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ORIGINAL RESEARCH

Functional Validation of Doxorubicin-Induced Cardiotoxicity-Related Genes

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ABSTRACT

BACKGROUND Genome-wide association studies and candidate gene association studies have identified more than 180 genetic variants statistically associated with anthracycline-induced cardiotoxicity (AIC). However, the lack of functional validation has hindered the clinical translation of these findings.

OBJECTIVES The aim of this study was to functionally validate all genes associated with AIC using human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs).

METHODS Through a systemic literature search, 80 genes containing variants significantly associated with AIC were identified. Additionally, 3 more genes with potential roles in AIC (*GSTM1*, *CBR1*, and *ERBB2*) were included. Of these, 38 genes exhibited expression in human fetal heart, adult heart, and hiPSC-CMs. Using clustered regularly interspaced short palindromic repeats/Cas9-based genome editing, each of these 38 genes was systematically knocked out in control hiPSC-CMs, and the resulting doxorubicin-induced cardiotoxicity (DIC) phenotype was assessed using hiPSC-CMs. Subsequently, functional assays were conducted for each gene knockout on the basis of hypothesized mechanistic implications in DIC.

RESULTS Knockout of 26 genes increased the susceptibility of hiPSC-CMs to DIC. Notable genes included efflux transporters (*ABCC10*, *ABCC2*, *ABCB4*, *ABCC5*, and *ABCC9*), well-established DIC-associated genes (*CBR1*, *CBR3*, and *RAC2*), and genome-wide association study–discovered genes (*RARG* and *CELF4*). Conversely, knockout of *ATP2B1*, *HNMT*, *POR*, *CYBA*, *WDR4*, and *COL1A2* had no significant effect on the in vitro DIC phenotype of hiPSC-CMs. Furthermore, knockout of the uptake transporters (*SLC28A3*, *SLC22A17*, and *SLC28A1*) demonstrated a protective effect against DIC.

CONCLUSIONS The present findings establish a comprehensive platform for the functional validation of DIC-associated genes, providing insights for future studies in DIC variant associations and potential mechanistic targets for the development of cardioprotective drugs. (J Am Coll Cardiol CardioOnc 2024;6:38-50) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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Anthracyclines, primarily doxorubicin, constitute a key component in about 60% of cancer treatment regimens,[1] with approximately 35% administered to patients with breast cancer. Despite their efficacy, doxorubicin-induced cardiotoxicity (DIC) occurs in a dose-dependent manner in about 9% of patients, and 98% of these cases emerge within the first year of treatment.[2] DIC encompasses 4 major interrelated molecular mechanisms: 1) generation of reactive oxygen species (ROS); 2) mitochondrial dysfunction; 3) DNA damage involving TOP2B; and 4) calcium overload leading to sarcomere damage. These mechanisms collectively lead to cardiomyocyte death, clinically determined by troponin detection in peripheral blood and reduced left ventricular ejection fraction.

To date, 5 genome-wide association studies (GWAS)[3-7] and 20 candidate gene association studies[8] have identified 80 genes with single-nucleotide polymorphisms (SNPs) significantly linked to anthracycline-induced cardiotoxicity (AIC).[8] Despite this, only 1 AIC-associated variant locus (in *SLC28A3*) has been independently replicated.[9,10] Thus, the functional validation of AIC-associated variants remains a critical prerequisite before incorporating this information into clinical practice.

The AIC-associated genes can be categorized into 6 major groups on the basis of their hypothesized mechanistic function. 1) Genes associated with ROS production and handling: doxorubicin induces ROS generation predominantly by reduction in the mitochondria, producing semiquinone-producing superoxide (O_2^+) free radicals. 2) Genes related to DNA damage: doxorubicin inflicts DNA damage on cardiomyocytes either by direct intercalation with DNA or by disruption of DNA repair after cleavage by TOP2B. 3) Genes associated with iron uptake: doxorubicin, with a high affinity for iron, can alter iron metabolism through interactions with iron regulatory proteins, stabilizing transferrin transcripts and inhibiting the expression of iron-sequestering proteins.[11] 4) Genes associated with transporters controlling doxorubicin uptake and efflux: generally, variants in uptake transporters (ie, members of the solute carrier [SLC] family) are protective against AIC by reducing doxorubicin transport into cardiomyocytes.[9,10,12,13] Conversely, variants in efflux transporters (adenosine triphosphate-binding cassette [ABC]) are linked to increased intracellular doxorubicin concentration and AIC.[14] 5) Genes involved in calcium handling: doxorubicin directly binds to ryanodine receptors, inducing calcium release from sarcoplasmic reticulum.[15] It also enhances L-type calcium channel activity,[16] leading to an increased level of intracellular calcium. 6) Genes related to altered electric currents in the cardiomyocytes: after doxorubicin treatment, this can result in impaired contractile function.[17]

Previously, we demonstrated that human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) accurately recapitulate patient-specific cardiotoxic responses to doxorubicin.[13,18,19] In the present study, we functionally validate the role of 38 genes associated with anthracycline cardiotoxicity in patients with cancer using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based knockout (KO) in hiPSC-CMs, followed by an array of in vitro characterization assays. Our work confirms that 31 genes, identified in GWAS and candidate gene association studies, play mechanistic roles in DIC at the cellular level. This approach provides insights into the impact of each gene KO on DIC development and their potential mechanistic pathways.

ABBREVIATIONS AND ACRONYMS

ABC = adenosine triphosphate–binding cassette

AIC = anthracycline-induced cardiotoxicity

CRISPR = clustered regularly interspaced short palindromic repeats

DIC = doxorubicin-induced cardiotoxicity

GWAS = genome-wide association study/studies

hiPSC-CM = human induced pluripotent stem cell-derived cardiomyocyte

IC_{50} = half maximal inhibitory concentration

KO = knockout

LD_{50} = median lethal dose

ROS = reactive oxygen species

SLC = solute carrier

SNP = single-nucleotide polymorphism

METHODS

VARIANT AND GENE CANDIDATE IDENTIFICATION. Following a comprehensive PubMed search involving both original and review papers investigating genetic risk factors associated with DIC, we compiled a list of potential candidates. These candidates underwent testing for their expression levels in hiPSC-CMs, adult human heart, and fetal human heart[19] (Supplemental Table 1).

HUMAN INDUCED PLURIPOTENT STEM CELL CULTURE, CRISPR/Cas9-MEDIATED KO GENERATION, AND DIFFERENTIATION TO CARDIOMYOCYTES. For detailed information, refer to the Supplemental Methods. We used a control male hiPSC line with an exogenous *TNNT2* promoter-driven phleomycin D1 resistance cassette for cardiomyocyte purification, as previously described.[13] All protocols received approval from the Northwestern University Institutional Review Board. Pairs of CRISPR/Cas9 guide RNAs were designed with a separation of >50 bp to induce a large deletion within the earliest common exon of each gene. Supplemental Tables 2 to 4 display all used primers for single guide RNA expression vector generation, a list of potential off-targets, and sequencing primers. We selected 1 KO hiPSC line with the lowest expression of each gene, which was then differentiated into cardiomyocytes and assessed on day 30.

