SUPPLEMENTARY DATA

SUPPLEMENTARY TEXT

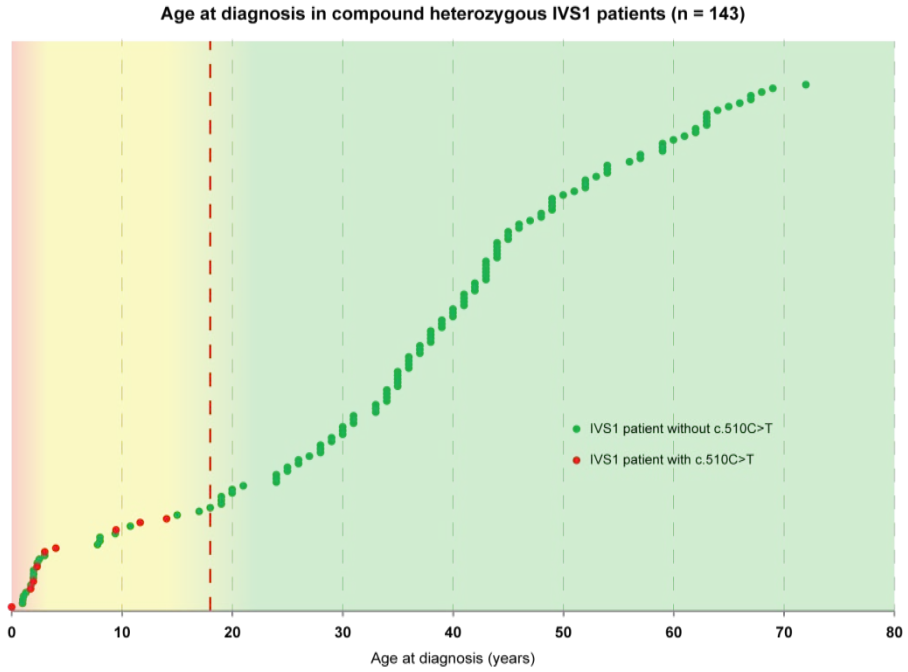
Pompe disease is an autosomal recessive disorder: two disease-associated variants from two different alleles are required to cause the disease. In the most severe classic infantile form of the disease, both disease-associated variants will result in a complete lack of residual GAA enzyme activity. However, GAA protein may still be expressed, but it will be enzymatically inactive for example due to misfolding. In the late onset form of the disease, usually one variant has no residual GAA enzyme activity at all, and one variant has some residual GAA enzyme activity. It is rare that late onset Pompe patients have two alleles that both express some residual GAA enzyme activity. Therefore, in the far majority of late onset Pompe patients, the contribution of one allele to the total GAA enzyme activity will be zero, and the entire residual GAA enzyme activity is contributed by one allele only. In the majority (90%) of late onset patients of Caucasian origin, this allele has the IVS1 disease-associated variant. The cases above describe compound heterozygous patients: patients with two different disease-associated variants.

In the case of homozygous patients, the situation is slightly different. When both alleles contain the same disease-associated variant with no residual GAA enzyme activity, the patient will have classic infantile Pompe disease. When both alleles contain the same disease-associated variant with some residual GAA enzyme activity, it will become important how high the total residual enzyme activity derived from both alleles is. If this is above the disease threshold of 20-30% of healthy control values, the individual will not have Pompe disease i.e. will remain asymptomatic. When this is below the disease threshold, the individual will develop symptoms of late onset Pompe disease. In the case of the IVS1 variant: this variant has 10-15% residual enzyme activity. When the IVS1 variant is combined with a second variant that has no residual enzyme activity in compound heterozygous IVS1 patients, the combined GAA enzyme activity will be 10-15%, which is below the disease threshold and the patient will develop symptoms of late onset Pompe disease. When the IVS1 variant is present at homozygous state, the combined GAA enzyme activity will be 20-30%, which is at or above the disease threshold. In practice, it has become clear that the majority of homozygous IVS1 individuals remain asymptomatic. Only a few homozygous IVS1 patients are known that have developed symptoms of late onset Pompe disease.

Pompe disease has over 400 disease-associated variants, that we maintain in the open access Pompe mutation database at www.pompecenter.nl (go to molecular aspects, mutations). We have identified a modifier that is present in a subset of patients that carry the common IVS1 variant. We suspect that also other putative modifying factors may exist that influence age at symptom onset. This is further discussed in the discussion section of the main manuscript..

