Science Translational Medicine
AAAS

stm.sciencemag.org/cgi/content/full/11/520/eaat6072/DC1

Supplementary Materials for

Titin splicing regulates cardiotoxicity associated with calpain 3 gene therapy for limb-girdle muscular dystrophy type 2A

William Lostal, Carinne Roudaut, Marine Faivre, Karine Charton, Laurence Suel, Nathalie Bourg, Heather Best, John Edward Smith, Jochen Gohlke, Guillaume Corre, Xidan Li, Zaher Elbeck, Ralph Knöll, Jack-Yves Deschamps, Henk Granzier, Isabelle Richard*

*Corresponding author. Email: richard@genethon.fr

Published 27 November 2019, *Sci. Transl. Med.* **11**, eaat6072 (2019)
DOI: 10.1126/scitranslmed.aat6072

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/11/520/eaat6072/DC1)

Data file S1 (Microsoft Excel format). Individual subject-level data.

SUPPLEMENTARY MATERIALS:

Materials and Methods

Vector design and production

An AAV vector expressing the human calpain 3 previously described (*18,19*) was used and two AAV plasmids (p.hDes-mfa*CAPN3*-V5-SV40 and p.hDes-mfa*CAPN3*-V5-SV40+2xmiR208aT) to express a V5-tagged-Macaca fascicularis calpain 3 coding sequence (mfa*CAPN3*-XM_005573606) under control of a human desmin (hDes) promoter were designed. The recombinant AAV vectors were produced and titrated as previously described (*40,41*).

Animal care and use

All animals were handled according to French and European legislation on animal care. Cynomolgus (macaca fascicularis) males of 2-3 years of age were obtained from the Bioprim facility in Baziege, France. The primate experiments were performed at the Veterinary school of Nantes/ONIRIS, Nantes, France and the mice experiments at the Centre d'exploration et de recherche fonctionnelle expérimentale (CERFE; Evry).

C57Bl6N (WT) mice were obtained from Charles River laboratories. The CAPN3-deficient (B6-*Capn3*$^{tm2.1Gnt}$; "C3KO"), the RBM20 KO and delMex5 (*Ttn*$^{gnt1.)}$mice) were previously described (*22,26,29*). The *CAPN3* KO model was crossed with dysferlin deficient model B6.A-Dysf^{prmd}/J (BlaJ; (*23*) to generate animals knock-out for both calpain 3 and dysferlin (B6.129-Dysf^{prmd}Capn3$^{tm2.1Gnt}$ or BDC, for BlaJ deficient in Calpain 3). Littermates with other genotypes (two deficient alleles for dysferlin and only one for calpain 3: HOHE) and (two deficient alleles for dysferlin or Blaj) were used as controls.

In vivo experiments

Vectors were delivered in primates by systemic injection into the saphenous vein in bolus at 3ml/min, for a total volume of 3 mL/kg [8-9 mL total (4.5-6 ml of rAAV preparation diluted in Ringer buffer)]. For intramuscular injections (IM) in mice, the animals were injected either into the TA muscle with a volume of 25 µL in one single injection or into the gastrocnemius or gluteus muscles with a total volume of 50 µL divided between 2 sites of injection (n = 4 muscles for each condition). For intravenous injection (IV) in mice, animals were injected into the tail vein with a dose of 2.5 x 10^{13} vg/kg or with Phosphate-Buffered Saline (PBS) in a 200 µl volume (n = 3-4 for each condition). All *in vivo* mouse experiments were performed at the age of 8 weeks. For histological and molecular analysis of mouse tissues, specimens were collected immediately after sacrifice, snap frozen in liquid-nitrogen-cooled isopentane and stored at −80°C.

