Paradoxical anti-arrhythmic effects of Zim3, a CRISPRi effector, in human iPSC-cardiomyocytes

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Abstract:
We show that Zim3, when used as Zim3-KRAB-dCas9 effector in interference CRISPR, without any guide RNAs, paradoxically upregulates key cardiac ion channel genes in human induced pluripotent stem-cell-derived cardiomyocytes, iPSC-CMs, responsible for healthy resting membrane potential, repolarization of the action potential and electrical transmission of signals. These were found to yield expected functional enhancements consistent with a more mature iPSC-CM phenotype, with anti-arrhythmic properties.

Zinc Finger imprinted 3 (Zim3 or ZNF657/264) is a protein-coding gene in humans, found at low levels in testis, brain, skeletal muscle and skin[1]. As other ZNF-KRAB (Krueppel associated box) domains, which bind to DNA and through complexing with KAP1 (KRAB-associated protein 1) affect chromatin organization[2,3], Zim3-KRAB may be involved in the regulation of transcription at multiple genomic loci. Recently, Zim3-KRAB has been identified as a potent effector (repressor) in combination with dCas9 for interference CRISPR (CRISPRi) gene regulation studies[4]. Indeed, using Zim3-KRAB-dCas9 adenoviral delivery in combination with suitable gRNAs in post-differentiated induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), we have shown robust suppression of the expression of select ion channel genes, e.g. KCNH2, with concomitant functional consequences[5]. While performing control experiments, we observed unexpected effects of Zim3-KRAB-dCas9 alone on cardiac electrophysiology without the addition of any gRNAs or with the use of scrambled/control gRNA. Namely, we found Zim3-KRAB-dCas9 expression to significantly upregulate the mRNA expression of several key cardiac ion channels: KCNJ2, encoding for the Kir2.1 protein – inward rectifier K+ channel, contributing to the maintenance of healthy negative resting membrane potential; KCNH2, encoding for the main repolarizing K+ channel in cardiomyocytes, the rapid delayed rectifier that controls action potential duration, APD; and GJA1, encoding for Cx43 – the main gap-junctional protein in ventricular cardiomyocytes; for Cx43 this was also confirmed at the protein level, **Figure 1**. Using all-optical electrophysiology[6-9], **Figure 1a**, we pursued functional studies, **Figure 1c-e**, which indeed corroborated physiologically relevant functional effects, expected for upregulated KCNH2 (APD shortening in paced conditions), **Figure 1c, f**; upregulated KCNJ2 (reduced or eliminated spontaneous firing), **Figure 1d, f**; and upregulated GJA1 (conduction velocity increase), **Figure 1e-h**. In combination, these electrophysiological changes are perceived as desirable and a signature of mature iPSC-CMs with anti-arrhythmic properties[10]. These gene expression changes and functional effects cannot be attributed to the expression of dCas9, as we have shown that regular KRAB-dCas9[11] without gRNAs does not alter these genes. The fluorescent reporter, mCherry, also does not have such effects. Related earlier-generation effector construct for CRISPRi – Mec2p-KRAB-dCa9[12] caused similar, but much milder changes when delivered in the same type of viral vector - adenovirus with an SFFV (spleen focus-forming virus) promoter and an mCherry reporter, at the same multiplicity of infection, MOI 1000, as Zim3-KRAB-dCas9. In fact, we are unaware of any pharmacological or other single perturbation acting as a potent agonist for all three genes. These findings should be taken into consideration when performing CRISPRi studies in iPSC-CMs. Zim3 also needs to be investigated further as a potential therapeutic tool for iPSC-CMs maturation for their use in drug screening, disease modeling and regenerative applications[13-15]; furthermore, it would be interesting to explore if Zim3 may exert similar effects *in vivo* in the adult human heart as an anti-arrhythmic therapy.

