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***FAST*TRACK CONGRESS**
Heart failure and cardiomyopathies

CRISPR activation to repair electrocardiogram abnormalities caused by a FLNC truncating variant in mice

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Introduction

Truncating variants in the *Filamin C* (*FLNC*) gene (*FLNCtv*) are one of the most frequent causes of genetic dilated cardiomyopathy (DCM) and non-dilated left ventricular (LV) cardiomyopathy.[1,2] In both cardiomyopathies, arrhythmic manifestations and sudden cardiac death are strong components of the disease and rarely improve after its onset.[1] There is no specific therapy for *FLNCtv*-induced cardiomyopathy and electrical manifestations of the disease.

CRISPR activation (CRISPRa) comprises catalytically inactive Cas9 proteins [dead Cas9 (dCas9)] fused to transcriptional activation domains, which are then directed to a gene-of-interest's promoter to induce transcription.[3] CRISPR activation–based therapies are of major interest for genes whose coding sequences exceed the packaging limits of conventional adeno-associated virus (AAV) and are therefore not suitable for gene replacement, a common feature among frequent cardiomyopathy-related genes like *FLNC*. While CRISPRa-based therapies have shown promising results in cell culture models of cardiomyopathy,[4] their potential to treat cardiomyopathy *in vivo* has not been explored.

Methods

To test the efficacy of CRISPRa for the treatment of *FLNC*tv-induced cardiomyopathy and to determine whether its pathological features can be reversed in adulthood, we generated a mouse model with a constitutive heterozygous deletion of *FLNC* exon 15 ($FLNC^{Ex15del/wt}$) mimicking a STOP codon identified in a FLNCtv patient (*Figure 1A*).[2] C57Bl/6 male and female mice generated at Centro Nacional de Investigaciones Cardiovasculares (CNIC) were used. To assess FLNC expression, we measured mRNA and protein levels in the LVs of mutant mice using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and western blot, respectively. Cardiac electrical activity was measured by electrocardiogram (ECG). Electrocardiograms were acquired for 90 s under 1.5% isoflurane anaesthesia as we recently described.[5] Recordings were acquired by a blinded operator. QRS duration and amplitude were analysed from three non-consecutive beats from Lead II. Where indicated, a 90-s baseline ECG was acquired, 40 mg/kg flecainide was administered i.p., and ECGs were recorded for 5 min.[5] Power calculations were conducted to detect a 20% reduction in QRS complex amplitude, assuming an standard deviation (SD) of 10%, with $\alpha = 0.05$ and power = 80%. χ^2, independent *t*-test, one-way, and two-way analysis of variance tests were used as appropriate.

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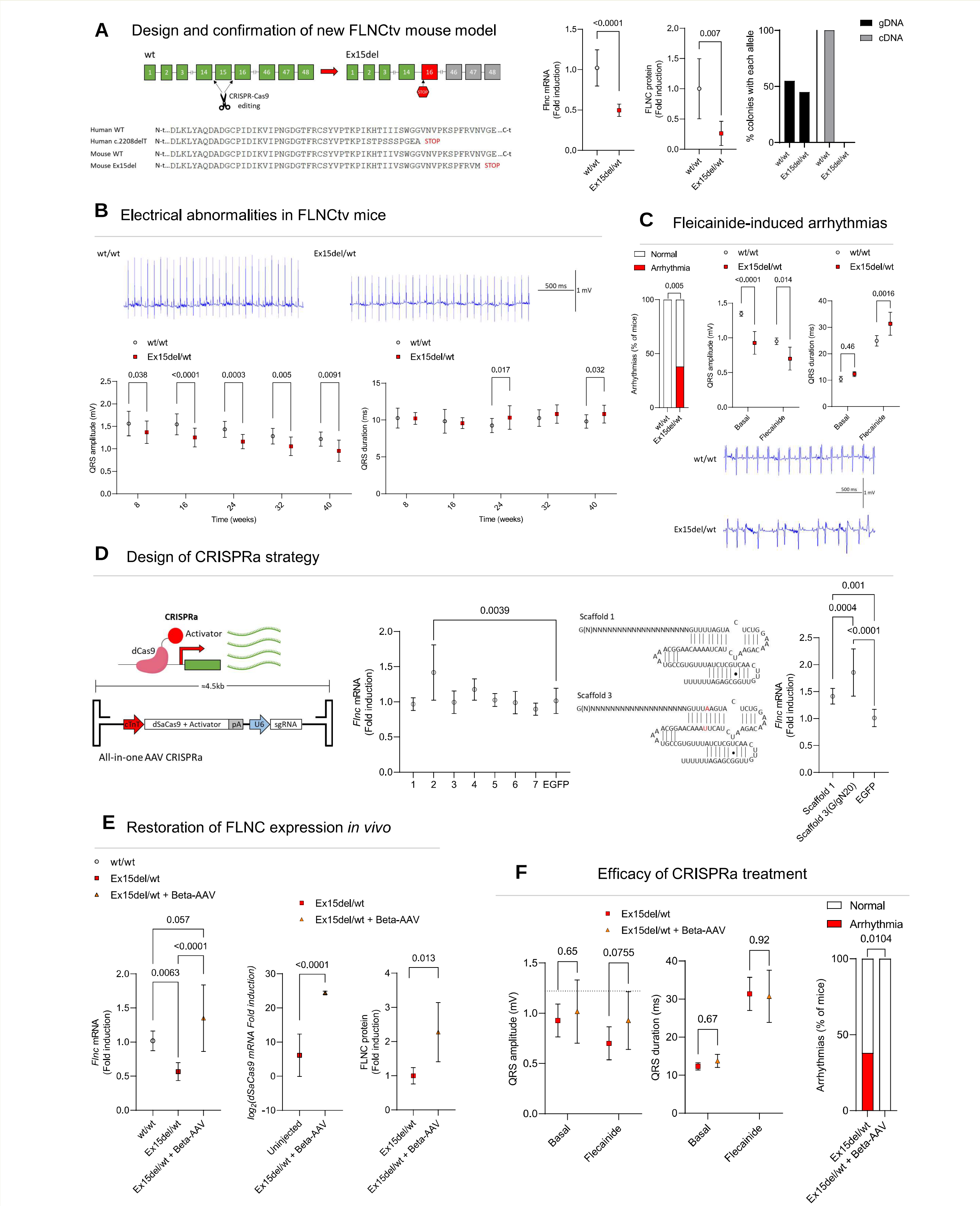