Histologic and immunostaining analyses

Mouse sections were immunostained using Cd11b (550282, BD Pharmingen, dilution 1/40) and developmental Myosin Heavy Chain (MHCd) primary antibodies (NCL-MHCd, Leica, 1/40) over night at 4°C. For Cd11b, after washing three times with PBS, muscle sections were incubated with a goat anti-rat secondary antibody conjugated with Alexa 594 dye (Molecular probe, dilution 1/1000) for 1 hour at room temperature (RT). Sections were mounted with fluoromount-G (SouthernBiotech) and visualized on a fluorescent microscope (Axio scan). For MHCd, muscle sections were incubated with a goat anti-rat secondary antibody conjugated with Horseradish Peroxidase (HRP;A 10549, Invitrogen, 1/600) for 1 hour at RT. After washing three times with PBS, muscle sections were incubated with 3,3'-diaminobenzidine (DAB) solution (K 3466, DAKO) for 2-3 min at RT. The muscle sections were dehydrated with ethanol solution

(70%, 95%, 100%). After xylene wash, the sections were mounted with Eukitt (05347505, LABONORD) and visualized on a microscope (Axio scan).

Transverse sections (8 µm) were processed for Hematoxylin-Phloxine-Saffron (HPS) or Sirius red histological staining. Quantification was performed by using Image J software.

ddPCR and RT-PCR analyses

For the biodistribution analysis, DNA extraction was performed with MagnaPure 96 DNA and Viral Nucleic acid kit (Roche). Genome copy titers were quantified by droplet digital PCR using ddPCR Supermix for Probes (Biorad) and primers (F, GGATGGAGACGGTATCATCAAACTCAA ; R, GCCACTGTGCTGGATATCTG) and probe (P, AGCTCACCATGTATGCCAAGGGTC) against mfaCAPN3/V5 and using primers/probe against Rplp0 (ribosomal protein) as a control (Rplp0; F, CTCCAAGCAGATGCAGCAGA; R, ATAGCCTTGCGCATCATGGT; P, CCGTGGTGCTGATGGGCAAGAA).

For RNA expression analysis, samples were resuspended in MagnaPure LC RNA Isolation tissue Lysis buffer and the extraction was performed with MagnaPure 96 cellular RNA kit (Roche). Reverse transcription was performed on 1 µg mRNA using RevertAid Fermentas Kit (ThermoFisher Scientific). Quantification of the primate calpain 3 transgene was performed by RT-dd PCR using the same primers used in the biodistribution study and the human transgene calpain using the primers [CAPN3sfr.f, 5'_CGCCTCCAAGGCCCGT_3'; CAPN3sfr.r, 5'_GGCGGAAGCGCTGGCT_3'; and the probe MGBTUCAPN3.p, 5'_CTACATCAACATGAGAGAGGT_3'] in the mouse experiments. Myogenin (Mm00446195_g1), MyoD1 (Mm00440387_m1), MHCd (Mm01332463_m1), Tmem8C (Mm00481256_m1), Cd11b (Mm00434455_m1), Fibronectin (Mm01256744_m1), Col1a1

(Mm00801666_g1), Col6a3 (Mm00711678_m1), Dysferlin (Mm00458042_m1), the endogenous mouse Calpain 3 (Mm00482985_m1) and the transgenic mfa Calpain 3 (Mf04363759_g1) were quantified by quantitative PCR with LC480 (Roche) according to the protocol Absolute QPCR Rox Mix (ABgene), using Taqman Gene Expression assays (ThermoFisher). A RT-qPCR for ubiquitous ribosomal phosphoprotein (Rplp0) was used to normalize the data across samples. Samples (heart and *biceps femoris*) of a control monkey (John) were kindly provided by Dr. F.Mingozzi. Quantification of miRNA in the serum of *macaca* was performed using the following Taqman MicroRNA assays (Life technology): miR93.5p (001090), miR-16-5p (000391), miR2308a (000511) and miR499-5p (0001352).

Seric and cardiac markers

Biochemistry, complete hematologic profile and additional cardiac markers: Creatine Kinase (CK), myoglobin, troponin and NT-proBNP were performed using human versions of the ELISA tests at the Centre Hospitalier Universitaire (CHU) of Nantes (France).

RNA-sequencing

Three WT mice, 3 RBM20 mice, 2 human and 2 primate samples were sequenced. RNA concentration was measured on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA quality (RIN≥8) was controlled using an Agilent RNA 6000 Pico Kit on a 2100 Bioanalyzer instrument (Agilent Technologies). The sequencing libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina) and sequenced according to the Illumina protocol. The reads were paired using Fastq-pair and aligned onto the mouse genome (mm10), human genome (hg38) and crab-eating macaque (Macaca fascicularis 5.0.92) using STAR aligner (*42*).