SUPPLEMENTARY FIGURES

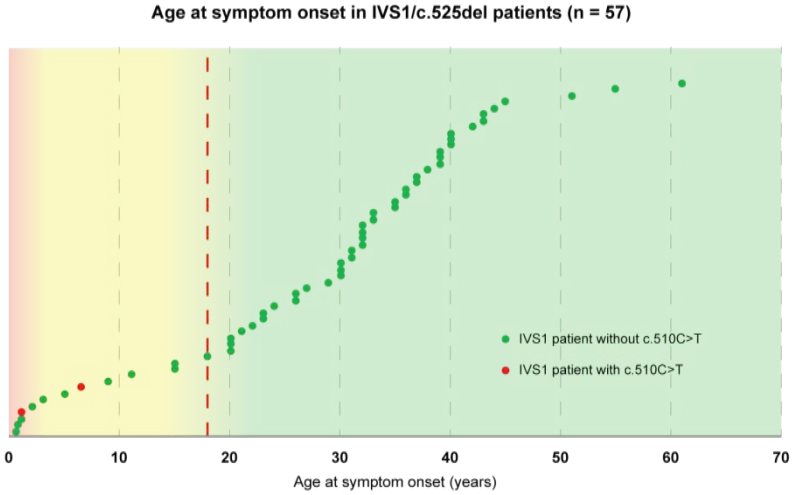
A



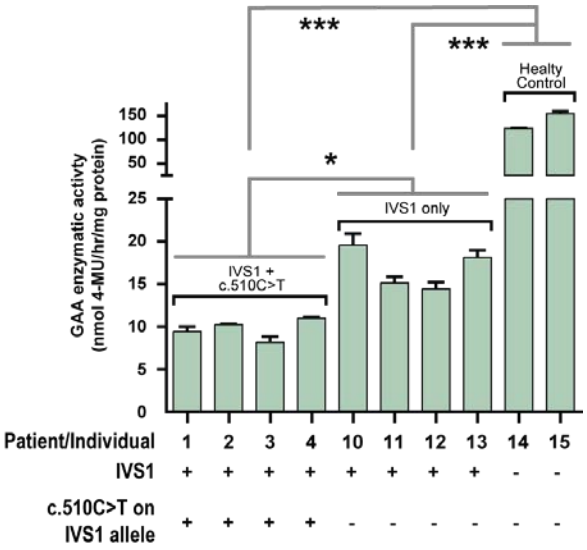
B

c.510C>T on IVS1 allele	*All patients (n)*	*Patients diagnosed at adulthood (n)*	*Patients diagnosed at childhood (n)*	*Median age at diagnosis (years)*	
+	9	0	9	5,4	***
-	134	116	18	37	

