CRISPR-Cas9 base editing of pathogenic CaMKIIδ improves cardiac function in a humanized mouse model

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Abstract

Cardiovascular diseases are the most common cause of worldwide morbidity and mortality, highlighting the necessity for advanced therapeutic strategies. Ca\(^{2+}\)/calmodulin-dependent protein kinase IIδ (CaMKIIδ) is a prominent inducer of various cardiac disorders, which is mediated by two oxidation-sensitive methionine residues within the regulatory domain. We previously showed that ablation of CaMKIIδ oxidation by CRISPR-Cas9 base editing enables the heart to recover function from otherwise severe damage following ischemia/reperfusion (IR) injury. Here, we extended this therapeutic concept toward potential clinical translation. We generated a humanized CAMK2D knockin mouse model, in which the genomic sequence encoding the entire regulatory domain was replaced with the human sequence. This enabled comparison and optimization of two different editing strategies for the human genome in mice. To edit CAMK2D in vivo, we packaged the optimized editing components into an engineered myotropic adeno-associated virus (MyoAAV 2A), which enabled efficient delivery at a very low AAV dose into the humanized mice at the time of IR injury. CAMK2D-edited mice recovered cardiac function, showed improved exercise performance, and were protected from myocardial fibrosis, which was otherwise observed in injured control mice post-IR. Our findings identify a potentially effective strategy for cardioprotection in response to oxidative damage.

Key Words: Ca\(^{2+}\)/calmodulin-dependent protein kinase IIδ, MyoAAV, ischemia/reperfusion injury, cardioprotection, cardiac disease
Cardiovascular diseases, in particular coronary artery heart disease with subsequent myocardial infarction, are the most common cause of death worldwide, highlighting the necessity for advanced therapeutic strategies (1). Chronic overactivation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II\(\delta\) (CaMKII\(\delta\)) has been shown to be a central indicator and inducer of cardiac disease (2-10). While regulating cellular homeostasis and signaling at normal activation levels, sustained increased CaMKII\(\delta\) activation has been linked to impaired excitation-contraction coupling, disturbances in cellular Ca\(^{2+}\) handling, inflammation, apoptosis, and fibrosis, which impair cardiac function (2, 4, 5, 8-14). Accordingly, CaMKII\(\delta\) overactivation has been linked to myocardial infarction and ischemia/reperfusion (IR) injury, heart failure, arrhythmias, cardiac hypertrophy, and sleep-disordered breathing (2, 3, 6-11, 15, 16). Mechanistically, oxidation of two methionine residues at position 281 and 282 has been shown to activate CaMKII\(\delta\) by preventing association of the autoinhibitory region with the catalytic domain (3, 10, 15-17). Indeed, Camk2d knockin mice, in which both oxidation-sensitive methionines were replaced with oxidation-resistant valines in the germline, were protected from severe cardiac damage (3, 15, 16). These studies suggested that preventing CaMKII\(\delta\) oxidation may offer a cardioprotective benefit to heart disease.

As knockin technology is not suitable for potential clinical application, we have previously developed a CRISPR-Cas9 adenine base editing strategy to ablate CaMKII\(\delta\) oxidation in vivo in adult mice (10). Base editing involves a fusion protein of Cas9 nickase or inactive Cas9 fused to a deaminase domain, which is directed to a genome sequence by a single-guide RNA (sgRNA) (18-23). Adenine base editing (ABE) allows the precise
conversion of adenine to guanine nucleotides without introducing double-stranded DNA breaks (10, 18-25). As methionines are encoded by ATG codons, we utilized ABE to convert the two methionine residues at position 281 and 282 to valines encoded by GTG codons in adult mice, thereby rendering CaMKIIδ insensitive to oxidative activation (10). Administration of Camk2d editing components at the day of IR injury to adult mice achieved successful editing and enabled the heart to recover function from otherwise severe damage (10). CAMK2D gene editing may thus represent a permanent and advanced strategy for heart disease therapy (10).

While our previous work demonstrated a potential intervention to protect the heart from IR damage, the approach has several limitations that could diminish its therapeutic application. First, the previous study used the mouse sgRNA sequence targeting the mouse genomic sequence of the CaMKIIδ oxidation sites, which is different from the human sequence. Therefore, the efficacy of the human sgRNA in vivo remains to be determined. Second, we administered a relatively high adeno-associated virus (AAV) dose to adult mice (10). However, high AAV doses have been associated with serious adverse effects, including acute liver damage, thrombocytopenia, and immunological responses with preexisting antibodies (18, 26, 27). Large-scale AAV production also poses substantial challenges. It is thus imperative to deliver AAV at the lowest effective dose. In addition, we performed a broad-windowed ablation of the oxidative activation sites of CaMKIIδ that contained bystander-editing in vivo (10).

The aim of the present study was to further advance our previous strategy of rendering CaMKIIδ resistant to oxidative activation toward a potential therapy for human cardiac disease. Therefore, we generated a CAMK2D knockin mouse model to humanize
the regulatory domain of CaMKIIδ, which allowed the use of human sgRNA-sequences in vivo in mice. We made efforts to reduce off-target editing and to decrease the AAV dose for delivery of the gene editing components in vivo. We further compared different editing patterns in vivo following IR, as narrow-windowed and bystander-free editing of only one methionine-encoding adenine may potentially be sufficient to confer cardioprotection (10, 17). In addition, we tested whether CAMK2D editing improves exercise performance following IR.
Results

Development of an optimized gene editing strategy in human iPSCs to reduce off-target editing

In our prior studies, we performed adenine base editing of methionine-encoding ATG codons of CAMK2D in human induced pluripotent stems (iPSCs) with high editing efficiencies. However, we observed off-target editing of 29.5% at an intronic site in the DAZL gene (10). Thus, to further develop an optimized base editing strategy for CAMK2D with reduced off-target editing, we used an engineered ABE8e variant (TadA-8e V106W) that causes less off-target editing (Figure 1A and B) (10, 19). Here, we compared two different editing strategies targeting the oxidative activation sites of CaMKIIδ (Figure 1A).

First, we deployed sgRNA1 that places CAMK2D c.A841 (p.M281) in protospacer position 13 (counted from the 5’ end of the protospacer adjacent motif [PAM] sequence) together with ABE8e(V106W) fused to SpCas9 nickase (targeting NGG PAMs; Figure 1C) (20). Following nucleofection of human induced pluripotent stem cells (iPSCs), we observed a mean editing efficiency of 64.7±1.5% for c.A841G (p.M281V) (Figure 1C and D). Notably, we detected no marked editing of c.A844 (p.M282) (Figure 1D) or any off-target editing at the top 8 predicted potential off-target sites in the human genome (Figure 1D).