Figure 1 CRISPR activation–based gene therapy improves cardiac function in a mouse model of cardiomyopathy caused by FLNCtv. (*A*) Schematic showing the design of the mutant mouse bearing a *Filamin C* truncating variant. Deletion of exon 15 (Ex15del) in the mouse *Filamin C* gene. Below, Filamin C amino acid sequence of the FLNCtv patient on which the model is based and our mouse model for Ex15del, compared with wild type in each species. Filamin C mRNA ($n = 8$–15/group) and protein ($n = 6$/group) expression were quantified in the left ventricle of wild-type (wt/wt) and Continued

Results

Mutant mice showed reduced levels of *FLNC* mRNA and protein overall, suggesting degradation of the mutant mRNA (*Figure 1A*). To confirm degradation of *FLNCtv* mRNAs in Ex15del mice, genomic DNA (gDNA) and RNA were extracted from LV myocardial samples from Ex15del mice, and RNA was reverse-transcribed into complementary DNA (cDNA). The *FLNC* region flanking exon 15 was amplified by PCR and the resulting product was cloned and transformed into bacteria. The plasmid in each colony was sequenced and the number of colonies (as % of the total) bearing a wild-type or mutant allele was quantified for gDNA and RNA. As shown in *Figure 1A*, while the number of colonies bearing the mutant DNA allele was similar to those bearing the wild-type allele, no colonies carrying the mutant cDNA were detected. These results suggested that the mutant *FLNC* mRNA was degraded, consistent with *FLNC* haploinsufficiency caused by *FLNCtv*.

Although no overt systolic dysfunction or myocardial fibrosis was found and survival was not affected in *FLNC* mutant mice, ECG revealed several abnormalities, including reduced QRS amplitude (*Figure 1B*). Administration of a supra-therapeutic dose of flecainide induced arrhythmias in ∼40% mutant mice, primarily consisting of premature ventricular contractions, and caused further reduction of the QRS amplitude and an increase in QRS duration compared to wild-type controls (*Figure 1C*).

Next, we developed a cardiac-specific all-in-one AAV CRISPRa system to restore FLNC expression. The vector comprised the following: (i) the chicken cardiac troponin T promoter to ensure cardiac-specific *dCas9* expression, (ii) a catalytically inactive version of the small Cas9 orthologue from *Staphylococcus aureus* (*dSaCas9*), (iii) the compact activator domain VP64 linked to dSaCas9,[6] (iv) a synthetic polyA, and (v) the single-guide RNA (sgRNA) driven by the U6 promoter. This resulted in a viral genome of ∼4.5 kb, well within AAV constraints (*Figure 1D*).

To optimize our viral system, we transfected the HL-1 cardiac muscle cell line with different constructs. First, we screened seven different protospacer sequences and found that sequence number 2 was the most efficient at activating mouse *FLNC* transcription, reaching *FLNC* mRNA expression levels of ∼1.5-fold compared with enhanced green frluorescent protein (EGFP)-transfected controls (*Figure 1D*). To further improve activation, we used a previously reported modified scaffold,[7] which was able to further increase FLNC activation, reaching *FLNC* mRNA expression levels of ∼1.8-fold compared with EGFP-transfected controls (*Figure 1D*). To maximize cardiomyocyte transduction, we packaged our CRISPRa system into a myotropic AAV capsid (AAVMYO).[5]

At 32 weeks of age, mutant mice with reduced QRS compared with the average of wild-type mice underwent baseline ECG recordings to document electrical abnormalities, before receiving an intravenous injection of 1×10^{13} vg of our AAV system ('Beta-AAV'). Five weeks post-injection, we performed follow-up ECGs with flecainide challenge and subsequently sacrificed the treated mice for expression analyses. Quantification of FLNC and dSaCas9 expression by qRT-PCR confirmed that the AAV system successfully increased FLNC mRNA levels in mutant mice, restoring FLNC expression levels to those of wild-type controls (*Figure 1E*). Remarkably, the treatment not only increased the QRS complex amplitude but also provided complete protection against ventricular arrhythmias during flecainide challenge, effectively rescuing the electrical phenotype in FLNC mutant mice (*Figure 1F*).