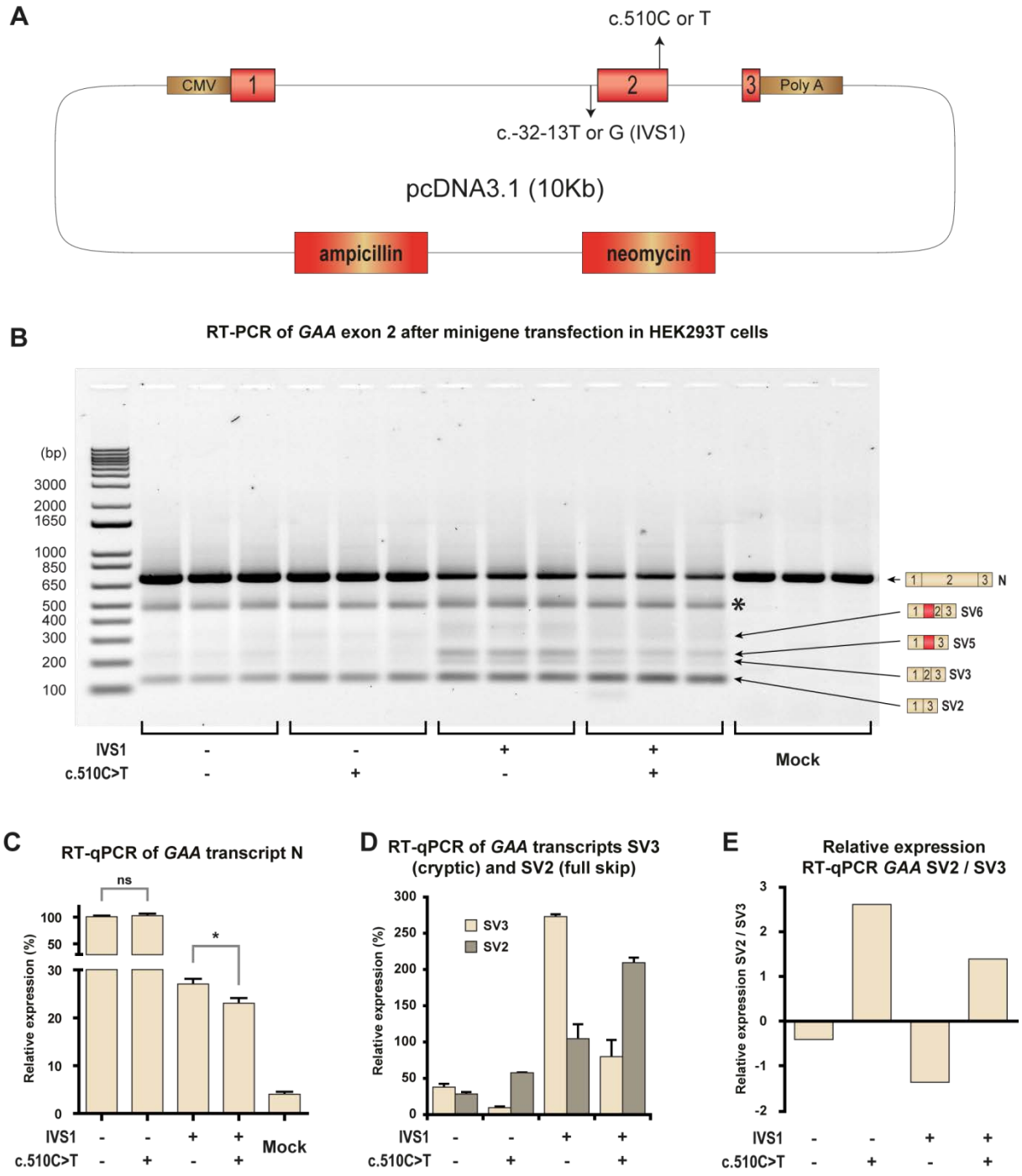
C



Supplementary Figure S1: Association of c.510C>T with age symptom onset and age at diagnosis in compound heterozygous IVS1 patients. (A) Distribution of ages at diagnosis in all compound heterozygous IVS1 patients with c.510C>T (red symbols) and without c.510C>T (green symbols). Each dot in the graph represents one patient. The dashed red line indicates the cut-off of 18 between patients with childhood onset and adult onset of symptoms. (B) Median age at diagnosis in all compound heterozygous IVS1 patients with and without c.510C>T. *** $p < 0.001$. (C) Distribution of ages at symptom onset in patients with the same IVS1/c.525del genotype. All patients in this graph have the IVS1 variant on one allele and the c.525del pathogenic variant on the other allele.