FIGURE 1 Prioritization of Doxorubicin-Induced Cardiotoxicity-Associated Loci

Rank	Gene	SNP ID	*P*-value	Odds Ratio	Cardiac Tissue Expression (0 2 4) Adult	Fetal	hiPSC-CMs	Reference
1	*RARG*	rs2229774	5.90E-08	4.7				Aminkeng *et al.* 2015 [3] - All combined
2	*PRDM2*	rs7542939	6.50E-07	n.a.				Wells *et al.* 2017 [5] - Combined
3	*WDR4*	rs15736	2.60E-06	4.4				Aminkeng *et al.* 2015 [3] - Discovery cohort
4	*ZNF521*	rs4381672	2.90E-06	4.3				Aminkeng *et al.* 2015 [3] - Discovery cohort
5	*SP4*	rs2282889	4.40E-06	0.2				Aminkeng *et al.* 2015 [3] - Discovery cohort
6	*RIN3*	rs9323880	6.80E-06	4.2				Aminkeng *et al.* 2015 [3] - Discovery cohort
7	*SLC28A3*	rs7853758	1.60E-05	0.36				Visscher *et al.* 2012+2013 [9,10] - Combined
8	*ABCC1*	rs4148350	2.80E-05	11.86				Visscher *et al.* 2012 [9] - Discovery cohort
9	*ABCC9*	rs11046217	7.10E-05	4.48				Visscher *et al.* 2015 [12] - Discovery cohort
10	*ABCC5*	rs7627754	1.00E-04	n.a.				Krajinovic *et al.* 2016 [14]
11	*HNMT*	rs17583889	3.40E-04	3.67				Visscher *et al.* 2012 [9] - Discovery cohort
12	*SLC22A17*	rs4982753	4.40E-04	0.5				Visscher *et al.* 2015 [12] - Combined
13	*GPX3*	rs2233302	7.40E-04	0.27				Visscher *et al.* 2015 [12] - Discovery cohort
14	*SLC28A1*	rs2305364	8.10E-04	2.49				Visscher *et al.* 2012 [9] - Replication cohort
15	*ERCC2*	rs13181	1.00E-03	n.a.				El-Tokhy *et al.* 2014 [60]
16	*MYH7*	rs3743527	1.00E-03	n.a.				Semsei *et al.* 2012 [62]
17	*CYP2J2*	rs2294950	1.40E-03	3.9				Visscher *et al.* 2015 [12] - Combined
18	*CBR3*	rs1056892	2.00E-03	6.19				Hertz *et al.* 2016 [62]
19	*COL1A2*	rs42524	2.00E-03	1.79				Visscher *et al.* 2015 [12] - Combined
20	*SPG7*	rs2019604	2.10E-03	0.39				Visscher *et al.* 2012 [9] - Combined
21	*HFE*	rs1800562	3.00E-03	n.a.				Lipshultz *et al.* 2013 [63]
22	*ABCC10*	rs1214763	3.10E-03	0.34				Visscher *et al.* 2015 [12] - Discovery cohort
23	*ABCB4*	rs1149222	5.40E-03	1.87				Visscher *et al.* 2012 [9] - Combined
24	*GSTP1*	rs1695	6.00E-03	9.4				Windsor *et al.* 2012 [64]
25	*PLCE1*	rs932764	6.80E-03	0.36				Hildebrandt *et al.* 2017 [48]
26	*GSTM1*	null	7.00E-03	2.7				Singh *et al.* 2020 [20]
27	*CELF4*	rs1786814	1.00E-02	22.2				Leger *et al.* 2016 [65]
28	*CYBA*	rs4673	1.00E-02	2				Wojnowski *et al.* 2005 [66]
29	*HAS3*	rs223228	1.00E-02	3.7				Wang *et al.* 2014 [45] - Combined
30	*MLH1*	rs1800734	1.00E-02	n.a.				Krajinovic *et al.* 2016 [14]
31	*POR*	rs13240755	1.38E-02	3.18				Lubieniecka *et al.* 2013 [67]
32	*RAC2*	rs13058338	1.90E-02	1.84				Rossi *et al.* 2009 [43]
33	*CAT*	rs10836235	2.00E-02	0.284				Rajic *et al.* 2009 [36]
34	*NOS3*	rs1799983	2.00E-02	n.a.				Krajinovic *et al.* 2016 [14]
35	*ABCC2*	rs8187710	2.10E-02	4.3				Aminkeng *et al.* 2015 [3] - Discovery cohort
36	*ATP2B1*	rs17249754	4.00E-02	0.07				Hildebrandt *et al.* 2017 [48]
37	*CBR1*	rs9024	3.00E-01	0.45				Hertz *et al.* 2016 [62]
38	*ERBB2*	rs1058808	3.00E-03	0.09				Boekhout *et al.* 2016 [40]

Table showing the 38 doxorubicin-induced cardiotoxicity–associated loci ranked on the basis of the single-nucleotide polymorphism (SNP) with highest *P* value from their respective publications.[23-30] The heat map of cardiac tissue expression shows the expression of anthracycline-induced cardiotoxicity–associated genes in adult human heart (n = 2), in fetal human heart (n = 2), and in human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) (n = 7) by RNA sequencing. n = number of distinct patient-specific samples.