We further analyzed sgRNA2 combined with ABE8e(V106W) fused to the engineered variant SpRY nickase that targets NRN (and NYN PAMs to a lesser extent; Figure 1E) (21). When using sgRNA2, adenines within the methionine-281 (c.A841) and -282 (c.A844) codons are placed in protospacer positions 17 and 14, respectively. Nucleofection of human iPSCs and subsequent DNA sequencing revealed an editing efficiency of 80.3±0.7% for c.A841G (p.M281V), 76.7±0.3% for c.A844G (p.M282V), and
76.3±0.7% for c.A848G (p.H283R) (Figure 1F). Deep amplicon sequencing of the top 8 predicted potential off-target sites revealed adenine to guanine editing of 9.2±1.1% in an intronic region of the DAZL gene, which was greatly reduced compared to the 29.5% off-target editing with the previous editing strategy (10). No other off-target editing was detected (Figure 1F).

**Editing oxidation-sensitive methionines of CaMKIIδ in a humanized mouse post-IR**

Efficient CRISPR-Cas9 gene editing is critically dependent on the sgRNA that corresponds to a specific target sequence in the genome (18, 20). While the amino acid sequence of CaMKIIδ is highly conserved across species, the DNA sequence differs. For example, for sgRNA1, there is a 10% mismatch between the human and the corresponding mouse sequence, which prevents highly efficient gene editing between species (18-20, 22-25). Thus, to enable possible translation to the human gene, we humanized the entire regulatory domain of the mouse CaMKIIδ by exchanging exons 11 and 12, intron 11, and portions of the flanking introns 10 and 12 with the human sequence, using CRISPR-Cas9-mediated homology-directed repair (Figure 2A and Supplemental Figure 1). This humanized mouse model enables deployment of gene editing strategies optimized for the human genome in vivo in mice. Compared to wildtype littermates, homozygous humanized mice showed normal body and heart weight at 12 weeks of age (Supplemental Figure 2A and B). Sequencing of cDNA showed that the humanized exons 11 and 12 were properly spliced between the mouse exons 10 and 13 (Supplemental Figure 2C). Western blot analyses revealed comparable CaMKII protein expression between mice with wildtype or humanized CAMK2D (Supplemental Figure 2D and E).
Accordingly, both genotypes had similar CaMKII activity at baseline, making the humanized \textit{CAMK2D} mouse a suitable model for in vivo gene editing (Supplemental Figure 2F).

In the present study, we used female and male mice that were homozygous for the humanized \textit{CAMK2D} knockin and both genders were represented equally within each group. One week before IR surgery, mice were subjected to baseline echocardiography (Figure 2B and Supplemental Figure 3). Importantly, all mice showed normal fractional shortening, left ventricular end-diastolic diameter, and left ventricular end-diastolic volume with no differences across all groups (Supplemental Figure 3). At 12 weeks of age, mice were subjected to either 45 min open chest surgery (Sham) or to 45 min ligation of the left anterior descending coronary artery (IR; Figure 2B). To deliver the \textit{CAMK2D} editing components in vivo into the heart, we used AAV with the myotropic AAV2/MyoAAV 2A serotype (28). Due to the limited packaging size of AAVs, which is exceeded by the ABE expression cassette, we used split-intein trans-splicing systems (Figure 2C and D). One pair of MyoAAVs carried ABE8e(V106W)-SpCas9 + sgRNA1 (Figure 2C). The second pair of MyoAAVs carried ABE8e(V106W)-SpRY + sgRNA2 (Figure 2D). Cardiomyocyte-specificity was ensured by using the cardiac troponin T (cTnT) promoter to drive the expression of the editing components. Mice subjected to IR received a single intracardiac injection post-reperfusion of either a non-editing control MyoAAV (IR+Control Virus), MyoAAV-ABE-sgRNA1 (IR+sgRNA1) or MyoAAV-ABE-sgRNA2 (IR+sgRNA2). Each mouse received a total virus dosage of $1.5 \times 10^{11}$ vg/kg body weight (equal amounts of N- and C-term for IR+sgRNA1 and IR+sgRNA2).
After 24 hours, all mice subjected to IR showed a substantial decline in cardiac function (Supplemental Figure 4). Compared to Sham mice with 57.7±1.1% fractional shortening, we observed a decrease in fractional shortening to 32.2±2.3% in IR+Control Virus (p<0.0001), 35.6±2.4% in IR+sgRNA1 (p<0.0001), and 37.0±0.9% in IR+sgRNA2 (p<0.0001) (Supplemental Figure 4B). Moreover, left ventricular end-diastolic diameter increased from 2.3±0.1 mm in Sham mice, to 3.3±0.1 mm in IR+Control Virus (p<0.0001), 3.1±0.1 mm in IR+sgRNA1 (p=0.0002), and 2.9±0.1 mm in IR+sgRNA2 (p=0.004) (Supplemental Figure 4C). Similarly, left ventricular end-diastolic volume was also significantly increased in all groups subjected to IR compared to Sham (Supplemental Figure 4D).

Three weeks post-IR, CAMK2D-edited mice displayed a significantly improved fractional shortening from 40.3±2.8% in IR+Control Virus to 51.6±3.4% in IR+sgRNA2 (p=0.02) (Figure 2E and F). Notably, mice subjected to IR+sgRNA1 only showed a non-significant intermediate recovery to 47.2±3.2% (p=0.09). Left ventricular end-diastolic diameter decreased from 3.3±0.2 mm in IR+Control Virus to 2.9±0.1 mm (p=0.03) and 2.8±0.1 mm (p=0.01) in IR+sgRNA1 and IR+sgRNA2, respectively (Figure 2G). Similarly, left ventricular end-diastolic volume was almost normalized from 46.5±7.0 μL in IR+Control Virus to 32.4±1.9 μL and 29.2±2.0 μL in IR+sgRNA1 (p=0.02) and IR+sgRNA2 (p=0.0095), respectively (Figure 2H).