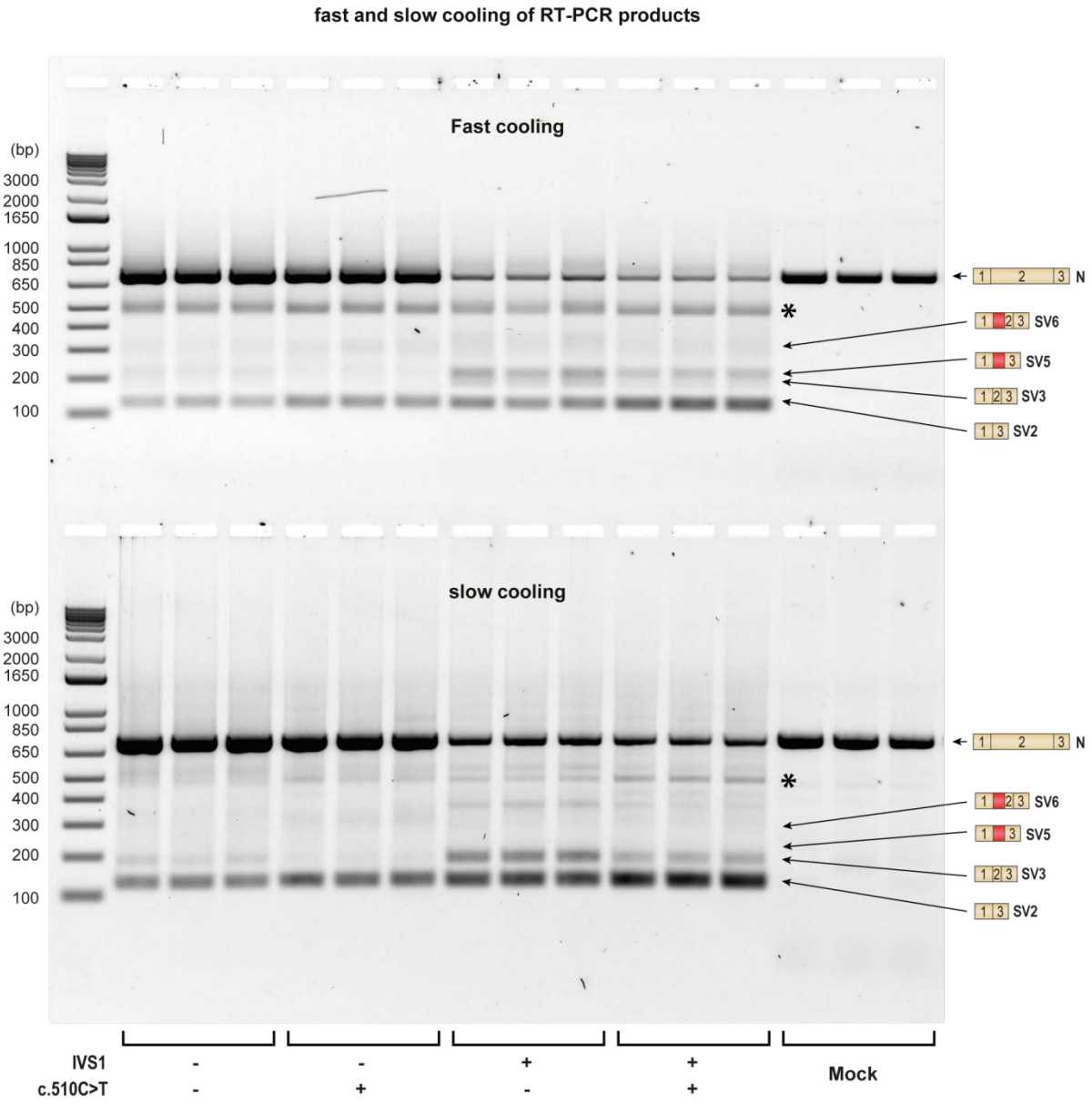


Supplementary Figure S2: Analysis GAA enzymatic activity in compound heterozygous patients. GAA enzymatic activity in fibroblasts from compound heterozygous IVS1 patients that were analyzed in Figure 3C-F. * $p < 0.05$, *** $p < 0.001$.



Supplementary Figure S3: Analysis of c.510C>T in a minigene construct. (A) Cartoon of the minigene. The full genomic DNA sequence of *GAA* exons 1-3 with or without the IVS1 variant were driven by the CMV promoter and contained a polyA signal from pcDNA3.1. c.510C>T was introduced by site-directed mutagenesis. (B) Flanking exon RT-PCR of *GAA* exon 2 in HEK293T cells transfected with *GAA* exon 1-3 minigenes containing the variants indicated below the gel. Mock: transfection of expression construct without minigene insert. Cartoons on the right of the figure depict five splice products (N, SV6, SV5, SV3 and SV2. *: structural variant (see Suppl. Figure S4)). Please note that the PCRs represent end-point PCRs in which abundant products were saturated: in the mock transfected cells, a strong wild-type *GAA* product was observed, which was derived from endogenous *GAA* expression, but quantitative analysis using RT-qPCR showed that minigene transfection caused 28-fold higher expression of *GAA* exons 1-3 relative to endogenous expression (see C). (C) Quantification of N (normally spliced product) using RT-qPCR. c.510C>T caused lower expression of N, but only when present together with the IVS1 variant. (D) Quantification of aberrant splice products SV2 and SV3 using RT-qPCR. In mock transfected cells, SV2 and SV3 expression was undetectable and could therefore not be

quantified. (E) As in (D), but plotted as the ratio of expression of SV2 and SV3. Data in C and D are means +/- SD (n= 3 biological replicates). *: $p<0.05$, ns = not significant.



