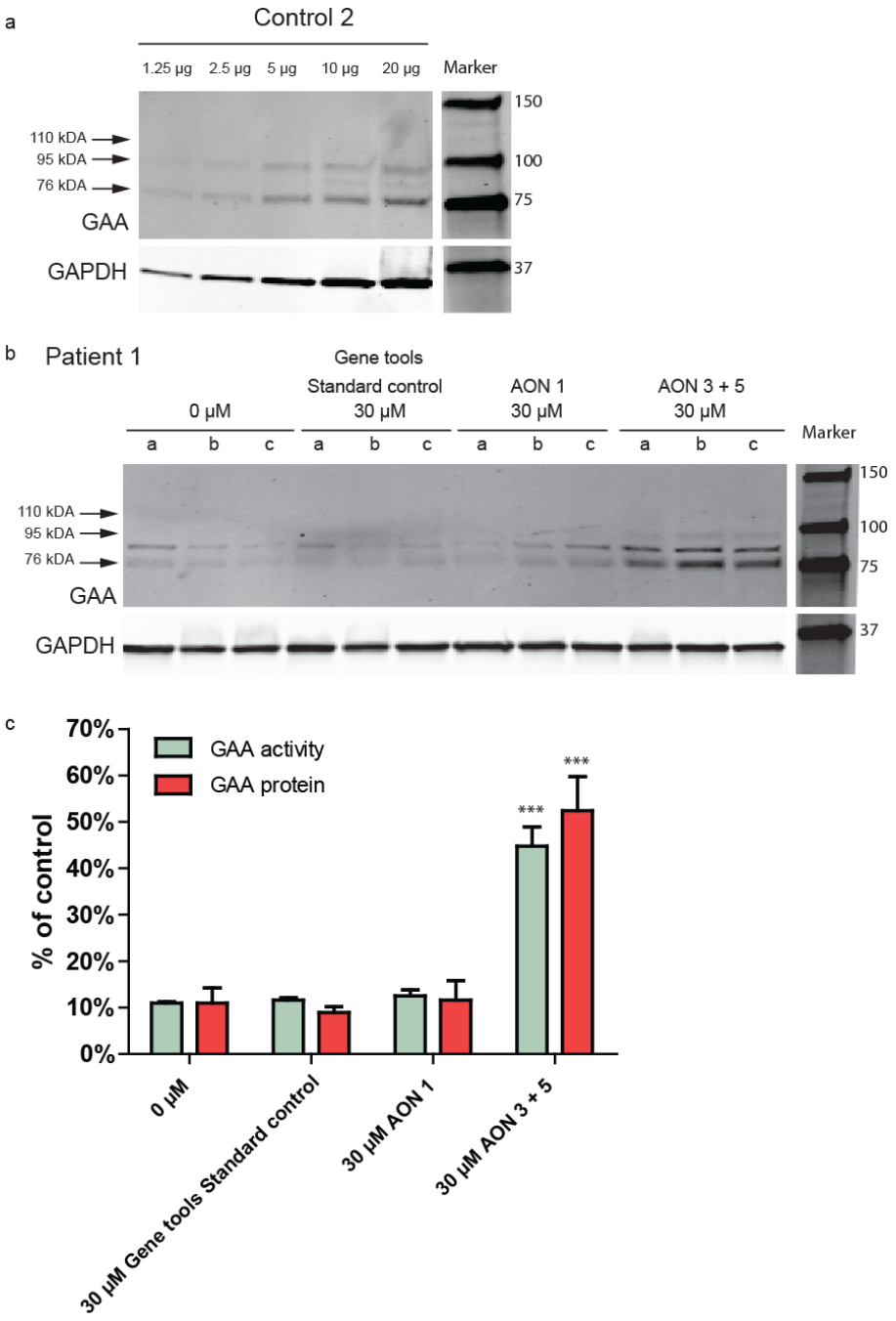


Figure S5. Quantification of GAA protein levels after treatment with AONs using immunoblot analysis.

(a) Linearities of GAA and GAPDH antibodies were tested by loading a concentration range of 1.25 µg – 20 µg of total protein obtained from iPS-derived skeletal muscle myotubes from control 2. Sizes of processed forms of GAA protein (110, 95, and 76 kDa) are indicated. The 76 kDa band is the active form of GAA after intracellular processing. The band in between the 76 kDa and 95 kDa bands was not identified and may represent an intermediate processing form of GAA. **(b)** iPS-derived skeletal muscle cells from patient 1 were differentiated to myotubes, treated with the indicated AONs, and 10 µg of total protein was used to detect GAA and GAPDH proteins. Two negative controls were used: a standard control from Gene tools, which targets *HBB*, and AON1, which targets the *GAA* pre-mRNA in intron 1 outside the pseudo exon. **(c)** The experiment from (b) was also analyzed for GAA enzyme activity, and the data from immunoblot analysis and GAA activity were compared. The active GAA protein form (76 kDa band) from (b) was quantified based on the linearities of the antibodies shown in (a). GAPDH was used for normalization. Data represent means +/- SD of three biological replicates. ***p < 0.001.