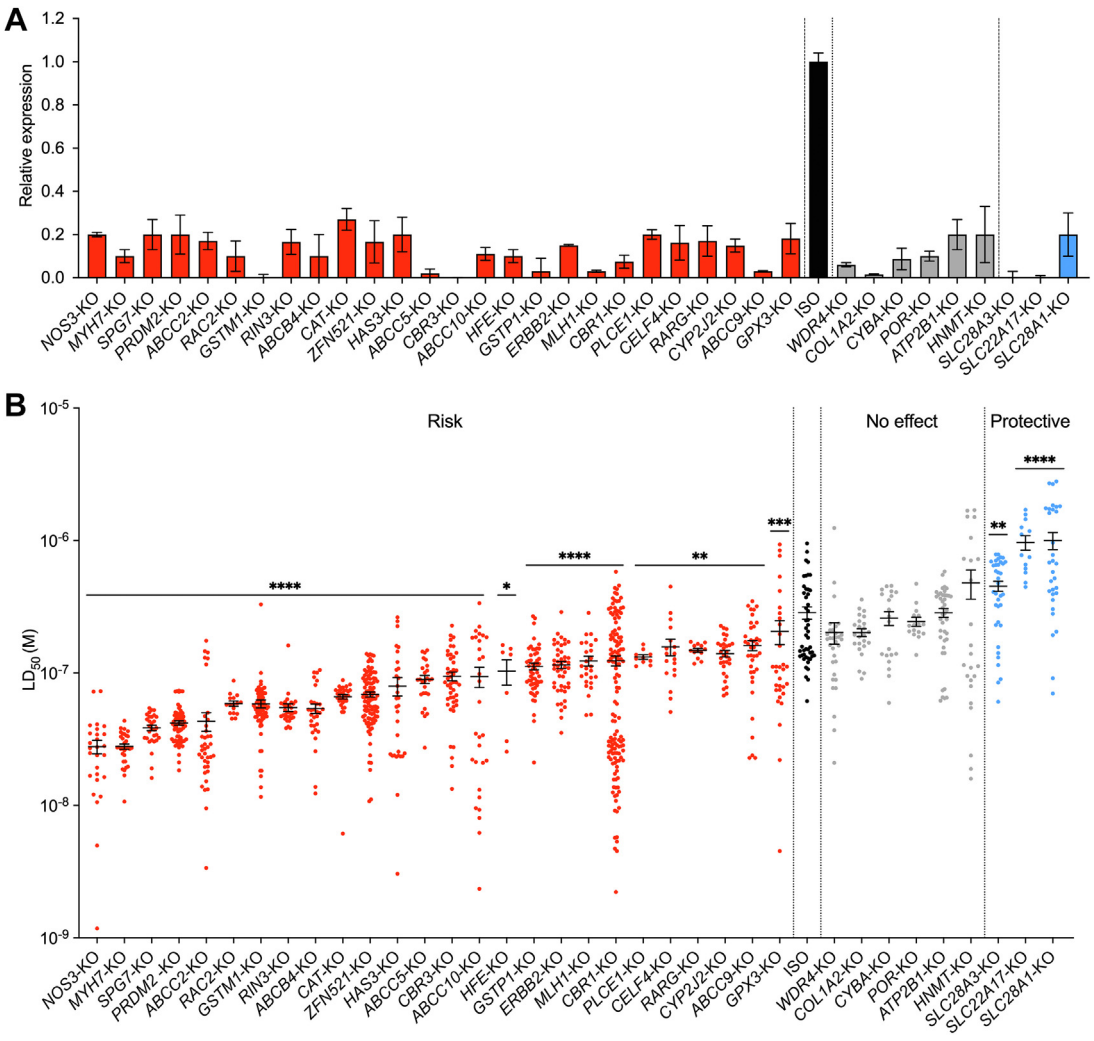
STATISTICAL METHODS. Data are expressed as mean ± SEM. Comparisons were conducted using 1- or 2-way analysis of variance or unpaired 2-tailed Student's *t*-tests, with significant differences defined as $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$.

For more detailed methods, refer to the Supplemental Appendix.

RESULTS

DIC-ASSOCIATED GENE PRIORITIZATION, KO GENERATION, AND IN VITRO DOXORUBICIN TOXICITY MEASUREMENT. We compiled a table of 429 SNPs associated with DIC, with 180 being unique (Supplemental Table 1). From this meta-analysis, we identified 80 SNP-harboring genes significantly linked to AIC. Our bioinformatic analysis did not predict any gain of function resulting from these SNPs. Additionally, we included 3 other potential candidates: *GSTM1*, associated with DIC through deletion rather than a SNP;[20] *CBR1*, considered one of the prototypical DIC-associated genes;[21] and *ERBB2*, implicated in the cardiotoxicity of trastuzumab plus doxorubicin regimens.[22] We assessed the expression of 83 genes using RNA sequencing in the fetal heart, adult human heart, and hiPSC-CMs. Subsequently, 38 genes consistently expressed (>10 transcripts per million for hiPSC-CMs) were chosen for KO generation (**Figure 1**, Supplemental Table 1).

FIGURE 2 **Validation of KOs of 36 Genes Significantly Associated With Doxorubicin-Induced Cardiotoxicity**



(A) Validation of successful clustered regularly interspaced short palindromic repeats/Cas9–based gene knockout (KO) in human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) using quantitative reverse transcriptase polymerase chain reaction. The data presented here are the relative expression of each studied gene in its own KO lines relative to isogenic control (ISO). (B) Effect of gene KOs on hiPSC-CM viability after doxorubicin treatments (72 hours). Each data point represents 1 median lethal dose (LD_{50}) calculation on the basis of an individual experimental replicate derived from a 5-log doxorubicin dosing. Central bar represents mean. Error bars represent ±SEM. *P* values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$) are derived from differences between gene KO and ISO (Mann-Whitney *U* test). Raw dose-response curves from which these LD_{50} values are derived are provided in Supplemental Figures 4 to 6.

We generated KO hiPSC-CM lines for each of the 38 genes, with all guide RNA sequences listed in Supplemental Table 2. All KOs were validated using Sanger sequencing (Supplemental Figures 1 to 4, Supplemental Tables 4 and 5). The absence of the knocked-out protein was confirmed using western blot analysis (Supplemental Figures 5 and 6, Supplemental Tables 6 and 7). Additionally, attenuation of the KO gene in the KO lines was further confirmed using quantitative reverse transcriptase polymerase chain reaction (**Figure 2A**, Supplemental Table 8). Relevant SNPs contained in the control human induced pluripotent stem cells are detailed in Supplemental Table 9. Of the 38 KOs attempted, KO of 2 genes (*ERCC2* and *ABCC1*) proved incompatible with hiPSC survival, and 1 gene (*SP4*) was incompatible with cardiac differentiation (Supplemental Table 1).