Functional and mechanistic analyses in CAMK2D-edited mice post-IR

As exercise performance depends on both skeletal muscle and cardiovascular function, we reasoned that recovery of cardiac function should improve exercise performance post-
IR. Thus, all mice were subjected to a treadmill exhaustion test four weeks post-IR, according to a standardized protocol (10). As expected, mice treated with a non-editing control virus achieved a substantially lower maximal velocity of 18.0±0.4 m/min compared to 28.0±0.5 m/min in Sham-treated mice (p<0.0001) (Figure 3A and B). Compared to IR+Control Virus, CAMK2D-edited mice showed a higher maximal velocity of 21.1±1.0 m/min (p=0.001) and 25.9±0.3 m/min (p<0.0001) with IR+sgRNA1 and IR+sgRNA2, respectively (Figure 3B). Notably, mice treated with sgRNA2 showed a significantly higher maximal velocity than mice treated with sgRNA1 (p<0.0001). Accordingly, the total distance run on the treadmill was ~4.6-fold shorter in mice treated with a control virus (109.2±12.3 m) compared to Sham (506.4±23.6 m, p<0.0001) (Figure 3C). Compared to IR+Control Virus, CAMK2D-edited mice achieved a longer distance of 218.1±37.3 m in IR+sgRNA1 (p=0.003) and an even higher recovery with 405.4±14.7 m in mice subjected IR+sgRNA2 (p<0.0001 vs. IR+Control Virus, p<0.0001 vs. IR+sgRNA1) (Figure 3C). Moreover, we found a significant correlation between fractional shortening and both the maximal velocity (p<0.0001, r²=0.52) and the total distance (p<0.0001, r²=0.50) achieved on the treadmill, further validating our findings (Figure 3D and E).

Five weeks post-IR, all mice were sacrificed for further analyses. Mice treated with the control virus post-IR showed a reduced body weight of 22.8±0.4 g compared to Sham with 28.0±0.7 g (p<0.0001), which is frequently observed in patients with critical illness (Figure 4A). Interestingly, only mice treated with sgRNA2 post-IR recovered in body weight to 26.7±0.7 g (p<0.0001) (Figure 4A). Additionally, control mice post-IR showed increased heart and lung weights compared to Sham, which were both significantly improved in mice treated with sgRNA2 but not with sgRNA1 post-IR (Figure 4B and C).
Importantly, there was no difference in liver weight across all groups, indicating that there was no severe liver failure due to the potential liver toxicity of AAV (Figure 4D). Analysis of picrosirius red-stained cardiac sections revealed a ~7.0-fold increase in fibrotic area in control mice post-IR, which was 9.3±0.2% in IR+Control Virus compared to 1.3±0.2% in Sham (p<0.0001) (Figure 4E and F). Importantly, only treatment with sgRNA2 but not with sgRNA1 resulted in a substantially reduced region of fibrotic tissue of 1.8±0.2% in IR+sgRNA2 (p<0.0001) and only a small residual fibrotic area was detectable (Figure 4E and F).

Analysis of editing efficiency in humanized mice upon IR

The editing efficiency was assessed in myocardial samples of the anterior cardiac wall, as this is the area of interest that was injured by IR and where the editing components were injected. In fact, we previously found that editing was restricted to the region of the intracardiac injection (10). In mice treated with sgRNA1, sequencing analyses revealed an editing efficiency of 36.2±0.6% at the DNA and 83.2±0.6% at the cDNA level for c.A841G (p.M281V) while no marked editing of c.A844 (p.M282) was detected (Figure 5A). In mice treated with sgRNA2, we measured an editing efficiency of 37.0±0.5%, 27.8±0.7%, and 36.4±0.7% at the DNA level, and 82.8±0.7%, 84.8±0.5%, and 83.4±0.4% at the cDNA level for c.A841G (p.M281V), c.A844G (p.M282V), and c.A848G (p.H283R), respectively (Figure 5B). The editing efficiency differs between the DNA and cDNA level since only DNA of cardiomyocytes is targeted when using a cardiac troponin T promoter and cardiomyocytes represent only ~40% of all cells in the heart (29). However, most of
the cardiac CaMKIIδ is expressed by cardiomyocytes, explaining the high editing efficiency at the cDNA level.

As expected, Western blot analyses revealed a ~4.7-fold increase in oxidized CaMKII in control mice post-IR, but not when the oxidative activation sites were ablated using ABE (Figure 5C-F). There was no difference in total CaMKII expression across all groups (Figure 5C and E). When normalized to total CaMKII expression, CaMKII oxidation levels increased from 0.13±0.03 in Sham to 0.63±0.04 in IR+Control Virus (p<0.0001; based on densitometric analysis) (Figure 5F). In contrast, we observed only minimal levels of oxidized CaMKII with 0.14±0.04 and 0.09±0.02 in IR+sgRNA1 and IR+sgRNA2, respectively (p<0.0001 for both groups vs. IR+Control Virus). This residual signal can either represent unedited CaMKIIδ, oxidized CaMKIIγ or unspecific background. Ablation of just methionine-281 with sgRNA1 was sufficient to decrease the binding affinity of the antibody recognizing oxidized CaMKII, which is in accordance with our previous data in iPSC-cardiomyocytes and also with other studies analyzing post-translational modification of one individual amino acid (10). Measurement of CaMKII activity showed a ~6.0-fold increased signal of 8.9±1.9 nmol/min/mg in IR+Control Virus compared to 1.5±0.1 nmol/min/mg in Sham (p=0.0001) (Figure 5G). Importantly, only treatment with sgRNA2, which ablates both oxidative activation sites, could normalize CaMKII activity to 1.5±0.1 nmol/min/mg post-IR (p=0.0001 vs. IR+Control Virus), which was substantially lower than 6.3±0.3 nmol/min/mg in IR+sgRNA1 (p=0.002) (Figure 5G).
The aim of the present study was to build on our previous work of rendering CaMKIIδ resistant to oxidative activation and to move one step closer to potential translation as a therapeutic strategy for IR injury (10). Highly efficient CRISPR-Cas9 gene editing is critically dependent on the sgRNAs that are designed to bind a specific DNA sequence (10, 18, 24). Even though the amino acid sequence is highly conserved between human and mouse, the CAMK2D DNA sequence varies between species, precluding the use of the human sgRNAs in vivo in wildtype mice. For this reason, we generated a humanized CAMK2D knockin mouse model, where the entire regulatory domain (exons 11 and 12 with parts of the flanking introns) was replaced with the human sequence. This humanized mouse model allowed the deployment of the gene editing strategies optimized for humans in vivo in mice.