Discussion

Our heterozygous *FLNCtv* model partially recapitulates the electrical abnormalities observed in *FLNCtv* carriers.[2] Most importantly, this study provides the first *in vivo* demonstration that CRISPRa-AAV-mediated gene activation can effectively treat an inherited cardiomyopathy driven by haploinsufficiency. We show that even after disease onset, electrical dysfunction can be reversed through this targeted therapeutic approach. Our findings open new avenues for broader applications of

Figure 1 Continued

heterozygous mutant mice (Ex15del/wt). Genomic DNA and RNA were extracted from left ventricle myocardial samples from Ex15del mice, and RNA was reverse-transcribed into complementary DNA. The *Filamin C* region flanking the exon 15 was amplified by polymerase chain reaction, and the resulting product was cloned and transformed into bacteria. The plasmid in each colony was sequenced, and the number of colonies (as % of the total) bearing a wild-type or mutant allele was quantified for genomic DNA and RNA. $n = 24$ colonies. (*B*) The amplitude and duration of the QRS complex were measured by electrocardiogram in wt/wt and Ex15del/wt mice ($n = 11$–28/group and time point). (*C*) Mice were injected intraperitoneally with 40 mg/kg flecainide at 37 weeks of age, and the incidence of arrhythmias, QRS amplitude, and duration were measured in wild-type and mutant mice electrocardiograms ($n = 4$–10/group). Arrhythmias were defined as the occurrence of more than three premature ventricular complexes and/or second-degree Type II atrioventricular blocks within a 10-s interval after flecainide administration. (*D*) Schematics showing the CRISPR activation approach comprising the dead Cas9 linked to the VP64 transcription activation domain guided to the promoter of interest and the adeno-associated virus vector design ('Beta-adeno-associated virus'). HL-1 cells were transfected with constructs bearing different protospacer sequences and *Filamin C* expression was quantified by qRT-PCR ($n = 6$/condition). The scaffold sequence of the sgRNA (#1) was modified (#3), and the effect on *Filamin C* induction was assessed by quantitative real-time reverse transcription polymerase chain reaction ($n = 14$–16/condition). Transfection with EGFP was used as a negative control. Protospacer: 5′-GCCAGGAATGCCGCCCGGCCC-3′; Scaffold: 5′-GTTTAAGTACTCTGGAAACAGAATCTACTTAAACAAGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGA-3′. (*E*) Exdel15/wt mice were infected with Beta-adeno-associated virus carrying the CRISPR activation machinery and the Filamin C promoter-targeting sgRNA, and the expressions of *Filamin C* and *dCas9-VP64* were determined by quantitative real-time reverse transcription polymerase chain reaction in the left ventricle myocardium ($n = 8$–10/condition). Filamin C protein expression was quantified by western blot. Wild-type (wt/wt) and uninfected Exdel15/wt mice (Ex15del/wt) were used as controls. (*F*) Mice were infected as in (*E*) at 32 weeks of age, and the QRS amplitude, QRS duration, and incidence of arrhythmias were determined at 40 weeks in the absence or presence of flecainide intraperitoneal injection ($n = 10$–14). Data shown as group mean ± standard deviation. Dashed line indicates wild-type average values at 40 weeks or age (*F*). Specific *P*-values shown for each comparison. Samples were tested for normality and homogeneity of variance. $\chi 2$ (*C* and *F*; no expected cell count was <1 and fewer than 20% were <5), independent *t*-test (*A* and *E*), one-way analysis of variance (*D* and *E*), and two-way analysis of variance plus Šídák's multiple comparisons test (*B*, genotype and time; *C* and *F*, genotype and treatment) were used

CRISPRa-AAV platforms in addressing cardiac disorders rooted in insufficient gene dosage.

Limitations: Our mouse model developed electrical abnormalities but not systolic dysfunction, which is present in around one-third of FLNCtv patients. The FLNC biodistribution profile in mutant mice treated with Beta-AAV was not checked outside the heart. A dose response curve for the virus was not generated, and follow-up was limited to 5 weeks. The CRISPRa system was not tested in human cells. Off-target effects were not analysed by RNA-seq, and we cannot exclude confounding effects of variables not tested.

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Declarations

Disclosure of Interest

A.C. has a series of CRISPR technology–related patents that are managed by Havard University and Columbia University. The results in this paper are part of a patent application filed by R.C.-A., E.L.-P., P.G.-P., and A.C.; patent application pending.

Data Availability

The data supporting the findings of this article are available from the corresponding author upon reasonable request.

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Ethical Approval

The procedures in this study were approved by the Ethics Committees of the CNIC and the Regional Government of Madrid (PROEX191.5/22).

Pre-registered Clinical Trial Number

Not applicable.

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