TABLE 1 Mechanistic Implications of Genes Associated With DIC

Group	Gene	First Author (Year) Ref#	Mechanistic Implication
1	*CAT*	Rajic et al (2009)[31]	ROS generation/handling[32]
	CBR1	Armenian et al (2013)[33]	ROS generation/handling[34]
	CBR3	Visscher et al (2012),[9] Armenian (2013)[33]	ROS generation/handling[34]
	ERBB2	Boekhout et al (2016)[35]	ROS generation/handling[36]
	GPX3	Visscher et al (2015)[12]	ROS generation/handling[37]
	GSTM1	Singh et al (2020)[20]	ROS generation/handling
	GSTP	Visscher et al (2012),[9] Rossi et al (2009)[38]	ROS generation/handling[39]
	HAS3	Wang et al (2014)[40]	ROS generation/handling[41]
	NOS3	Krajinovic et al (2016)[14]	ROS generation/handling[42]
	PLCE1	Hildebrandt et al (2017)[43]	ROS generation/handling[44]
	RAC2	Armenian et al (2013),[33] Rossi et al (2009)[38]	ROS generation/handling[45]
	SPG7	Visscher et al (2013)[10]	ROS generation/handling[46]
2	*PRDM2*	Wells et al (2017)[5]	DNA damage[47]
	MLH1	Krajinovic et al (2016)[14]	DNA damage[48]
	RARG	Aminkeng et al (2015)[3]	DNA damage[3]
3	*HFE*	Armenian et al (2013)[33]	Iron uptake and homeostasis[49]
4	*SLC22A17*	Visscher et al (2015)[12]	DOX uptake[13]
	SLC28A1	Visscher et al (2012)[9]	DOX uptake[50]
	SLC28A3	Visscher et al (2012),[9] Visscher et al (2013)[10]	DOX uptake[19]
	ABCB4	Visscher et al (2012)[9]	DOX efflux[51]
	ABCC2	Aminkeng et al (2015)[3]	DOX efflux[52]
	ABCC5	Krajinovic et al (2016)[14]	DOX efflux[14]
	ABCC9	Visscher et al (2015)[12]	DOX efflux[53]
	ABCC10	Visscher et al (2015)[12]	DOX efflux[53]
5	*CELF4*	Wang et al (2016)[6]	Calcium handling[54]
	MYH7	Wasielewski et al (2014)[55]	Calcium handling[56]
6	*CYP2J2*	Visscher et al (2015)[12]	Cardiac electrical activity[57]
	RIN3	Aminkeng et al (2015)[3]	Cardiac electrical activity[58]
	ZFN521	Aminkeng et al (2015)[3]	Cardiac electrical activity[59]

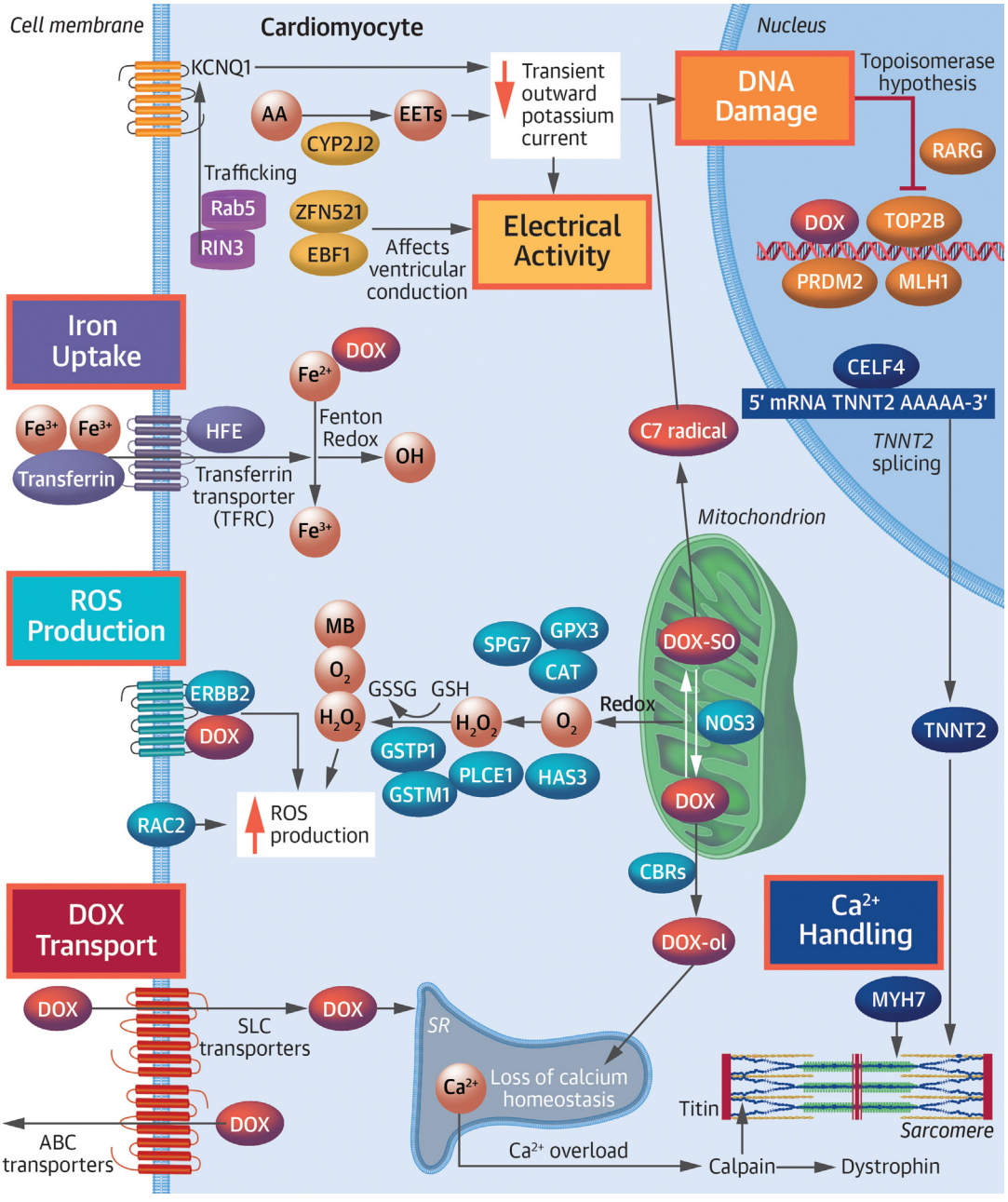
DIC = doxorubicin-induced cardiotoxicity; DOX = doxorubicin; ROS = reactive oxygen species.

Next, the remaining 35 KO hiPSC lines were differentiated into cardiomyocytes and exposed to 5-log doses of doxorubicin (10^{-8} to 10^{-4} M) for 72 hours, followed by viability assessment to establish the dose required to kill 50% of the cells (median lethal dose [LD_{50}]) (**Figure 2B**). KO of the uptake drug transporters *SLC28A3*, *SCL22A17*, and *SLC28A1* increased viability after treatments with doxorubicin, with LD_{50} values between 6.35 and 11.2 μM compared with 3.78 μM in isogenic control hiPSC-CMs (**Figure 2B**, Supplemental Figure 7). KO of *ATP2B1*, *HMNT*, *POR*, *CYBA*, *WDR4*, and *COL1A2* did not alter viability after doxorubicin treatments (**Figure 2B**, Supplemental Figure 8). KO of the rest of the genes (n = 26) increased the hiPSC-CMs' sensitivity to doxorubicin (**Figure 2B**, Supplemental Figures 9 and 10). LD_{50} values for KOs with increased sensitivity to doxorubicin ranged between 0.32 μM (*NOS3*-KO and *MYH7*-KO cardiomyocytes; $P < 0.0001$) and 2 μM (*GSTM1*-KO cardiomyocytes; $P = 0.0018$) compared with 3.78 μM for isogenic control hiPSC-CMs.

