Cardiac stereotactic body radiotherapy to treat malignant ventricular arrhythmias directly affects the cardiomyocyte electrophysiology

Christine Mages, MD, Heike Gampp, Ann-Kathrin Rahm, MD, Juline Hackbarth, Julia Pfeiffer, Finn Petersenn, Xenia Kramp, Fatemeh Kermani, Juan Zhang, PhD, Daniel A. Pijnappels, PhD, Antoine A.F. de Vries, PhD, Katharina Seidensaal, MD, Bernhard Rhein, PhD, Jürgen Debus, MD, Nina D. Ullrich, PhD, Norbert Frey, MD, Dierk Thomas, MD, Patrick Lugenbiel, MD

PII: S1547-5271(24)02817-0

DOI: https://doi.org/10.1016/j.hrthm.2024.06.043

Reference: HRTHM 10579

To appear in: Heart Rhythm

Received Date: 19 May 2024

Revised Date: 21 June 2024

Accepted Date: 22 June 2024

Please cite this article as: Mages C, Gampp H, Rahm A-K, Hackbarth J, Pfeiffer J, Petersenn F, Kramp X, Kermani F, Zhang J, Pijnappels DA, de Vries AAF, Seidensaal K, Rhein B, Debus J, Ullrich ND, Frey N, Thomas D, Lugenbiel P, Cardiac stereotactic body radiotherapy to treat malignant ventricular arrhythmias directly affects the cardiomyocyte electrophysiology, *Heart Rhythm* (2024), doi: https://doi.org/10.1016/j.hrthm.2024.06.043.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2024 Published by Elsevier Inc. on behalf of Heart Rhythm Society.



1 Cardiac stereotactic body radiotherapy to treat malignant ventricular 2 arrhythmias directly affects the cardiomyocyte electrophysiology

3

4 **Running Title:** Cardiac SBRT Directly Modulates Cardiomyocyte Electrophysiology

5

Authors: Christine Mages, MD ^{a,b,c,d#}, Heike Gampp ^{a,b#}, Ann-Kathrin Rahm, MD ^{a,b,c,d},
Juline Hackbarth ^{a,b}, Julia Pfeiffer ^{a,b}, Finn Petersenn ^{a,b}, Xenia Kramp ^{a,b}, Fatemeh
Kermani ^e, Juan Zhang, PhD ^f, Daniel A. Pijnappels, PhD ^f, Antoine A. F de Vries, PhD^f,
Katharina Seidensaal, MD ^{g,h}, Bernhard Rhein, PhD ^{g,h}, Jürgen Debus, MD ^{g,h}, Nina
D. Ullrich, PhD ^{c,e,i}, Norbert Frey, MD ^{a,b,c,d}, Dierk Thomas, MD ^{a,b,c}, Patrick Lugenbiel,
MD ^{a,b,c*}

12

13 **Total Word Count: 5041**

- ¹⁴ ^aDepartment of Cardiology, University Hospital Heidelberg, Heidelberg, Germany;
- ¹⁵ ^bHeidelberg Center for Heart Rhythm Disorders (HCR), University Hospital
 ¹⁶ Heidelberg, Heidelberg, Germany;
- ¹⁷ ^cGerman Centre for Cardiovascular Research (DZHK), Partner site
 ¹⁸ Heidelberg/Mannheim, University of Heidelberg, Heidelberg, Germany;
- ¹⁹ ^dInformatics4Life Consortium (Institute for Informatics Heidelberg), Heidelberg,
 20 Germany;

^eDivision of Cardiovascular Physiology, Institute of Physiology and Pathophysiology,
 Heidelberg, Germany;

23 fDepartment of Cardiology, Leiden University Medical Center, Leiden, the Netherlands

^gDepartment of Radiation Oncology, Heidelberg University Hospital, Heidelberg,
 Germany;

²⁶ ^hHeidelberg Institute of Radiation Oncology (HIRO), Heidelberg, Germany;

²⁷ ⁱDepartment of Physiology, University of Bern, Switzerland

28

29 # authors contributed equally

*Address for correspondence: Patrick Lugenbiel, MD, FEHRA; Department of
 Cardiology, Heidelberg University, Im Neuenheimer Feld 410, 69120 Heidelberg,
 Germany; E-Mail: <u>patrick.lugenbiel@med.uni-heidelberg.de</u>