Exon numbering is according to Cardiodb (https://www.cardiodb.org/titin/titin_transcripts.php)

with titin coordinates from Locus Reference Genomic sequences (LRG_391). Primate and mouse exons are numbered according to their homology to human exons. For calculating the inclusion percentage of all exons from titin transcripts, inclusion reads (IR) and exclusion reads (ER) were counted for each exon. IR are reads overlapping the exon being investigated, normalized by exon length. ER are reads either upstream or downstream that support exclusions of the read. From these factors, the following equations were used to calculate the percent spliced in index (PSI) as a measure of exon expression (*i*: exon number / *n*: normalized read counts):

$$IR_{i,n} = \frac{IR_i}{length\ exon_i + read\ length - 1}$$

$$ER_{i,n} = \frac{ER_i}{read\ length - 1}$$

$$PSI_i = \frac{IR_{i,n}}{IR_{i,n} + ER_{i,n}}\%$$

Western Blot

Proteins were extracted from tissues by cell lysis buffer (RIPA Buffer, Thermo Scientific) and EDTA free-Proteases Inhibitors (Complete PIC, Roche). The samples were prepared and separated following the NuPAGE Gel protocol (ThermoFisher). Detection of transgenic calpain 3 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were performed using standard Odyssey protocol with primary specific monoclonal anti-calpain3 antibodies: Mouse calpain 3 (NCL-CALP-12A2, Novocastra, Leica Biosystems) diluted 1/200, Goat calpain 3 (COP-SQP-080049, Cosmo Bio CO, LTD) diluted 1/1000, Rabbit anti–GAPDH (FL-335) antibody (Santa Cruz Biotechnology) diluted 1:500.

Proximity ligation assay

The proximity ligation assay (PLA) was performed using the Duolink kit (Olink Bioscience; Sigma-Aldrich) and primary V5 antibodies (mouse P/N 4607-05; Invitrogen), rabbit ab9116 (abcam) 1/400 over-night at +4°C. A colabeling staining was performed with a goat anti-SGCG primary antibody (sc14181d; Santa Cruz, Clinisciences) 1/100. Amplified products were detected as dots with a Leica SP8 confocal microscope. Confocal microscopy images were acquired using a 40X oil immersion objective and intensity signal counted using ImageJ software (https://imagej.nih.gov/ij/). For quantification analysis, 10 fields of 74.236 µm2 were acquired under the same conditions (laser power, photomultiplier tube gain and pinhole) for each experiment and analyzed as a whole. A supervised threshold was determined on 8-bit images for the intense dots corresponding to Duolink amplification (ImageJ). Then, an analysis of dots was performed with the following parameters: size: 3-30 pixels² / "bare outlines", to determine the sum of positive signal area normalized by the total area of the image.

Immunological profiling

Primate sera (1:3 and 1:10 dilutions) were analyzed using an ELISA for the AAV9 capsid as previously described (*43*). Briefly, a plate coated with AAV particles or with contaminants from AAV preparation as control was incubated with heat-inactivated sera dilutions. After three washes, the plate was incubated by Horseradish peroxidase (HRP) purified antibody against immunoglobulin for 1 h at 37°. Then, the plates were washed three times and revealed with tetramethylbenzidine (TMB) substrate solution (BD Biosciences), 30 min in the dark. The reaction was stopped with H_2SO_4 solution and measurements were made at 450 nm. A neutralizing antibody test based on an AAV9-CMV-luciferase transfection assay on HeLa cells was also performed as previously described (*43*). Briefly, mixtures of AAV9-CMV-Luciferase and heat-inactivated sera dilutions were added to HeLa cells. After 2 days of culture, the

luciferase activity was read with a luminometer (VICOTR2, Perkin Elmer). Data are expressed in dilution concentration of the sample where neutralizing activity was still observed.

Echocardiogram

Two-dimensional echocardiographic measurements were performed on standard apical 2- and 4-chamber views with three consecutive cardiac cycles. Left-ventricular end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were recorded. The ejection fraction (EF) of the left ventricle was evaluated with the Simpson's single-plane method as EF = (LVEDV−LVESV)/LVEDV×100 %.