FUNCTIONAL VALIDATION OF DIC-ASSOCIATED GENES. Next, for each gene KO, we performed a functional study to investigate their potential mechanistic implications for DIC. We categorized the genes into 6 functional groups (**Table 1**) and adopted a functional assay relevant to their mechanisms of action (**Central Illustration**).

Genes involved in ROS production and handling. The largest group of genes in our list (12 of 35) is associated with ROS production and handling (**Table 1**). We assessed the H_2O_2 levels in hiPSC-CMs after 24 hours of doxorubicin treatment (**Figures 3A to 3C**). After doxorubicin treatment, our analysis indicated that ROS levels were lower in *CBR1*-KO (half maximal inhibitory concentration [IC_{50}] = 20.6 mM; $P = 0.0024$) and *CBR3*-KO (IC_{50} = 31 mM; $P = 0.0069$) hiPSC-CMs compared with isogenic control hiPSC-CMs (IC_{50} = 4.1 mM) (**Figure 3A**). However, after doxorubicin treatment, ROS levels were increased in *SPG7*-KO (IC_{50} = 0.3 mM; $P = 0.0006$), *HAS3*-KO (IC_{50} = 0.1 mM; $P = 0.0023$), *GSTM1*-KO (IC_{50} = 0.26 mM; $P = 0.039$), and *RAC2*-KO

CENTRAL ILLUSTRATION **Mechanistic Implications of Relevant Candidate Genes in DOX-Induced Cardiotoxicity**



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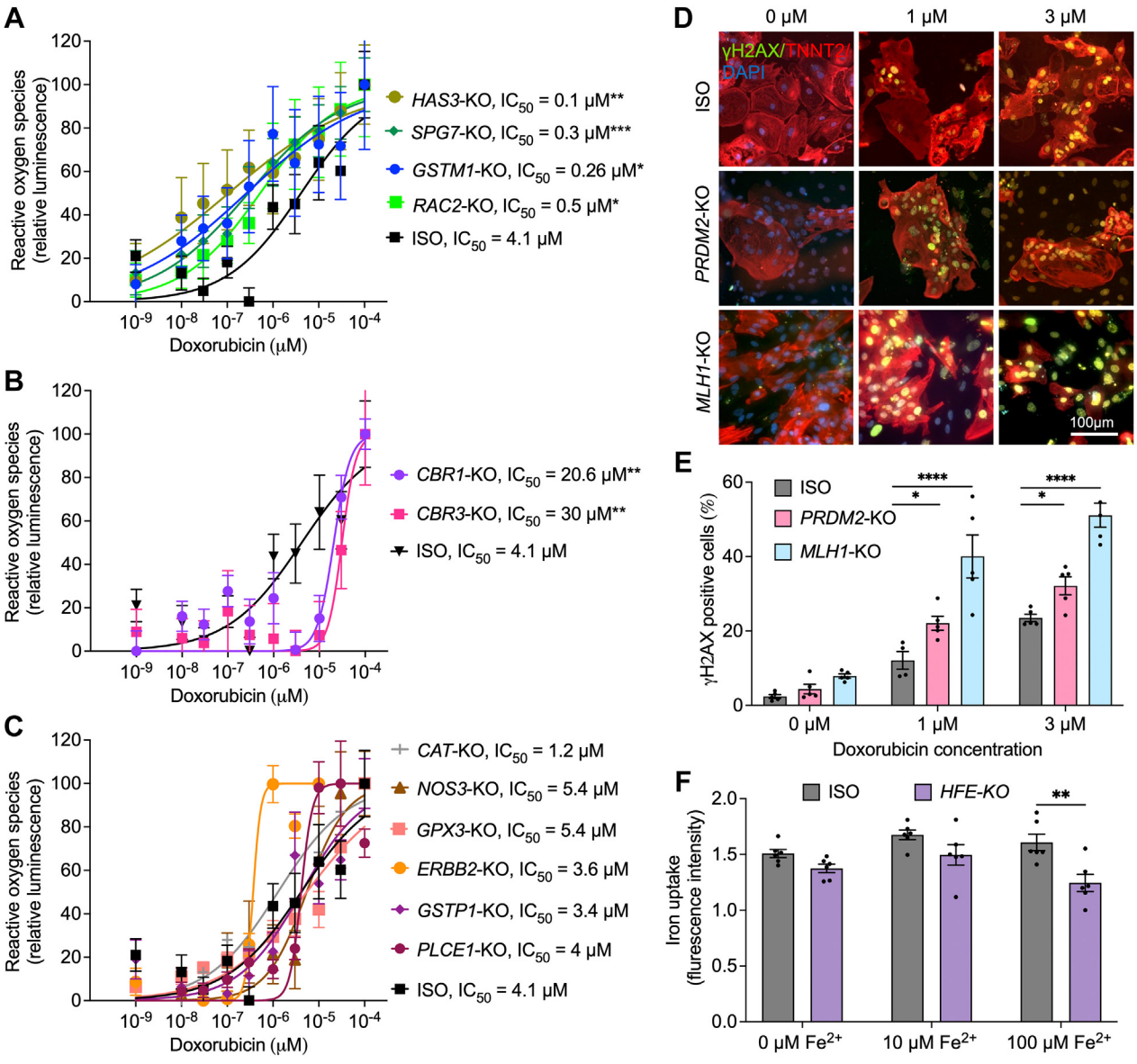
The different mechanisms of cardiotoxicity after exposure to doxorubicin (DOX) are highlighted in colored boxes. AA = arachidonic acid; ABC = adenosine triphosphate-binding cassette; EET = epoxyeicosatrienoic acid; mRNA = messenger RNA; ROS = reactive oxygen species; SLC = solute carrier; SR = sarcoplasmic reticulum.

FIGURE 3 Reactive Oxygen Species Production, DNA Damage, and Iron Uptake



(A-C) Hydrogen peroxide levels measured by ROS-Glo assay (luminescence) in hiPSC-CMs after doxorubicin treatments (24 hours). (D) Representative images for γH2AX immunofluorescent staining in hiPSC-CMs after treatments with doxorubicin (24 hours, 1 and 3 μM). (E) Quantification of DNA damage on the basis of γH2AX staining in hiPSC-CMs using flow cytometry (ISO, n = 5; *PRDM2*-KO, n = 5; and *MLH1*-KO, n = 5). (F) Effect of *HFE* knockout on hiPSC-CM iron uptake (ISO, n = 6; and *HFE*-KO, n = 6) measured using calcein staining. Error bars represent ±SEM. n = full independent experimental replicates. *$P < 0.05$, **$P \leq 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by Mann-Whitney *U* test (A-C) and 2-way analysis of variance (E and F). DAPI = 4′,6-diamidino-2-phenylindole; IC_{50} = half maximal inhibitory concentration; other abbreviations as in Figure 2.