Up to now, it was unclear whether ablation of both oxidative activation sites of CaMKIIδ (methionine-281 and methionine-282) was required to confer resistance to IR injury or whether the full cardioprotective effect could be achieved by modifying one residue. Therefore, in the current work, we applied and compared two different CAMK2D editing strategies in vivo that we previously identified as lead-candidates in human iPSCs in vitro (10). One editing strategy represents a narrow-windowed approach that only modifies methionine-281 (M281V; sgRNA1). The other strategy introduces a broader editing pattern that modifies both methionines together with a bystander mutation (MMH281/282/283VVR; sgRNA2). We found that ablating both oxidative activation sites (sgRNA2) conferred a higher degree of cardioprotection upon IR than ablation of just methionine-281 (sgRNA1). This difference is less pronounced in the echocardiographic
analyses, which may be due to technical variability. We conclude that the presence of one oxidized methionine is still sufficient to disrupt the reassociation of the autoinhibitory region with the catalytic domain of CaMKIIδ, even though to a lesser extent than the presence of both oxidized methionines. This conclusion is further supported by our data showing a CaMKII activity level in sgRNA1-treated mice intermediate between mice treated with a control virus or with sgRNA2. Future studies will include work comparing specific editing of the CaMKIIδ oxidative activation sites with cardiomyocyte-restricted ablation of the complete enzyme, which could also be achieved with CRISPR-Cas9 technology. As CaMKIIδ is an important regulator of cardiac physiology at a normal activation level, complete ablation of this enzyme might not be the most beneficial strategy, but this remains to be tested (14, 30).

Base editing may also cause off-target editing and indeed, we previously observed off-target editing at an intronic site in the DAZL gene when using ABE8e-SpRY + sgRNA2. Therefore, we further developed and optimized our editing strategy by introducing a V106W substitution into the TadA domain of the base editor, which has been shown to reduce off-target editing (19). Indeed, we observed no marked off-target editing at any of the top 8 potential off-target sites when using ABE8e(V106W) + sgRNA1. We were further able to substantially decrease off-target editing in the DAZL gene from previously 29.5% to now 9.2% when using ABE8e(V106W)-SpRY + sgRNA2 (10). This off-target site is located in an intronic region of DAZL, meaning that this edit would not be expressed in an mRNA transcript. Plus, using a cardiomyocyte-specific troponin T (cTnT) promoter restricts potential off-target editing to cardiomyocytes, where DAZL expression is very low (31). We thus think that the clinical consequences of editing DAZL would be minimal.
Prior to a potential first-in-human clinical trial, a broader analysis of potential off-target editing will be necessary (e.g., whole-genome sequencing of human myocardial biopsies treated with CAMK2D editing). We did not assess potential off-target editing in the mouse genome. As the DNA sequences differ between the human and mouse genomes, also the potential genomic off-target sites differ across species and are difficult to compare. For example, the off-target sequence in the human DAZL gene does not exist in the mouse Dazl gene. Interpreting the relevance of potential off-target editing in the mouse genome following treatment with a human sgRNA would be even more difficult.

Another challenge of efficient in vivo gene editing is the choice of the delivery modality. To date, most approaches have relied on AAV, which shows good infectivity of cardiac tissue (18, 24, 25, 29, 32). However, systemic administration of high AAV doses has been associated with serious adverse effects, including acute liver damage, thrombocytopenia, and immunological responses with preexisting antibodies (18, 26, 27). Large-scale AAV production also poses substantial challenges. It is thus imperative to keep the viral dose as low as possible or to deploy other delivery strategies like lipid nanoparticles or virus-like particles (18, 28, 33-35). We opted for an engineered AAV vector with a modified capsid protein following two generations of directed evolution (MyoAAV 2A) (28). Compared to AAV9, the transduction efficiency of MyoAAV 2A has been shown to be ~17-fold higher in the heart and ~2.5 times lower in the liver following systemic administration in mice (28). To achieve therapeutic thresholds in muscle tissue with natural AAV capsid variants, current strategies require virus doses of up to ~2x10^{14} vg/kg body weight (28, 36, 37). By performing intracardiac injection, we were previously able to obtain high local editing efficiency of ~85% at the cDNA level with an AAV9 dose.
of 1.5x10^{12} \text{vg/kg body weight} \ (10). Utilizing the engineered MyoAAV 2A vector enabled further reduction of the virus dose by 10-fold to 1.5x10^{11} \text{vg/kg body weight}, while maintaining a similar high editing efficiency. The substantially reduced viral dose and the use of an engineered AAV capsid with higher muscle tropism and lower liver tropism decreases the risk of liver toxicity or AAV-related immunological effects (28, 35, 38).

CRISPR-Cas9 gene editing to render CaMKIIδ insensitive to oxidative activation overcomes many of the challenges of traditional compound-based strategies. Careful design of a sgRNA corresponding to the \textit{CAMK2D} gene substantially reduces the risk of targeting other enzymes or ion channels. Utilizing a cardiomyocyte-specific promoter (e.g., the troponin T promoter) to drive the expression of the gene editing components exclusively in cardiomyocytes prevents gene editing in organs other than the heart. This decreases the risk of adverse side effects, which are frequently observed with common heart failure medications. Administration of one \textit{CAMK2D} editing dosage on the day of cardiac injury was sufficient to confer sustained cardioprotection over the entire observation period of 5 weeks, thereby overcoming the requirement of daily administration. As CRISPR-Cas9 gene editing is permanent, the beneficial effects are expected to be maintained longer than 5 weeks (39). This issue as well as applying \textit{CAMK2D} editing in a setting of a more severe IR injury will be tested in future studies.

Identifying the optimal timepoint to administer the gene editing components will be critical as \textit{CAMK2D} editing is unlikely to convey therapeutic benefits once cardiomyocytes have died. We administered the \textit{CAMK2D} editing components immediately post-reperfusion, but it will be important to test whether administration at later timepoints is still beneficial since a myocardial infarction is not always diagnosed and treated immediately.
In this study, we opted for an intracardiac injection of the editing components, which enabled substantial reduction of the viral dose. In the clinic, intracardiac injection of the editing components could be achieved with catheter techniques in conjunction with coronary angiography and revascularization of the infarct artery, which might incur other challenges. Thus, it will be imperative to also explore other delivery modalities like intravenous injections. Another potential limitation of CRISPR-Cas9 gene editing is its irreversibility. However, permanent silencing of a pathomechanism might be suitable for patients with chronic diseases (e.g., coronary artery heart disease), where chronic disturbance of a pathogenic signaling cascade is perpetuated for many years. Future studies will aim to test additional non-viral delivery strategies and to determine whether CAMK2D editing is also beneficial to a broader range of cardiovascular diseases, as oxidized CaMKIIδ has been linked to numerous disorders like atrial fibrillation, diabetes mellitus, and sleep-disordered breathing (3, 15, 16, 40).
Methods

Plasmids

Plasmids were ordered from Addgene and adapted using oligonucleotides (IDT) or PCR products template sequences (PrimeStar GXL Polymerase, Takara), as appropriate. NEBuilder HiFi DNA Assembly (NEB) was used to clone oligonucleotides and PCR products into restriction enzyme-digested vectors.