Comparison of the desmin promoter in murine and macaca cells

Murine and primate primary cells were prepared according to the following protocol. Biospies from the triceps and quadriceps of two macaca fascicularis and all limb muscles of two mice were excised and placed in cold Opti-MEM (Life technologies). They were mechanically dissociated and the fragments were placed on culture plates coated with Fetal Bovine Serum (FBS) in DMEM/F-12 (1 :1) (Gibco), Fetal Bovine Serum (FBS) 20% (Eurobio), gentamicin at 30 µg/ml (Life technologies). The cells were incubated at 15°C for the first day of culture and at 37°C thereafter. Washing and medium changes were done every two days during culture. Cells were scraped off using trypsin (Life technologies), washed in media and filtrated on cell strainer (40 µM; Corning) to remove debris. Cells were amplified in T75 flasks coated with Collagen (bovine collagen type I, StemCell Techn.).

Twenty-four hours before transduction, cells were plated at 70% confluency on 6-wells plates coated with collagen. Viral preparations of lentivirus expressing GFP under the control of the desmin promoter were added to the media (2.5×10^5 or 1.5×10^6 infectious genomes). Two days

after, the cells were washed and trypsined for cell sorting. Non transduced cells were used as controls.

Cell sorting and flow cytometry analysis

Cultured cells were high-speed sorted on a Beckman Coulter MoFlo Astrios-EQ. The gating strategy involved three steps: (1) Exclusion of cellular debris and dead cells; (2) GFP+ selection; (3) exclusion of doublets. Data was analyzed using Kaluza software (Beckman Coulter).