(IC_{50} = 0.5 mM; P = 0.04) hiPSC-CMs (Figure 3B). No significant differences in ROS production were detected in *PLCE1*-KO, *ERBB2*-KO, *CAT*-KO, *GSTP1*-KO, *NOS3*-KO, and *GPX3*-KO hiPSC-CMs (Figure 3C).

Genes involved in DNA damage. *RARG*, *PRDM2*, and *MLH1* were linked to DNA damage response[3,19,47,48,60] (Table 1). Given our comprehensive study of the role of *RARG* in DIC,[19] we directed our focus to *PRDM2* and *MLH1* to quantify double-

stranded DNA breaks in hiPSC-CMs after doxorubicin treatment. Our immunofluorescence analysis suggests increased γH2AX staining in *PRDM2*-KO and *MLH1*-KO compared with isogenic hiPSC-CMs (Figure 3D). The elevated levels of $\gamma H2AX^{+}$ cells were further confirmed using flow cytometry after 1- and 3-μM doxorubicin treatments (Figure 3E). *PRDM2* (22.1% ± 1.88% [$P = 0.0283$] and 32.16% ± 2.43% [$P = 0.047$]) and *MLH1* (40.07% ± 5.79% [$P < 0.0001$] and 51.11% ± 3.2% [$P < 0.0001$]) KOs expressed higher levels of γH2AX after 1- and 3-μM doxorubicin treatments compared with isogenic control hiPSC-CMs (12.1% ± 2.37% and 23.52% ± 0.98%, respectively).

Genes involved in iron uptake and homeostasis. *HFE* encodes the homeostatic iron regulator protein, which controls iron transport and metabolism. The intracellular iron disposition in cardiomyocytes was examined by measuring iron-calcein quenching. No significant differences in iron uptake were observed in the presence of 0 and 10 μM Fe^{2+}. However, *HFE*-KO hiPSC-CMs exposed to 100 μM of Fe^{2+} exhibited significantly reduced iron uptake (Figure 3F).

Genes involved in doxorubicin uptake and efflux. Doxorubicin uptake was assessed by measuring intracellular doxorubicin autofluorescence using flow cytometry after drug treatments.[13] After the 1-μM doxorubicin treatment, *SLC28A1*-KO cardiomyocytes exhibited significantly reduced doxorubicin uptake (4.6% ± 2.5%; $P = 0.0039$) compared with isogenic control hiPSC-CMs (18.63% ± 3.24%) (Figure 4A). Conversely, doxorubicin uptake was significantly higher in *ABCB4*-KO (52.73% ± 3.2%; $P < 0.0001$), *ABCC5*-KO (48.45% ± 5.5%; $P < 0.0001$), *ABCC9*-KO (29.98% ± 2.7%; $P = 0.0327$), and *ABCC10*-KO hiPSC-CMs (60.18% ± 2.6%; $P < 0.0001$) (Figure 4A). After 1-μM treatments, no significant differences in doxorubicin uptake were observed in *SLC22A17*-KO, *SLC28A3*-KO, and *ABCC2*-KO hiPSC-CMs (Figure 4A).

Upon increasing the doxorubicin concentration to 3 μM, all the cells, except for *ABCC10*-KO, exhibited significant differences in doxorubicin uptake compared with control. Doxorubicin uptake was significantly lower in *SLC22A17*-KO (43.55% ± 3.04%; $P = 0.0005$), *SLC28A1*-KO (35.11% ± 2.63%; $P < 0.0001$), and *SLC28A3*-KO (35.24% ± 1.36%; $P < 0.0001$) hiPSC-CMs compared with isogenic control hiPSC-CMs (57.68% ± 3.11%) (Figure 4A). Conversely, 3-μM doxorubicin treatment led to significantly higher doxorubicin uptake in *ABCB4*-KO (71.77% ± 3.02%; $P = 0.0001$), *ABCC2*-KO (74.52% ± 1.57%; $P = 0.0003$), *ABCC5*-KO (79.52% ± 3.79%; $P < 0.0001$), and *ABCC9*-KO (68.57% ± 1.02%; $P = 0.04$) hiPSC-CMs (Figure 4A).

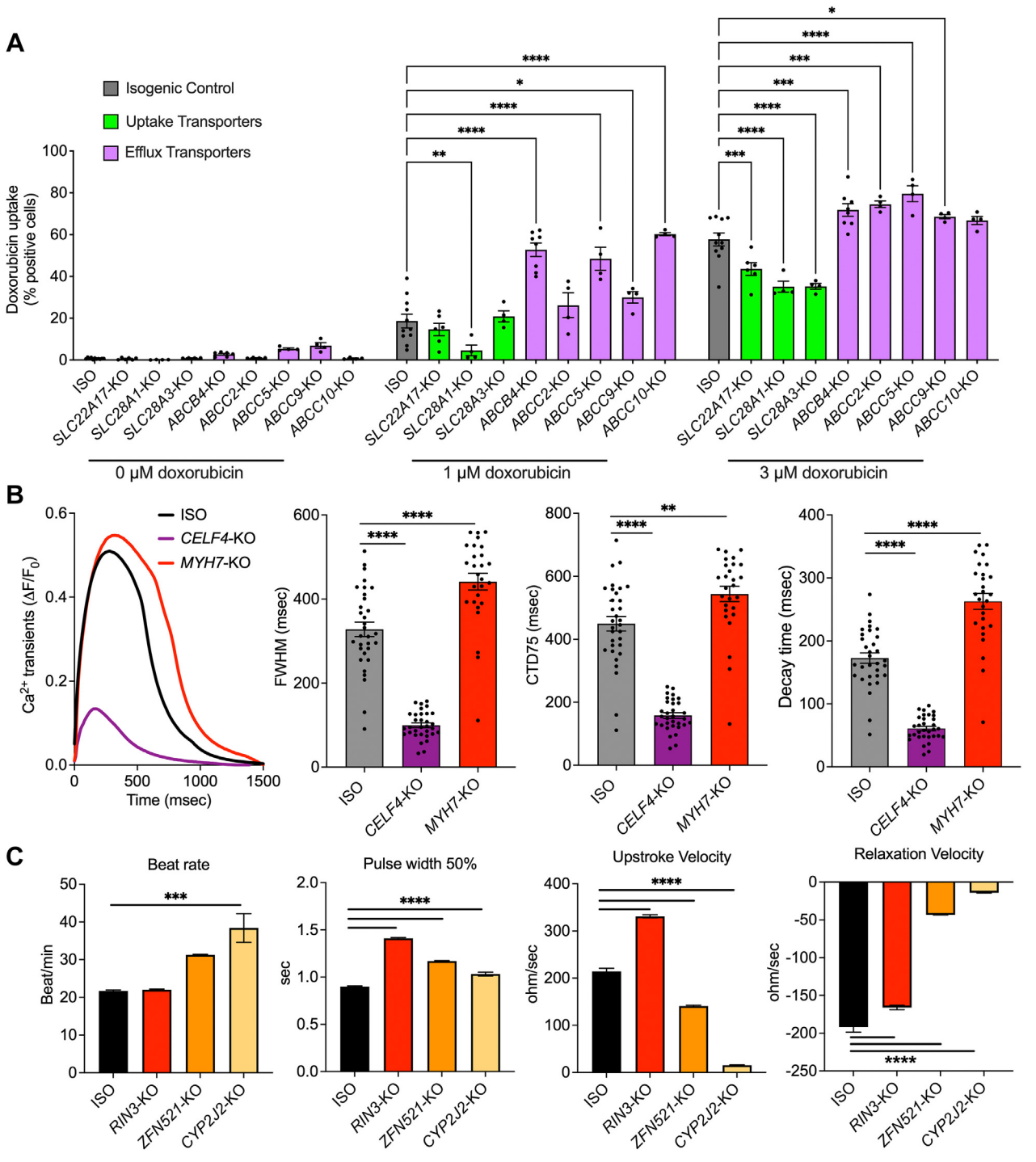
Genes involved in calcium handling. *CELF4* and *MYH7* play crucial roles in controlling cardiomyocyte function by affecting calcium transients. KO of *CELF4* resulted in a significant shortening of full width at half maximum (99.89% ± 5.67%; $P < 0.0001$), calcium transient duration at 75% (158.04% ± 8.79%; $P < 0.0001$), and decay time (60.83% ± 3.5%; $P < 0.0001$) of calcium transients compared with isogenic control hiPSC-CMs (328.15% ± 17.27%, 449.41% ± 23.04%, and 172.73% ± 8.27%, respectively) (Figure 4B). On the contrary, KO of *MYH7* resulted in a significant prolongation in full width at half maximum (441.44% ± 18.21%; $P < 0.0001$), calcium transient duration at 75% (543.82% ± 22.4%; $P < 0.0023$), and decay time (262.82% ± 11.56%; $P < 0.0001$) compared with isogenic control hiPSC-CMs (Figure 4B).