SgRNAs were cloned into a pmCherry_gRNA plasmid containing a U6-driven sgRNA scaffold and a cytomegalovirus (CMV)–driven pmCherry fluorescent protein (gift from Ervin Welker, Research Centre for Natural Sciences of the Hungarian Academy of Sciences, Budapest, Hungary; Addgene plasmid: #80457, http://n2t.net/addgene:80457, RRID: Addgene_80457) (10). ABE8e(TadA-8e V106W) was a gift from David Liu (Harvard University, Cambridge, USA; Addgene plasmid: #138495, http://n2t.net/addgene:138495, RRID: Addgene_138495) (19). pCMV-T7-ABEmax(7.10)-SpRY-P2A-EGFP (RTW5025) was a gift from Benjamin Kleinstiver (Massachusetts General Hospital, Boston, USA; Addgene plasmid: #140003, http://n2t.net/addgene:140003, RRID: Addgene_140003) (21). ABE8e(TadA-8e V106W)-SpRY was obtained by adapting pCMV-T7-ABEmax(7.10)-SpRY-P2A-EGFP (RTW5025). Other plasmids used to produce AAVs are described in the corresponding paragraph.
Human induced pluripotent stem cells (iPSCs)

Human iPSCs were previously generated and used in our laboratory (10, 24). We used Matrigel (Corning)-coated 6-well polystyrene culture plates to maintain iPSCs in mTeSR\textsuperscript{TM}1 media (STEMCELL). iPSCs were passaged at 70-80% confluency using Versene (Thermo Fisher Scientific).

Approximately $8 \times 10^5$ iPSCs were treated with 10 μM ROCK inhibitor (Y-27632, Selleckchem) one hour before the nucleofection experiments. Accutase (Innovative Cell Technologies) was used to obtain single cell status. We mixed the iPSCs either with 1.5 μg of pmCherry\_gRNA plasmid carrying sgRNA1 and 4.5 μg ABE8e(TadA-8e V106W) plasmid or with 1.5 μg of pmCherry\_gRNA plasmid carrying sgRNA2 and 4.5 μg ABE8e(TadA-8e V106W)-SpRY plasmid. iPSCs were nucleofected using the P3 Primary Cell 4D-Nucleofector X Kit (Lonza), according to the manufacturer’s protocol. The culture media was then supplemented for one day with ROCK inhibitor (10 μM) and Primocin (100 μg/mL) (InvivoGen). Using fluorescence-activated cell sorting, we collected pmCherry-positive cells two days after the nucleofection experiment.

Off-target analyses in human iPSCs

The editing efficiency was assessed in human iPSCs after nucleofection of ABE components and either sgRNA1 or sgRNA2. The cutting frequency determination (CFD) score of CRISPOR was used to identify the top 8 genomic sites for potential off-target editing in the human genome for both ABE8e(TadA-8e V106W) + sgRNA1 and ABE8e(TadA-8e V106W)-SpRY + sgRNA2 (10, 41). The predicted sites for sgRNA2 have
been analyzed previously following nucleofection together with ABE8e-SpRY (10). ABE8e(TadA-8e V106W)-SpRY is a modified adenine base editor and less prone to potential off-target editing (10, 19).

Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) and we PCR-amplified the targets using PrimeStar GXL Polymerase (Takara, primers listed in Supplemental Table 1). In a second PCR round, we added the Illumina flow cell binding sequences and barcodes. After that, we purified the PCR products with AMPure XP Beads (Beckman Coulter), tested them for integrity on a 2200 TapeStation System (Agilent), and measured the DNA concentration using a QuBit dsDNA high-sensitivity assay (Invitrogen). After sample pooling and sequencing by an Illumina MiSeq, we demultiplexed the samples and analyzed the amplicon reads using CRISPResso2 (42).

We reported the background-corrected adenine to guanine editing efficiency for each adenine along the 20-base pair target DNA sequence corresponding to either sgRNA1 or sgRNA2.

Generation of a humanized CAMK2D knockin mouse model

To be able to use the sgRNAs optimized for the human genome, we humanized the regulatory domain of CaMKIIδ, which is encoded by exons 11 and 12. Therefore, we replaced 1,386 base pairs (300 base pairs of the 3’ end of intron 10, 84 base pairs of exon 11, 459 base pairs of intron 11, 43 base pairs of exon 12, and 500 base pairs of the 5’ end of intron 12) of the mouse Camk2d gene with the corresponding human sequence using CRISPR-Cas9-mediated homology-directed repair (HDR). We used 5’- and 3’-
homology arms of 1,200 base pairs corresponding to the mouse genome (Supplemental Figure 1). The HDR template was obtained by PCR-amplification of the corresponding mouse and human genomic segments and cloning into a plasmid backbone (Supplemental Table 2). We designed two sgRNAs that corresponded to mouse genomic segments of either intron 10 or intron 12 and were both within the region that was later replaced with the human sequence (Supplemental Table 2). The sgRNAs were ordered and synthesized from IDT.

We injected both sgRNAs (each 15 ng/μL), the HDR template (12.5 ng/μL), and Cas9 mRNA (50 ng/μL, TriLink BioTechnologies) into the pronucleus and cytoplasm of mouse zygotes to humanize the regulatory domain of CaMKIIδ. We treated 6-weeks old C57BL/6N (Charles River Laboratories) female mice for superovulation and mated them with C57BL/6N stud males to induce zygote production. After that, we isolated zygotes, transferred them to M16 (Brinster’s medium for ovum culture supplemented with 100 units/mL penicillin and 50 mg/mL streptomycin), and injected in M2 medium (M16 medium and 20 mM HEPES). After culture in M16 medium for 1 h at 37º C, we transferred the injected zygotes into the oviducts of pseudo-pregnant female ICR mice.

For genotyping, we selected two primers that were both outside the homology arms of the template (Supplemental Table 1). Using these primers, we PCR-amplified ear genomic DNA and digested the PCR product with SbfI (NEB). Since there is no SbfI restriction site in the wildtype PCR product, there was a single product of 3,898 base pairs (Supplemental Figure 1B). In contrast, the PCR product of humanized CAMK2D knockin mice contains one restriction site for SbfI, resulting in two products of 2,473 and 1,432 base pairs (Supplemental Figure 1B). Since the human intron 11 is 7 base pairs longer
than the mouse intron 11, the undigested PCR product of humanized CAMK2D knockin mice is slightly longer (3,905 base pairs) than that of wildtype mice. One mouse of the F₀ generation with a high knockin level was selected as a founder for the humanized CAMK2D knockin line and backcrossed for at least three generations. Successful integration of the knockin in the genome as well as successful transcription and splicing were confirmed by Sanger sequencing of the DNA and cDNA (after RT-PCR), respectively.