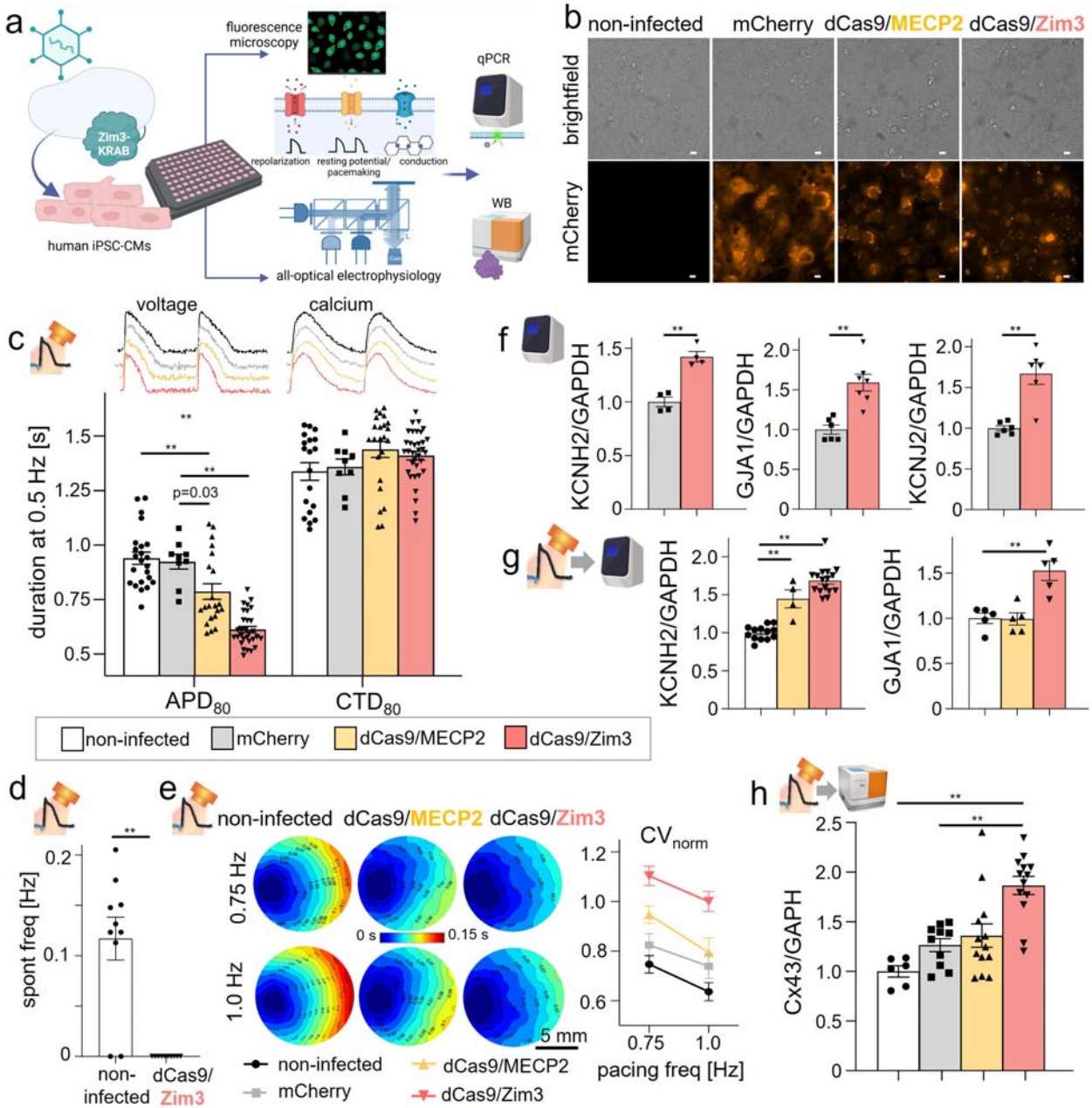

Figure 1. Effects of dCas9/Zim3 expression in human iPSC-CMs: RNA, protein, and electrophysiological consequences. a. Schematic illustrating the experimental workflow with all-optical electrophysiology and molecular characterization. b. Validation of virally-mediated delivery of mCherry tagged constructs (mCherry, Mec2p-KRAB-dCa9-mCherry, Zim3-KRAB-dCa9-mCherry) in human iPSC-CMs. Scale bar 10 μmc. Functional changes in action potential duration (APD) and calcium transient duration (CTD) under 0.5 Hz pacing upon expression of the various constructs (n=9-34 biologically independent samples; one-way ANOVA with post-hoc Tukey correction, **p<0.01). d. Changes on spontaneous beat frequency in dCas9/Zim3 expressing cells compared to non-infected control. (n=10 biologically independent samples; unpaired t-test, **p<0.01). e. Activation maps of hiPSC-CMs expressing dCas9/MECP2 and dCas9/Zim3 compared to non-infected controls; representative images and summary of

normalized conduction velocity (CV_{norm}) changes in the differentially treated groups at 0.75 Hz and 1.0 Hz pacing frequencies (n=2-9 biologically independent samples). Data are represented as mean ± S.E.M. f. qPCR analysis of KCNH2, GJA1, and KCHJ2 of pristine samples expressing dCas9/Zim3 compared to mCherry control (n=4-7 biologically independent samples, n=3 technical replicates; unpaired t-test, **p<0.01). g. qPCR analysis of KCNH2 and GJA1 mRNA changes upon expression of dCas9/MECP2 and dCas9/Zim3 compared to non-infected control post functional experiments (n=4-16 biologically independent samples, n=3 technical replicates; one-way ANOVA, **p<0.01). h. Western blot (Wes by ProteinSimple) protein quantification of Cx43 upon the different treatments post all-optical electrophysiology (n=6-13 biologically independent samples; one-way ANOVA, **p<0.01). Biorender was used for some of the panels.

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