Supplementary Figure S4: Characterization of the additional product of approximately 500 bp (*). This product appears to be a PCR artefact. We performed the PCR using the conventional PCR protocol (fast cooling) and a PCR with an additional slow cooling step (slow cooling, 1°C per 3 min. from 95°C to 4°C) at the end of the protocol. The 500 bp product (*) was reduced significantly by slow cooling, indicating that it is likely the product of secondary structure formation. This is confirmed by our previous observation using topo cloning and sequencing of the PCR products, which identified all annotated products but failed to identify any product that matched the size of product *. [1; 2]

SUPPLEMENTARY TABLES

Supplementary Table S1

Reference	*Country of study*	*Total no. of late onset patients with reported genotype*	*Late onset patients with IVS1 variant*
Scheidegger et al., 2018[3]	Switzerland	7	7
Witkowski et al., 2018[4]	Poland	5	3
Löscher et al., 2018[5]	Austria	21	17
Montalvo et al., 2006[6]	Italy	40	34
Angelini et al., 2009[7]	Italy	6	5
Regnery et al., 2012[8]	Germany	38	32
Semplicini et al., 2018[9]	France	170	151
van der Meijden et al., 2017[10]	International	17	14
Kuperus et al., 2017[11]	Netherlands	102	99
Figueroa-Bonaparte et al., 2018[12]	Spain	32	27
Mori et al., 2017[13]	United States	51	49
Papadimas et al., 2011[14]	Greece	9	9
Total no. of patients		498 (100%)	447 (90%)

A literature study was performed to estimate the prevalence of the c.-32-13T>G (IVS1) variant by identifying Caucasian patients in various countries. Caution was taken to avoid duplicate entries. Studies were considered that provided genotype information and had not selected patients on the basis of the IVS1 genotype.

Supplementary Table S2

Patient / individual	*c.-32-13T>G (IVS1) (allele 1 / allele 2)*	*c.510C>T (allele 1 / allele 2)*	*Disease-associated variant on the second allele*	*Cell type used for further investigation*	*Age at symptom onset (years)*	*Age at diagnosis (years)*
1	+ / -	+ / -	c.2331+2T>A	Fibroblasts	2.5	3
2	+ / -	+ / -	c.525delT	Fibroblasts	6.5	11.7
3	+ / -	+ / -	c.525delT	Fibroblasts	1	1.7
4	+ / -	+ / -	c.1548G>A	Fibroblasts and myoblasts	7	9.5
5	+ / -	+ / -	c.2135T>C	-	0.8	0
6	+ / -	+ / -	c.2135T>C	-	0.8	2.3
7	+ / -	+ / -	c.1441T>C	-	12	4
8	+ / -	+ / -	c.1933G>A	-	13	14.1
9	+ / -	+ / -	c.1051delG	-	1	2.0

Patient information on the nine compound heterozygous IVS1 patients who carry the c.510C>T variant on the IVS1 allele. Molecular analysis of patients 1 to 4 was performed in more detail in Figures 3 and 5.

Supplementary Table S3

Patient / individual	*c.-32-13T>G (IVS1) (allele 1 / allele 2)*	*c.510C>T (allele 1 / allele 2)*	*Disease-associated variant on the second allele*	*Cell type used for further investigation*	*Age at symptom onset (years)*	*Age at diagnosis (years)*
10	+ / -	- / -	c.525delT	Fibroblasts	5	10.7
11	+ / -	- / -	c.525delT	Fibroblasts	8.9	9.4
12	+ / -	- / -	c.2331+2T>A	Fibroblasts	5	7.8
13	+ / -	- / -	c.525delT	Fibroblasts	15	25
14	- / -	- / -	-	Fibroblasts	Healthy control	Healthy control
15	- / -	- / -	-	Fibroblasts	Healthy control	Healthy control
16	+ / -	- / -	c.2481+102_2646+31del *	Myoblasts	-	-
17	+ / +	+ / +	IVS1	Fibroblasts	59	60
18	+ / +	+ / +	IVS1	Myoblasts	48	50
19	+ / +	+ / -	IVS1	-	12	-
20	+ / +	- / -	IVS1	Fibroblasts	49	51
21	+ / +	- / -	IVS1	-	58	-
22	+ / +	- / -	IVS1	-	42	-
23	+ / +	- / -	IVS1	-	Asymptomatic	Asymptomatic
24	+ / +	- / -	IVS1	-	Asymptomatic	Asymptomatic
25	+ / +	- / -	IVS1	-	Asymptomatic	Asymptomatic
26	+ / +	- / -	IVS1	-	Asymptomatic	Asymptomatic