**Virus production**

To deliver the optimized gene editing constructs in vivo, we used AAV with the AAV2/MyoAAV 2A serotype (28). Since an ABE system exceeds the packaging limit of AAV, we designed a split-virus system encoding the N- and C-terminal halves of the base editor, as previously described (10, 24). Utilizing a split-intein trans-splicing system enabled reassembly of both parts to a functional ABE system in vivo. Therefore, we adapted the N- and C-terminal ABE constructs from Cbh_v5 AAV-ABE N-terminal (gift from David Liu, Harvard University, Cambridge, USA; Addgene plasmid: #137177, http://n2t.net/addgene:137177, RRID: Addgene_137177) (43) and Cbh_v5 AAV-ABE C-terminal (gift from David Liu, Harvard University, Cambridge, USA; Addgene plasmid: #137178, http://n2t.net/addgene:137178, RRID: Addgene_137178) (43), respectively. The modified plasmids carried either ABE8e(TadA-8e V106W) combined with sgRNA1 or ABE8e(TadA-8e V106W)-SpRY combined with sgRNA2. A cardiac troponin T promoter was used to drive the expression of the base editors exclusively in cardiomyocytes. The expression of the sgRNAs was driven by a U6 promoter.
To produce AAVs, near-confluent HEK293T cells (ATCC) were transfected with 6 μg of either AAV-ABE plasmid, 12 μg of pHelper plasmid (Cell Biolabs), and 24 μg of pRepCap Myo2A plasmid (kindly provided by Dr. Jan-Bernd Funcke, University of Texas Southwestern Medical Center, Dallas, USA). Transfection was performed in DMEM supplemented with 5% FBS, 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX), 100 U/mL penicillin, and 100 mg/mL streptomycin, utilizing PEI (linear, MW25000; Polysciences) at a PEI:DNA mass ratio of 3:1. After three days, culture supernatant was harvested and stored at 4° C. Fresh DMEM containing 5% FBS, 2 mM GlutaMAX, 100 U/mL penicillin, and 100 mg/mL streptomycin was then replenished. Five days following the transfection, both culture supernatants and cells were collected, combined with the stored supernatants, and subjected to centrifugation to isolate the cellular pellets.

AAVs were purified according to a recognized three-phase partitioning technique (44). The separated supernatants were reserved, while cells were lysed using a buffer composed of 50 mM Tris-HCl, 150 mM NaCl, and 2 mM MgCl₂ at pH 8.0. Lysis was achieved through a series of three freeze-thaw cycles involving liquid nitrogen and a 37° C water bath. The resulting cell lysates were supplemented with 50 U/mL of Benzonase (Sigma-Aldrich) and 10 U/mL of RNase I (Thermo Fisher Scientific). Following 30 min incubation at 37° C, 0.5% (w/v) SDS was introduced, and the mixture was incubated for another 30 min at 37° C. Debris was eliminated through centrifugation from the lysates, which was then merged with the previously separated supernatants to which 500 mM NaCl and 8% (w/w) PEG-8000 were added. After incubation at 4° C overnight and subsequent centrifugation at 4,000 x g and 4° C for 30 min, the ensuing pellets were resuspended in purification buffer II (50 mM Tris-HCl, 500 mM NaCl, 2 mM MgCl₂, 1%
(w/w) sarkosyl, and 1% (v/v) Triton X-100 at pH 7.5). Samples were saturated with
(NH₄)₂SO₄ at 20% and incubated for 5 min at 37° C and 300 rpm. After that, tert-butanol
was added, samples were incubated for another 5 min at 37° C and 300 rpm, and
subsequently centrifuged for 10 minutes at 4,000 x g and room temperature to facilitate
collection of the lower aqueous phases. Amicon Ultra-15 centrifugal filter units with a
molecular weight cut-off of 100 kDa (Thermo Fisher Scientific) were prewashed with
DPBS containing 0.01% (w/v) Pluronic F-68 and used for the following step. The collected
aqueous phases were subjected to three washing steps with an excess of injection buffer
(DPBS containing 200 mM NaCl and 0.001% (w/v) Pluronic F-68) prior to concentration.
The resulting purified AAVs were divided into aliquots and stored at -80° C until use. AAV
titers were quantified with qPCR according to a recognized protocol (primers listed in
Supplemental Table 1) (45).

Ischemia/reperfusion injury

All mice were housed and bred at the Animal Resource Center at the UT Southwestern
Medical Center, which is a pathogen-free facility with regular 12 h light/dark cycle
(temperature of 18-24° C and humidity of 35-60%). There was a maximum of 5 mice per
cage with ad libitum access to food and water. All mice were monitored daily for potential
health problems and all mice received standard chow (2916 Teklad Global).

For all experiments, we used female and male mice that were homozygous for the
humanized CAMK2D knockin. Ischemia/reperfusion (IR) surgery was performed in 12-
weeks old mice, as previously described (10). Ketamine/Xylazine complex was used for
anesthesia. Mice were intubated and ventilated with a MiniVent mouse ventilator (Hugo
Sachs Elektronik, 250 μL stroke volume, 105 breaths/min). The body temperature was monitored with a rectal probe and kept close to 37.0° C. After opening the chest between the left fourth and fifth ribs, a 7-0 nylon suture was put below the left anterior descending coronary artery, and a non-traumatic occluder was placed on the artery. After 45 min of ischemia, the suture and occluder were removed (reperfusion) and we injected the MyoAAV 2A carrying the CRISPR-Cas9 components directly into the left anterior wall of the heart, which is the area of injury following ligation of the left anterior descending coronary artery.

All mice were injected with a total virus dose of 1.5x10^{11} vg/kg body weight that was diluted with 0.9% sodium chloride solution (Sigma-Aldrich) to an injection volume of 30 μL. Control mice received either only the N- or only the C-term of the virus, which has previously been shown to be a suitable non-editing control virus (IR+Control Virus) (10). Mice subjected to CAMK2D editing received equal amounts of N- and C-term with either sgRNA1 (IR+sgRNA1) or sgRNA2 (IR+sgRNA2). Sham-treated mice were subjected to 45 min open chest without IR and without any injection (Sham). All surgeries and intracardiac injections were performed by the same experienced surgeon in a standardized manner and blinded to the content of liquid (control virus, sgRNA1 or sgRNA2). Mice were euthanized after 5 weeks for further histological and molecular analyses.