GENE INVOLVED IN CARDIAC ELECTRIC ACTIVITY. We identified 3 genes, *RIN3*, *ZFN521*, and *CYP2J2*, associated with cardiac electric activity[57-59] (Table 1). Impedance measurement revealed that KO of *RIN3* increased the pulse width at 50% (1.41% ± 0.007% vs 0.89% ± 0.006%; $P < 0.0001$) and upstroke velocity (331% ± 3.37% vs 214% ± 7.02%; $P < 0.0001$) and reduced the relaxation velocity (−166% ± 2.94% vs −192% ± 6.74%; $P < 0.0001$), compared with isogenic control hiPSC-CMs (Figure 4B). KO of *ZFN521* resulted in hiPSC-CMs with increased pulse width at 50% (1.17% ± 0.005%; $P < 0.0001$) and upstroke velocity (141% ± 1.55%; $P < 0.0001$) and reduced relaxation velocity (−43.2% ± 0.32%; $P < 0.0001$). KO of *CYP2J2* resulted in increased beat rate (38.39% ± 3.82% vs 21.7% ± 0.26%; $P < 0.0004$) and pulse width at 50% (1.03% ± 0.01%; $P < 0.0001$) and decreased upstroke velocity (15.53% ± 0.5%; $P < 0.0001$) and relaxation velocity (−13.98% ± 0.58%; $P < 0.0001$), compared with isogenic control hiPSC-CMs (Figure 4B).

DISCUSSION

Inter-individual cardiotoxic response to doxorubicin is variable, indicating that genetics plays an important role in DIC response. The functional validation of genes and variants associated with cardiotoxicity risk is essential to improve the outcome of anthracycline regimens and to develop cardioprotective treatments.

FIGURE 4 Doxorubicin Uptake, Calcium Handling, and Contractility



(A) Doxorubicin uptake in hiPSC-CMs with knockouts of *SLC* and *ABC* transporters (isotype, n = 11; *SLC28A3*-KO, n = 4; *SLC22A17*-KO, n = 6; *SLC28A1*-KO, n = 4; *ABCC2*-KO, n = 4; *ABCB4*-KO, n = 7; *ABCC5*-KO, n = 4; *ABCC9*-KO, n = 4; and *ABCC10*-KO, n = 4). n = full independent experimental replicates. (B) Effect of knocking out *MYH7* and *CELF4* on calcium transients. Left: representative calcium transients. Middle left: full width at half maximum (FWHM). Middle right: calcium transient duration 75% (CTD_{75}). Right: decay time. (C) Contractility analysis using impedance measurement in *RIN3*-KO, *ZFN521*-KO, and *CYP2J2*-KO hiPSC-CMs (ISO, n = 41; *RIN3*-KO, n = 48; *ZFN521*-KO, n = 48; and *CYP2J2*-KO, n = 89). n = number of assessed wells of 96-well plate derived from at least 3 independent rounds of differentiation (B and C). Error bars represent ±SEM. *$P < 0.05$, **$P \leq 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by 2-way analysis of variance (A) and 1-way analysis of variance (B and C). Abbreviations as in Figure 2.

KNOCKING OUT DIC-RELATED GENES ALTERS THE hiPSC-CM RESPONSE TO DOXORUBICIN TREATMENT. Of the 35 genes studied, 3 gene KOs were protective of DIC (*SLC28A3*, *SLC22A17*, and *SLC28A1*), 6 gene KOs did not have a significant effect on DIC (*ATP2B1*, *HNMT*, *POR*, *CYBA*, *WDR4*, and *COL1A2*), and 26 KOs increased DIC (*ABCC10*, *ABCC2*, *ABCB4*, *ABCC5*, *ABCC9*, *CAT*, *CBR1*, *CBR3*, *CYP2J2*, *ERBB2*, *GPX3*, *GSTM1*, *GSTP1*, *HAS3*, *HFE*, *MLH1*, *MYH7*, *NOS3*, *PLCE1*, *PRDM2*, *RAC2*, *RARG*, *RIN3*, *SPG7*, *CELF4*, and *ZFN521*).