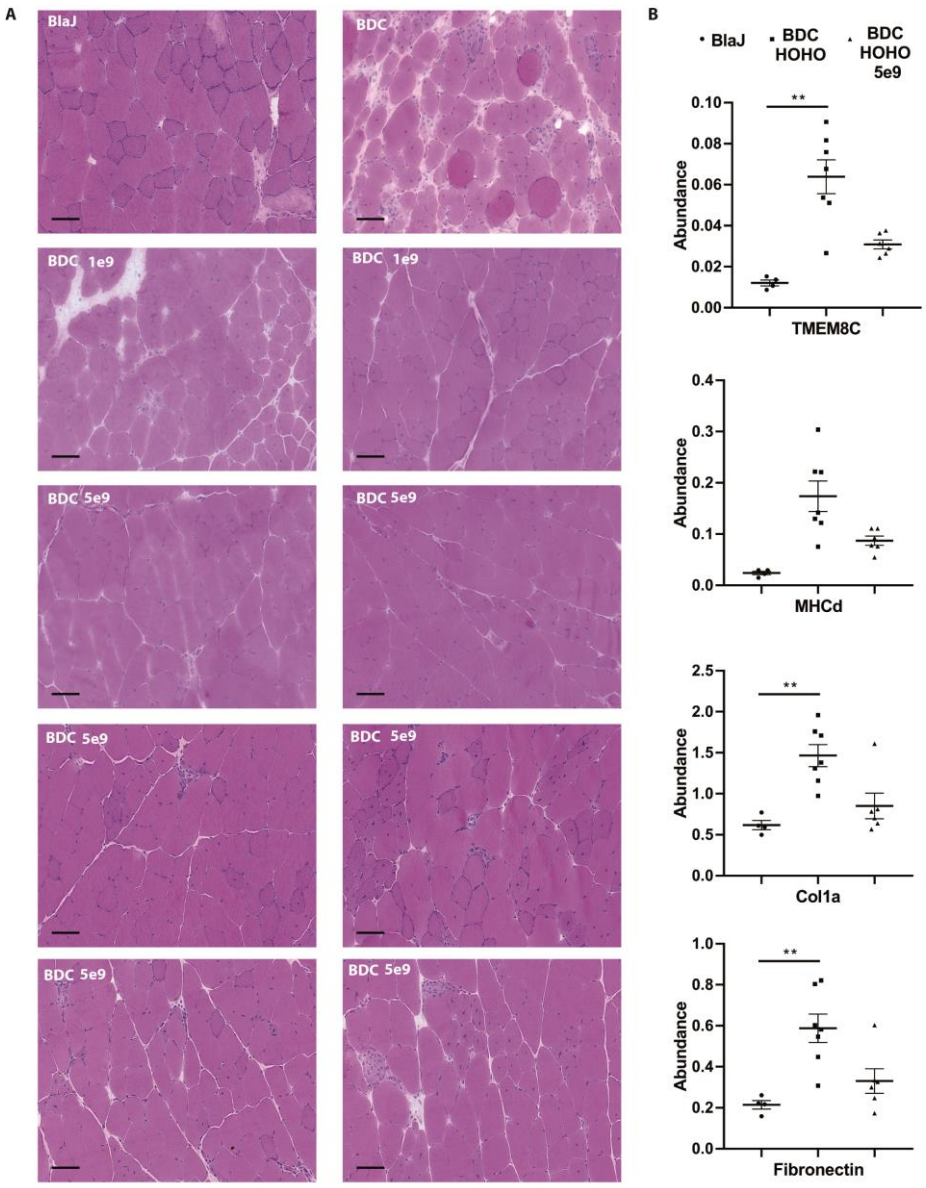

Fig. S1. Supplementary data for the dKO experiments. (A) HPS staining of *gluteus* muscle sections from non-injected BlaJ and BDC mice; BDC mice injected with 1×10^9 vg ($n = 2$); and with 5×10^9 vg ($n = 6$). Scale bar = 100 µm. **(B)** Quantification of dystrophic markers. qRT-PCR were performed to quantify genes representative of several dystrophic features in muscles of Blaj, BDC HOHO and injected BDC HOHO. The data are expressed as abundance normalized

by *Rplpo*. Statistics analyses were performed using non-parametric Kruskal Wallis test and the post-hoc Multiple Comparison Dunn's. $*P < 0.05$; $**P < 0.01$.