Table S1. Cell culture media tested for sustaining expansion of myogenic progenitors

Medium no	Composition
1	DMEM/F12, 1% ITS-X and 1% P/S/G
2	HAM F10, 20% FBS and 1% P/S/G
3	HAM F10, 20% FBS, 1% P/S/G and 100 ng/ml FGF2
4	DMEM HG, 10% FBS and 1% P/S/G
5	DMEM HG, 10% FBS, 1% P/S/G and 100 ng/ml FGF2

Table S2. Antibodies used in experiments

Name	Dilution	Company
Goat-α-NANOG	1:50	R&D systems (AF1997)
Goat-α-OCT4	1:100	Santa Cruz (sc-8629)
Mouse-α-SSEA4	1:100	Millipore (SCR001)
Mouse-α-TRA-1-60	1:100	Millipore (SCR001)
Mouse-α-TRA-1-81	1:100	Millipore (SCR001)
Mouse-α-SMA	1:50	Dako (M0851)
Mouse-α-AFP	1:200	Sigma (A8452)
Mouse-α-UJ1	1:1000	Sigma (T8660)
Mouse-α-MF20	1:50	DSHB
Rabbit-α-Myogenin	1:100	Santa Cruz (sc-576)
Rabbit-α-MyoD	1:100	Santa Cruz (sc-304)
Mouse-α-Pax7	1:100	DSHB
Mouse-α-C-MET-APC	1:50	R&D systems (FAB3582A)
Mouse-α-HNK1-FITC	1:100	AVIVA SYSTEMS BIOLOGY (OASA02271)
Mouse-α-GAPDH	1:1000	Millipore (MAB374)
Rabbit-α-GAA	1:1000	Abcam (137068)

Table S3. Composition of in vitro differentiation medium into three germ layer derivatives

Medium	Component	Supplier
Endoderm/Mesoderm	DMEM High Glucose	Gibco
	20% FBS	Thermo scientific
	1% PSG	Gibco
	α-Thioglycerol (4µl/100ml)	Sigma
	1x NEAA	PAA
	0.1% ß-mercaptoethanol	Life technologies
Ectoderm	50% Neurobasal medium	Gibco
	50% DMEM/F12	Gibco
	1% PSG	Gibco
	0.1% ß-mercaptoethanol	Life technologies
	1:500 7.5% BSA fraction V	Gibco
	1:200 N2	Gibco
	1:100 B27 w/o VitA	Gibco

Table S4. AONs used in this study

Name:	cDNA location:	Sequence 5' to 3':	Nucleotides:
Gene Tools Standard control	*HBB* c.316-162_138	CCTCTTACCTCAGTTACAATTTATA	25
AON 1	*GAA* c.-32-219_-200	GAGTGCAGAGCACTTGCACA	20
AON 3	*GAA* c.-32-187_-167	CCAGAAGGAAGGGCGAGAAAA	21
AON 4	*GAA* c.-32-190_-166	GCCAGAAGGAAGGGCGAGAAAAGCT	25
AON 5	*GAA* c.-32-64_-40	GGGCGGCACTCACGGGGCTCTCAAA	25

Table S5. Primers used for qRT-PCR, RT-PCR, cloning and sequencing

Primer target	Sequence (5'-3')	Used for
ß-*Actin* fw	AACCGCGAGAAGATGACCC	qPCR/RT-PCR
ß-*Actin* rv	GCCAGAGGCGTACAGGGATAG	qPCR/RT-PCR
GAA Exon 1-2 fw	AAACTGAGGCACGGAGCG	qPCR
GAA Exon 1-2 rv	GAGTGCAGCGGTTGCCAA	qPCR
GAA Cryptic Exon 2 fw	GGCACGGAGCGGGACA	qPCR
GAA Cryptic Exon 2 rv	CTGTTAGCTGGATCTTTGATCGTG	qPCR
GAA Full Skip Exon 2 fw	AGGCACGGAGCGGATCA	qPCR
GAA Full Skip Exon 2 rv	TCGGAGAACTCCACGCTGTA	qPCR
GAA Pseudo Exon fw	AAACTGAGGCACGGAGCG	qPCR
GAA Pseudo Exon rv	GCAGCTCTGAGACATCAACCG	qPCR
α-*Actinin* fw	GAGACAGCGGCTAACAGGAT	qPCR
α-*Actinin* fw	ATTCCAAAAGCTCACTCGCT	qPCR
Six1 fw	GTCCAGAACCTCCCCTACTCC	qPCR
Six1 rv	CGAAAACCGGAGTCGGAACTT	qPCR
Six4 fw	CCATGCTGCTGGCTGTGGGAT	qPCR
Six4 rv	AGCAGTACAACACAGGTGCTCTTGC	qPCR
FGF2 fw	CAAAAACGGGGGCTTCTTCC	qPCR
FGF2 rv	GCCAGGTAACGGTTAGCACA	qPCR
Sox1 fw	GAGCTGCAACTTGGCCACGAC	qPCR
Sox1 rv	GAGACGGAGAGGAATTCAGAC	qPCR
MyoD fw	CACTCCGGTCCCAAATGTAG	qPCR
MyoD rv	TTCCCTGTAGCACCACACAC	qPCR
Myog fw	CACTCCCTCACCTCCATCGT	qPCR
Myog rv	CATCTGGGAAGGCCACAGA	qPCR
LAMP1 fw	GTGTTAGTGGCACCCAGGTC	qPCR
LAMP1 rv	GGAAGGCCTGTCTTGTTCAC	qPCR
LAMP2 fw	CCTGGATTGCGAATTTTACC	qPCR
LAMP2 rv	ATGGAATTCTGATGGCCAAA	qPCR
M13 fw	GTAAAACGACGGGCCAG	Sequencing
M13 rv	CAGGAAACAGCTATGAC	Sequencing
GC GAA Exon1-3 fw	AGGTTCTCCTCGTCCGCCCGTTGTTCA	RT-PCR
GC GAA Exon1-3 rv	TCCAAGGGCACCTCGTAGCGCCTGTTA	RT-PCR

Supplemental References

1. Dambrot, C, van de Pas, S, van Zijl, L, Brandl, B, Wang, JW, Schalij, MJ, et al. (2013). Polycistronic lentivirus induced pluripotent stem cells from skin biopsies after long term storage, blood outgrowth endothelial cells and cells from milk teeth. Differentiation 85: 101-109.