ROS PRODUCTION IS THE MAJOR MECHANISM CAUSING DIC. The largest functional group studied comprised genes related to ROS production. H_2O_2 production analysis in cardiomyocytes exposed to doxorubicin revealed reduced production in *CBR1*-KO and *CBR3*-KO hiPSC-CMs, indicating a potential link to cardiotoxicity via reduced metabolism of doxorubicin to doxorubicinol by carbonyl reductases. In contrast, *HAS3*-KO, *SPG7*-KO, *GSTM1*-KO, and *RAC2*-KO exhibited higher ROS production and increased sensitivity to doxorubicin, indicating a role of these genes in H_2O_2 handling. Notably, no significant differences in H_2O_2 production were observed in *CAT*-KO, *NOS3*-KO, *PLCE1*-KO, *GPX3*-KO, *ERBB2*-KO, and *GSTP1*-KO. This might suggest that the DIC observed in the KO of these genes is due to the defects in the handling and detoxification of additional ROS products and/or participation in other mechanisms related to doxorubicin toxicity besides ROS generation. In fact, it has been shown that *CAT*, glutathione S-transferases, and glutathione peroxidases have played crucial roles in detoxifying metabolites generated during oxidative stress.[61]

DNA DAMAGE RESPONSE IS ELEVATED IN *PRDM2*-KO AND *MHL1*-KO. *RARG* inhibits *TOP2B*, which binds to DNA and stabilizes the intermediate TOP2B-mediated double-stranded DNA breaks. KOs of the *RARG* gene activate this DNA damage pathway, leading to increased cardiac cell death. Similar mechanisms are thought to exist in *PRDM2* and *MLH1* gene KOs. We detected higher levels of DNA damage after doxorubicin treatments *PRDM2*-KO and *MHL1*-KO, suggesting a protective role for these genes against doxorubicin-induced DNA damage.

***HFE*-KO CARDIOMYOCYTES EXHIBIT REDUCED IRON UPTAKE.** Our results indicate reduced iron uptake in *HFE*-KO cardiomyocytes compared with isogenic control, particularly evident at high Fe^{2+} concentrations (100 μM). Considering the important role of iron in mitochondrial function and the energy-demanding nature of cardiomyocytes, this impaired iron uptake may contribute to the heightened DIC observed in *HFE*-KO cardiomyocytes, as evidenced by the exacerbated risk for heart failure in the context of iron deficiency.[62]

KOs OF SLC TRANSPORTERS REDUCE AND ABC TRANSPORTERS KOs INCREASE THE DOXORUBICIN UPTAKE. KOs of SLC family members in hiPSC-CMs showed reduced doxorubicin uptake compared with isogenic controls. In addition, KO of ABC family members in hiPSC-CMs resulted in an elevation of doxorubicin uptake. Our results indicate that SLC transporters play a crucial role in facilitating the influx of doxorubicin into the cardiomyocytes, thereby contributing to an increased risk for DIC. Conversely, ABC transporters are implicated in the efflux of doxorubicin out of the cells, reducing intracellular doxorubicin levels and establishing them as potential cardioprotective gene targets.

***CELF4*-KO AND *MYH7*-KO hiPSC-CMs DEMONSTRATE ALTERED CALCIUM TRANSIENTS.** *CELF4* is involved in regulatory splicing events essential for the proper functioning of cardiac troponin T, which in turn plays an essential role in proper calcium signaling in cardiomyocytes.[63] *MYH7*, a gene encoding myosin heavy chain beta isoform, is associated with increased intracellular calcium levels in patients with hypertrophic cardiomyopathy.[64] Our analysis revealed alterations in multiple aspects of calcium transients in both *CELF4*-KO and *MYH7*-KO, suggesting impaired calcium handling as one of the mechanisms underlying DIC.

CONTRACTILE PROPERTIES OF *ZFN521*-KO, *RIN3*- KO, AND *CYP2J2*-KO hiPSC-CMs ARE AFFECTED. *ZFN521* regulates the ventricular conduction system in the heart,[65] while *RIN3* interacts with and regulates RAB5, facilitating membrane trafficking of the voltage-gated potassium channel KCNQ1 and thereby regulating potassium currents.[66] *CYP2J2* KO has been shown to result in QT-interval prolongation on echocardiography.[67] Impedance analysis of *ZFN521*-KO, *RIN3*-KO, and *CYP2J2*-KO hiPSC-CMs revealed significant differences in contractile properties, including beat rate, pulse width at 50%, upstroke velocity, and relaxation velocity, compared with the isogenic control.

STUDY LIMITATIONS. Although we investigated the KO of gene candidates from GWAS and candidate gene association studies, these studies identified SNPs linked to AIC. Because not all the SNPs cause loss of function, studying individual SNP corrections and/or patient-specific hiPSC-CMs can shed further light on this matter. Additionally, we generated KOs of selected candidate genes on the basis of their potential link to AIC and expression in hiPSC-CMs. Although not all the generated KOs showed

significant differences in LD_{50} after doxorubicin treatment, repeating this process using KO of a gene with no proven function in AIC could further validate these findings. Additionally, the application of more mature cardiomyocytes could assist to harness the differences in a more physiologically relevant system. The present study was focused only on the effect of KOs on cardiomyocytes, but cardiotoxicity might stem from malfunction in other cell types, including endothelial cells and cardiac fibroblasts. Finally, for each KO, only 1 functional study was performed; additional functional studies can provide a more detailed understanding of each gene contribution to the formation of DIC.

CONCLUSIONS

This study demonstrates that genomic analysis, coupled with human induced pluripotent stem cell modeling, is an efficient platform for assessing the mechanisms of DIC. Through high-throughput assays, we validate the influence of DIC genes on cell viability, ROS production, DNA damage, doxorubicin uptake, iron uptake, calcium handling, and electric activity in response to doxorubicin. Our results confirm that more than 55% of the DIC genes identified to date in association studies are expressed in cardiac cells, reliably recapitulating alterations in DIC phenotype in the hiPSC-CM model. Furthermore, for each gene, we provide a functional assay to validate its role in DIC.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In this study, we have functionally validated all current DIC-associated genes that are expressed in cardiomyocytes. This comprehensive validation represents the first step in translating functionally validated genotype-phenotype correlations into clinical tests. Once the relevance of a gene in DIC is identified, and its mechanism of action is known, the next step involves validating the role of genetic variants in modulating the response to doxorubicin. This information will serve as a unique platform for designing polygenic risk scores that can benefit patients undergoing doxorubicin treatment. In addition, a precise understanding of the genetic basis of DIC is crucial for identifying druggable targets and discovering innovative cardioprotective therapies.

TRANSLATIONAL OUTLOOK: One major barrier in the clinical application of this study lies in determining the genetic contribution to DIC risk for each gene for all sexes and ancestries. Currently, there is a scarcity of studies exploring the effects of sex and diverse genetic backgrounds on the mechanisms of DIC. Establishing patient-specific risk factors is a crucial prerequisite before the clinical implementation of the findings from this study can be applied.

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APPENDIX **For supplemental methods, tables, and figures, please see the online version of this paper.**