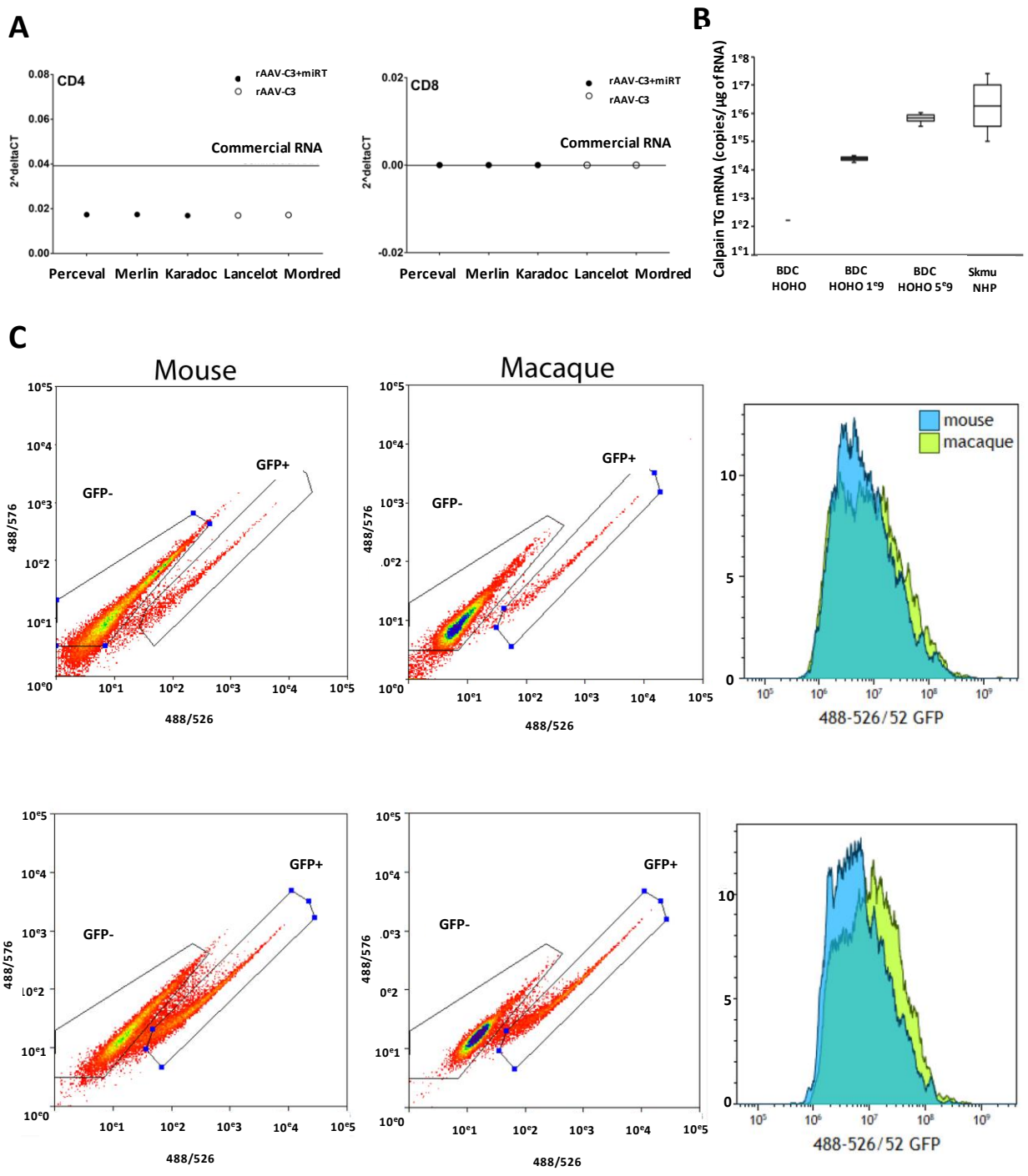

Fig. S2. Supplementary data for the primate experiments. (A) Expression of CD4 and CD8 mRNA in the liver of injected primates normalized to *Rplpo* relative to the quantification in a commercial primate RNA. **(B)** Expression of the transgenic *CAPN3* mRNA in dKO mice non-

injected, injected (1×10^9 and 5×10^9 vg) and injected primates. The 5×10^9 vg in dKO showed a similar expression of mRNA to the 3×10^{13} vg/kg in systemic injection in NHP. **(C)** Flow cytometry analysis of mouse and macaque primary muscle cells transduced with a vector expressing GFP under the desmin promotor; performed in duplicate experiments.

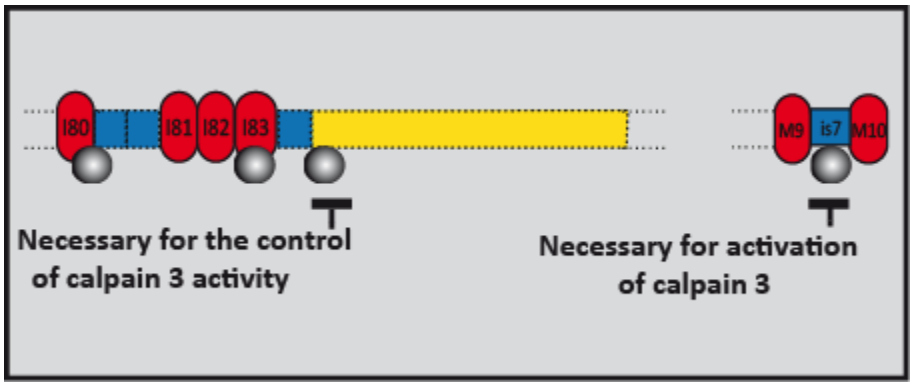

Fig. S3. Proposed model of calpain 3 activation. There are four calpain binding sites on titin: the PEVK is necessary for the control of calpain 3 activity and the binding site in the M-line participates in the activation of calpain 3.

Table S1. Screening of primates for AAV9 seronegativity. First column: Identity number (ID) and name of the primate. Second column: total immunoglobulin binding antibody titer obtained in an ELISA test for the AAV9 capsid expressed as the lowest dilution where binding to AAV9 were detected. - = no binding was observed at the lowest dilution (1:3) in the sample; + = binding is observed starting with a 1:10 dilution. Third column: neutralizing antibody (Nab) titer value obtained on an AAV9-luciferase transfection assay expressed as the highest dilution where at least 50% decrease of expression of a rAAV9-Luc cassette was observed. In the grey background are indicated the primates that were tested for the Nab titer (all primates negative in ELISA together with 4 additional positive ones) and in bold, the ones which were included in the study. We named the primates according to characters from the legend of the "Knights of the Round Table". A pool of human IVIG was used as positive control (Pos. Ctrl). ND= not detected