Echocardiography

Cardiac function was assessed by two-dimensional transthoracic echocardiography (Vevo2100 imaging system, VisualSonics) in conscious mice one week before as well as
24 h and three weeks after the surgery. M-mode traces were acquired to average three consecutive heart beats. Left ventricular end-diastolic (LVIDd) and end-systolic (LVIDs) internal diameter were analyzed to calculate fractional shortening (%) using the equation 

\[ \frac{(LVIDd - LVIDs)}{LVIDd} \times 100 \]

All echocardiographic measurements were performed and analyzed by the same experienced investigator, who was blinded to the treatment group.

**Treadmill exhaustion test**

Exercise capacity was investigated 4 weeks after the IR injury (without or with editing *CAMK2D*) on an Exer-3/6 rodent treadmill with 10° inclination (Columbus Instrument) (10, 46). There was an electric shock grid at the rear end with a stimulation intensity of 10 at a frequency of 3 Hz. All mice were acclimated to the treadmill on three consecutive days by subjecting them to 10 min sessions with a treadmill velocity of 0, 5, and 10 m/min for the first, second, and third day, respectively. After a warm-up of 10 m/min for 2 min, the velocity was set to 15 m/min. The velocity was accelerated at a rate of 0.6 m/min per minute until the mouse was exhausted, which was defined by continuous standing for 5 s on the electrical shock grid. All treadmill exhaustion tests were performed by the same investigator, who was blinded to the surgery (Sham vs. IR) and the treatment (control virus vs. sgRNA1 vs. sgRNA2).
Western blot analysis

For Western blot analysis, snap-frozen mouse cardiac tissue was pulverized using a tissue crusher and proteins were isolated with RIPA buffer (Sigma-Aldrich) supplemented with protease- and phosphatase-inhibitors (Roche). After the genomic DNA was broken by sonication with a Bioruptor Pico (10 on/off-cycles of 30 s sonication, Diagenode), we centrifuged the samples at 4º C for 15 min at 10,000 x g. The protein concentration was measured by a BCA assay (Thermo Fisher Scientific) and equal amounts were loaded on a Mini-PROTEAN® TGX™ gel (Bio-Rad). Proteins were transferred onto a polyvinylidene fluoride membrane (Millipore), blocked in 5% milk supplemented with TBS-Tween 0.1%, and incubated with the primary antibody at 4º C overnight. Primary antibodies were rabbit polyclonal anti-oxCaMKII (1:1,000, Sigma-Aldrich, catalog number 07-1387), mouse monoclonal anti-CaMKII (1:1,000, BD Biosciences, catalog number 611293), and mouse monoclonal anti-GAPDH (1:1,000, Sigma-Aldrich, catalog number MAB374). After that, membranes were incubated for one hour at room temperature with either HRP-conjugated goat anti-rabbit (1:10,000, Bio-Rad, catalog number 1706515) or HRP-conjugated goat anti-mouse (1:10,000, Bio-Rad, catalog number 1706516), which were used as secondary antibodies. Immunodetection was performed in the presence of Western Blotting Luminol Reagent (Santa Cruz Biotechnology) on a ChemiDoc MP Imaging System (Bio-Rad). The densitometric analysis was performed using ImageJ.

CaMKII activity assay

Snap-frozen mouse cardiac tissue was pulverized using a tissue crusher and samples were lysed in a buffer containing 1% (v/v) Triton X-100, 20 mM Tris, and 100 mM NaCl.
supplemented with protease- and phosphatase-inhibitors (Roche) at a pH of 7.4. After centrifugation for 15 min at 10,000 x g at 4°C, equal volumes of the supernatant were loaded onto the CycLex® CaM-kinase II assay kit (MBL International Corporation). The assay was performed according to the manufacturer's recommendations. The absorbance was measured at a wavelength of 450 nm on a CLARIOstar microplate reader (BMG LABTECH). A standard curve with dilutions of the CaM-kinase II Positive Control (MBL International Corporation) was used to calculate the CaMKII activity of each sample, which was then normalized to the protein concentration of the lysate (BCA assay, Thermo Fisher Scientific).

Routine histology

Mouse hearts were carefully explanted and cleaned for 5 minutes in phosphate-buffered saline (PBS) supplemented with 0.2 M KCl for cardioplegia. After fix in 10% neutral-buffered formalin (Sigma-Aldrich) at room temperature overnight, the samples were dehydrated in 70% ethanol, embedded in paraffin, and subjected to routine histology (picrosirius red staining). A BZ-X700 microscope (Keyence) was used to capture images of transverse cross-sections at 10x magnification (1,500 μm below the expected normoxic area). The collagen positive area of the section was determined using ImageJ and was divided by the total area of the heart to obtain the percentage of fibrotic tissue.
Statistics

For this study, we used female and male mice that were homozygous for the humanized CAMK2D knockin. Mice were randomly assigned to the respective groups while keeping the gender distribution within the groups at a balanced ratio. All experiments were conducted in replicates. We dedicated three mice per group to histological analyses and five mice per group to further molecular analyses, which has previously been shown to be a sufficient sample size to reach statistical significance (10). All data are included in this study and reported as mean ± standard error of the mean (SEM).

Distribution of the data (normal vs. non-normal) was assessed using the Shapiro-Wilk normality test. If the data was not normally distributed or if the sample size was too small to assess normality, non-parametric tests were applied. When comparing two groups, two-tailed Student’s t or Mann-Whitney test were applied for either normally or not normally distributed data, respectively. One-way ANOVA with Holm-Sidak’s post-hoc correction was applied for the comparison of more than two groups and a variable that was normally distributed. The Kruskal-Wallis test with Dunn’s post-hoc correction was used for the comparison of more than two groups and a variable that was not normally distributed. Post-hoc multiple comparisons were only performed when the ANOVA/Kruskal-Wallis test was significant. Linear regression analysis was used to test for correlations. Statistical comparisons were performed using GraphPad Prism 10 and two-sided p-values below 0.05 were considered statistically significant (* − p<0.05, ** − p<0.01, *** − p<0.001, **** − p<0.0001).
Study approval

All iPSC experiments complied with the regulations of the UT Southwestern Stem Cell Research Oversight Committee. Animal work described in this manuscript has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee.

Data availability

All data from this study are available either in the main manuscript or the supplemental material. Supporting data values associated with the main manuscript and supplemental material can be found in the Supporting data values file.
**Author contributions**

SL and ENO conceptualized the overall objective of this study and designed the experiments. LGS and PES produced the MyoAAV 2A. SL, XMC, DA, WT, HL and JM performed the experiments. SL, WT, KC, and LX analyzed the data. SL wrote the initial version of the manuscript that was reviewed and edited by LGS, PES, NL, RBD, and ENO.