Patient information on other patients analyzed in more detail in this paper (Figures 3, 4 and 5). For clarity, patients in gray are homozygous for the IVS1 variant.

* The c.2481+102_2646+31del (deletion of exon 18) variant allows for normal expression of *GAA* exon 1-3 mRNA (product N).

Supplementary Table S4

Primer name	*Primer sequence*	*Purpose*
IVS1_spec_downstream_fw	TCCCTGCTGAGCCCGCTTG	Allele-(a)specific primers *GAA* exon 1-3
aspec_downstream_rv	GAAGGGCTCCTCGGAGAA	
nonIVS1_spec_downstream_fw	TCCCTGCTGAGCCCGCTTT	
aspec_upstream_fw	CGAGCTCCCGCCGGTCACGTGACCC	
IVS1_spec_upstream_rv	GCTCCTACAGGCCTGCGGGAGAAGC	
SQ_intron1_rv1	CGGGATTTTGCCATGTTACC	Sequencing of PCR products
SQ_intron1_rv2	GGTTAACAAGTACCAACGACC	
SQ_intron1_rv3	TGTTACAGAAGGCTTGGCTGG	
SQ_intron1_rv4	TGCCTTGGTGTGTTCCACAAC	
SQ_intron1_rv5	GACTGAGCACTGCGTCGATC	
SQ_intron1_rv6	CCTCAGTTTCCCCGTCAGCTG	
SQ_intron2_rv1	TGAGGTGCGTGGGTGTCGATGTC	
*GAA*_flank_Exon2_fw	AGGTTCTCCTCGTCCGCCCGTTGTTCA	Flanking exon PCR *GAA* exon 2
*GAA*_flank_Exon2_rv	TCCAAGGGCACCTCGTAGCGCCTGTTA	
qPCR_*Actin*_fw	AACCGCGAGAAGATGACCC	Normalization of fibroblast cDNA input
qPCR_*Actin*_rv	GCCAGAGGCGTACAGGGATAG	
qPCR_*Neomycin*_fw	TCATCTCACCTTGCTCCTGC	Normalization of minigene cDNA input
qPCR_*Neomycin*_rv	GTGGTCGAATGGGCAGGTAG	
qPCR_*GAA*_Exon1-2_fw	AAACTGAGGCACGGAGCG	Quantification splice product N
qPCR_*GAA*_Exon1-2_rv	GAGTGCAGCGGTTGCCAA	
qPCR_*GAA*_CrypticE2_fw	GGCACGGAGCGGGACA	Quantification splice product SV3
qPCR_*GAA*_CrypticE2_rv	CTGTTAGCTGGATCTTTGATCGTG	
qPCR_*GAA*_SkipE2_fw	AGGCACGGAGCGGATCA	Quantification splice product SV2
qPCR_*GAA*_SkipE2_rv	TCGGAGAACTCCACGCTGTA	
SDM_*GAA*_c.-32-13T>G_fw	CTGCTGAGCCCGCTTGCTTCTCCCGCAGGCC	Primers for Site Directed Mutagenesis minigenes
SDM_*GAA*_c.-32-13T>G_rv	GGCCTGCGGGAGAAGCAAGCGGGCTCAGCAG	
SDM_*GAA*_c.510C>T_fw	ACCCTGCGGCTGGATGTGATGATGGAGACT	
SDM_*GAA*_c.510C>T_rv	AGTCTCCATCATCACATCCAGCCGCAGGGT	

Primers used for PCR, sequencing, and Site Directed Mutagenesis.

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