ID	ELISA AA9	Nab titer	ID	ELISA AA9	Nab titer	ID	ELISA AA9	Nab titer
1	+		18	+		35	+	
2	+		19	+		36	+	
3	+		20	+		37	+	
4	+		21	+	1:3160	38	+	
5	-	ND	**22 Perceval**	**-**	**ND**	39	+	
6	+		23	+		**40 Karadoc**	**-**	**ND**
7	+		**24 Mordred**	**-**	**ND**	41	+	
8	+	1:10	25	+		42	+	1:31.6

9	+		26	+		43	+	
10	+	1:31.6	27	-	1:1	44	+	
11	+		28	+		45	+	
12	+		29	-	1:1	46	+	
13	+		30	+		47	+	
14 Merlin	**-**	**ND**	31	+		48	+	
15	+		32	+	1:31.6	49	-	ND
16	+		33	+		50	+	
17 Lancelot	**-**	**ND**	34	+		Pos.Ctrl	+	1:316

Table S2. Antibody response against the AAV9 serotype on sera (preimmune and sacrifice).

Sera collected at D-4 and at D31. All primates developed an immune response against the vector.

Primate name	Anti-AAV9 IgG (µg/mL)	
	D-4 before AAV9 injection (1:10)	D31 after AAV9 injection (1:12,800)
Merlin	**0.1**	**1381.5**
Lancelot	**0.09**	**379.7**
Perceval	**0.08**	**885.8**
Mordred	**0.08**	**966.3**
Karadoc	**0.11**	**3092.4**

Table S3. **Blood parameters of primates**. **(A)** Biochemistry parameters measured in the blood of the primates. ALT: alanine transaminase; AST: aspartate aminotransferase. **(B)** Hematology parameters measured in the blood of the primates. To serve as references, the third column indicates the normal values in human. No variation in the values was observed before and at the end of the protocol. Note that one primate (Karadoc) has a preexisting platelet deficiency. RBC: red blood cells; MCV: mean corpuscle volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cells.

A

			Perceval		Merlin		Karadoc		Lancelot		Mordred	
	Unit	Normal Values	D-4	D+30	D-4	D+30	D-4	D+30	D-4	D+30	D-4	D+30
Urea	g/L	[0.2-0.6]	0.2	0.3	0.3	0.3	0.3	0.5	0.2	0.4	0.3	0.3
Creatinine	mg/L	[4-9]	8	7	9	9	8	8	8	7	7	8
Alkaline phosphatase	U/L	[<2000]	1577	1448	1789	1050	1551	1300	1696	1541	>2000	>2000
ALT/SGPT	U/L	[16-120]	38	40	43	64	40	49	42	37	27	34
AST/SGOT	U/L	[15-70]	77	51	40	64	55	93	55	57	31	49
Total serum proteins	g/L	[65-85]	71	70	72	64	58	63	63	65	74	67
Bilirubin (total)	mg/L	[1-4]	<1	2	1	3	1	2	2	2	1	2
Sodium	mmol/L	[143-163]	153	160	155	154	152	158	150	158	156	155
Potassium	mmol/L	[3.8-6.0]	4.2	2.8	3.9	3.4	2.8	3.3	3.3	3.1	3.3	2.8
Chloride	mmol/L	[101-121]	116	118	112	116	112	111	115	117	114	115

B

			Perceval		Merlin		Karadoc		Lancelot		Mordred	
	Unit	Normal Values	D-4	D+30	D-‘	D+30	D-4	D+30	D-4	D+30	D-4	D+30
RBC	10^6/µL	[4.5-6.5]	6.2	6.4	6.3	5.7	5.6	5.6	6.6	6.5	6.8	6.1
Hemoglobin	g/L	[108-150]	116	124	125	117	107	108	133	130	126	113
Hematocrit	%	[36-48]	40	41	42	40	34	36	42	42	42	38
MCV	fL	[58-71]	63.5	67.7	66.9	71.5	60.8	63.6	65.0	66.1	62.6	63.0
MCH	pg	[19.5-27.0]	18.6	19.3	19.7	20.7	19.1	19.4	20.1	20.0	18.5	18.5