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Conflict of interest

ENO is a consultant for Cardurion Pharmaceuticals, Vertex Pharmaceuticals, Tenaya Therapeutics, and Prime Medicine. The other authors have declared that no conflict of interest exists.
**References**


Figures

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Figure 1. Analysis of on- and off-target editing in human iPSCs. A) Schematic showing the structure of CaMKIIδ with its three domains (blue – catalytic domain, green – regulatory domain, brown – association domain). Upon oxidative stress, two critical methionine residues at position 281 and 282 become oxidized, thereby preventing association of the regulatory with the catalytic domain, and resulting in cardiac disease.

We deployed two adenine base editing strategies to ablate either one (sgRNA1) or both (sgRNA2) oxidative activation sites of CaMKIIδ. B) Human induced pluripotent stem cells (iPSCs) were nucleofected with ABE8e(V106W) and either sgRNA1 or sgRNA2. C) Sequence of sgRNA1 (CAMK2D on-target, ON) and the corresponding DNA and PAM sequences of the top 8 predicted potential off-target sites (OT), as predicted by CRISPOR. Nucleotides highlighted in yellow are different from sgRNA1. D) Percentage of adenine (A) to guanine (G) editing for all adenines within the on- and off-target sites (ordered from 5’ to 3’) following adenine base editing with ABE8e(V106W)-SpCas9 + sgRNA1 (n=3). E) Sequence of sgRNA2 (CAMK2D on-target, ON) and the corresponding DNA and PAM sequences of the top 8 predicted potential off-target sites (OT), as predicted by CRISPOR. Nucleotides highlighted in yellow are different from sgRNA2. F) Percentage of adenine (A) to guanine (G) editing for all adenines within the on- and off-target sites (ordered from 5’ to 3’) following adenine base editing with ABE8e(V106W)-SpRY + sgRNA2 (n=3). All data are individual data points with mean ± SEM. Replicates are human iPSCs following three independent nucleofections.
Figure 2. Editing CAMK2D in a humanized mouse model upon ischemia/reperfusion injury (IR). A) Schematic showing humanization of the mouse sequence of the regulatory domain of CaMKIIδ, which is encoded by exons 11 and 12. B) Flowchart showing the experimental design for subjecting 12-weeks old humanized CAMK2D female and male mice to IR. Cardiac function was assessed one week before as well as 24 h and three weeks after IR by echocardiography. At 4 weeks post-IR, mice were subjected to a treadmill exhaustion test. At 5 weeks post-IR, mice were sacrificed, and tissue collected for further analyses. C) Illustration of the split-MyoAAV 2A that was used to deliver ABE8e(V106W)-SpCas9 + sgRNA1 in vivo. D) Illustration of the split-MyoAAV 2A that was used to deliver ABE8e(V106W)-SpRY + sgRNA2 in vivo. E) Representative M-mode traces of hearts from mice subjected to either Sham, IR+Control Virus, IR+sgRNA1 or IR+sgRNA2 (echocardiography; three weeks post-IR; in total n=8 per group). F) Mean
fractional shortening three weeks post-IR (n=8 per group). **G) Mean left ventricular end-diastolic diameter three weeks post-IR (n=8 per group). **H) Mean left ventricular end-diastolic volume three weeks post-IR (n=8 per group). All data are individual data points with mean ± SEM and all replicates are individual mice. Statistical comparisons are based on one-way ANOVA post-hoc corrected by Holm-Sidak (F-H); * – p<0.05, ** – p<0.01, *** – p<0.001, **** – p<0.0001.
Figure 3. **CAMK2D-edited mice show improved exercise performance post-IR.** A) Protocol used for the treadmill exhaustion test. B) Mean maximal velocity achieved on the treadmill prior to exhaustion (n=8 per group). C) Mean total distance achieved on the treadmill prior to exhaustion (n=8 per group). D) Linear regression analysis of fractional shortening and the corresponding maximal velocity achieved on the treadmill (n=8 per group, n=32 in total). E) Linear regression analysis of fractional shortening and the corresponding total distance achieved on the treadmill (n=8 per group, n=32 in total). All data are individual data points with mean ± SEM and all replicates are individual mice. Statistical comparisons are based on one-way ANOVA post-hoc corrected by Holm-Sidak (B and C) and linear regression analysis (D and E); ** – p<0.01, **** – p<0.0001.
Figure 4. Normal organ weights and prevention of fibrosis in sgRNA2-treated mice post-IR. A) Mean body weight for mice subjected to Sham, IR+Control Virus, IR+sgRNA1 or IR+sgRNA2 (n=8 per group). B) Mean heart weight normalized to tibia length (n=8 per group). C) Mean lung weight normalized to tibia length (n=8 per group). D) Mean liver weight normalized to tibia length (n=8 per group). E) Representative picrosirius red staining of transverse cardiac sections from all groups (scale bar 1,000 μm). F) Mean percentage of fibrotic tissue (n=3 per group). All data are individual data points with mean ± SEM and all replicates are individual mice. Statistical comparisons are based on one-
way ANOVA post-hoc corrected by Holm-Sidak (A-D,F); n.s. – not statistically significant,

** – p<0.01, *** – p<0.001, **** – p<0.0001.
**Figure 5. Analysis of editing efficiency.** A) Percentage of adenine (A) to guanine (G) editing at the DNA and cDNA level in the anterior cardiac wall of mice following treatment with ABE8e(V106W)-SpCas9 + sgRNA1 (n=5 per group). B) Percentage of adenine (A) to guanine (G) editing at the DNA and cDNA level in the anterior cardiac wall of mice following treatment with ABE8e(V106W)-SpRY + sgRNA2 (n=5 per group). C) Western blot analysis of oxidized CaMKII, total CaMKII, and GAPDH in mice subjected to either Sham, IR+Control Virus, IR+sgRNA1 or IR+sgRNA2 (in total n=5 per group). D) Mean densitometric analysis for oxidized CaMKII normalized to GAPDH (n=5 per group).
Mean densitometric analysis for total CaMKII normalized to GAPDH (n=5 per group). **
Mean densitometric analysis for oxidized CaMKII normalized to total CaMKII (n=5 per group). G) Mean CaMKII activity for all groups (n=5 per group). All data are individual data points with mean ± SEM and all replicates are individual mice. Statistical comparisons are based on one-way ANOVA post-hoc corrected by Holm-Sidak (D-G);
n.s. – not statistically significant, ** – p<0.01, *** – p<0.001, **** – p<0.0001.