MCHC	%	[28-33]	29.0	30.2	29.8	29.3	31.5	30.0	31.7	31.0	30.0	29.7
Reticulocytes	10^3/µL	[<80]	28.7	50.0	12.0	28.8	135.3	93.2	24.5	26.0	41.6	31.8
WBC	10^3/µL	[5.7-21.0]	16.9	13.6	8.4	5.3	6.3	6.4	6.1	12.2	8.2	7.0
Neutrophils	10^3/µL	[3.5-8.5]	4.7	5.2	1.9	1.6	1.6	3.3	1.3	8.0	0.4	3.9
Lymphocytes	10^3/µL	[3.5-7.8]	10.9	7.4	6.2	3.4	4.4	2.6	4.5	4.0	7.7	3.0
Monocytes	10^3/µL	[0.4-1.3]	0.9	0.7	0.2	0.2	0	0.2	0.1	0.1	0	0.1
Eosinophis	10^3/µL	[<0.7]	0.2	0.3	0.1	0.1	0.3	0.3	0.2	0.1	0.1	0
Basophils	10^3/µL	[<0.1]	0.2	0	0	0	0	0	0	0	0	0
Platelets	10^3/µL	[270-580]	342	171	185	250	1	2	391	363	424	272

Table S4. Summary of the echocardiogram analysis before and after systemic injection in primates. Analysis was performed on injected primates before the injection (D-2), and at the end of the study (D+34). (**A)** Bidimension (morphological features), Time-Movement (diastole and systole measurements) Ratio LA/Ao: Left Atrium diameter/Aorta diameter; E-Septum: Septum thickness; RVd: Right Ventricular diameter at end-diastole; IVSd: intraventricular septal thickness at end-diastole; LVDd: Left Ventricular diameter at end-diastole; LVFWd: Left ventricular free-wall thickness at end-diastole; IVSs: interventricular septal thickness at end-systole; LVDs: Left Ventricular diameter at end-systole; LVFWs: Left ventricular free-wall thickness at end-systole; FS (%): Fractional shortening. (**B)** Doppler analyses. Vpulm: transpulmonary flow velocity; VAo: transaortic flow velocity; VE: early transmitral flow velocity; VA: late transmitral flow velocity. Bpm: bit per minute

A		**BD**		**TM**							
		LA/Ao	**E-Septum (mm)**	**RVd (mm)**	**IVSd (mm)**	**LVDd (mm)**	**LVFWd (mm)**	**IVSs (mm)**	**LVDs (mm)**	**LVFWs (mm)**	**FS (%)**
Perceval	D-4	1.04	0.43	1.7	2.4	8.8	3.8	3.5	4.7	5.4	47
	D30	1.33	1.6	2.1	3.1	11.6	4.1	3.6	8.5	5.4	27
Merlin	D-4	1.16	1.8	2.3	3.9	11.6	4.4	4.7	8.5	6.0	27
	D30	1.26	1.5	1.9	2.2	12.9	3.2	3	8.6	4.5	33
Karadoc	D-4	1.18	0.65	0.86	2.4	10.8	3.2	3.4	5.0	5.6	54
	D30	1.25	0.86	1.2	2.4	7.4	3.3	2.2	4.3	2.8	42
Lancelot	D-4	1.25	0.52	1.3	2.6	9.1	4.4	3.4	5.7	4.4	37
	D30	1.0	1.5	0.86	2.4	10.3	3.9	2.8	7.8	3.9	25
Mordred	D-4	1.14	1.6	1.6	2.8	12.4	3.9	3.6	8.3	5.7	33
	D30	1.0	1.8	1.5	2.2	9.9	2.8	2.6	5.6	3.2	43

B

		Doppler				
		Vpulm flow (m/s)	**VAo flow (m/s)**	**VE (m/s)**	**VA (m/s)**	**bpm**
Perceval	D-4	0.89	1.07	0.92	-	184
	D30	0.71	0.77	0.65	0.36	183
Merlin	D-4	0.86	1.00	0.89	0.25	134
	D30	1.09	0.99	1.03	0.80	178
Karadoc	D-4	0.85	0.98	0.76	0.39	157
	D30	0.93	0.93	1.05	0.94	175
Lancelot	D-4	0.68	1.07	0.66	0.48	163
	D30	0.78	0.66	0.54	0.36	170
Mordred	D-4	0.98	0.74	0.74	0.41	142
	D30	0.98	-	0.87	0.65	169