34

Funding: This work was supported in part by research grants from the University of 35 Heidelberg, Faculty of Medicine (Postdoctoral Fellowships to P.L. and A.K.R.), from 36 the German Cardiac Society (Fellowships to A.K.R. and P.L., Otto-Hess-37 Promotionsstipendium to H.G.), from the Elisabeth und Rudolf-Hirsch Stiftung für 38 Medizinische Forschung (to A.K.R), from the Ernst und Berta Grimmke-Stiftung (to 39 P.L.), from the Tschira-Foundation for InformaticsForLife to A.K.R. (research funding 40 of C.M.), from the German Heart Foundation/German Foundation of Heart Research 41 (Fellowship to A.K.R), from the German Internal Medicine Society (Clinician-Scientist-42 Program to A.K.R.), from the German Research Foundation (UL 466/2-1 to N.D.U.), 43 and from the Joachim Siebeneicher Foundation (to D.T.). 44

45

Disclosures: C.M. acknowledges educational support from Biotronik and Johnson & 46 Johnson, while A.K.R. acknowledges support from Boston Scientific, Johnson & 47 Johnson, Abbott, and Medtronic. D.T. has received lecture fees/honoraria from various 48 pharmaceutical and medical device companies, including Abbott, AstraZeneca, Bayer 49 Vital, Boehringer Ingelheim Pharma, Bristol-Myers Squibb, Daiichi Sankyo, Johnson 50 and Johnson Medical, Medtronic, Novartis, Pfizer Pharma, Sanofi-Aventis, and ZOLL 51 CMS. P.L. has received lecture fees from Bayer Vital, Pfizer Pharma, and Bristol-52 Myers-Squibb, as well as educational support from Boston Scientific and 53 fees/honoraria from Johnson & Johnson and Boston Scientific. The remaining authors 54 have declared no relevant relationships. 55

56 Abstract

57 Background

Promising as a treatment option for life-threatening ventricular arrhythmias, cardiac
stereotactic body radiotherapy (cSBRT) has demonstrated early antiarrhythmic effects
within days of treatment. The mechanisms underlying the immediate and short-term
antiarrhythmic effects are poorly understood.

62 **Objectives**

We hypothesize that cSBRT has a direct antiarrhythmic effect on cellular electrophysiology through reprogramming of ion channel and gap junction protein expression.

66 Methods

Following exposure to 20Gy of X-rays in a single fraction, neonatal rat ventricular cardiomyocytes (NRVCs) were analyzed 24 and 96h post-radiation to determine changes in conduction velocity, beating frequency, calcium transients, and action potential duration (APD) in both monolayers and single cells. Additionally, the expression of gap junction proteins, ion channels, and calcium handling proteins was evaluated at protein and mRNA levels.

73 **Results**

Following irradiation with 20Gy, NRVCs exhibited increased beat rate and conduction velocities 24 and 96h after treatment. mRNA and protein levels of ion channels were altered, with the most significant changes observed at the 96h-mark. Upregulation of *Cacna1c* (Cav1.2), *Kcnd3* (Kv4.3), *Kcnh2* (Kv11.1), *Kcnq1* (Kv7.1), *Kcnk2* (K₂P2.1), *Kcnj2* (Kir2.1), and Gja1 (Cx43) was noted, along with improved gap junctional coupling. Calcium handling was affected, with increased *Ryr2* (RYR2) and *Slc8a1*

80 (NCX) expression and altered properties 96h post-treatment. Fibroblast and
 81 myofibroblast levels remained unchanged.

82 Conclusions

cSBRT modulates expression of various ion channels, calcium handling proteins, and
gap-junction proteins. The described alterations in cellular electrophysiology may be
the underlying cause of the immediate antiarrhythmic effects observed following
cSBRT.

- 87 **Keywords:** radiation; ion channel; remodeling; neonatal rat cardiomyocytes;
- 88 ventricular arrhythmia, sudden cardiac death.
- 89
- 90 Abbreviations list:
- 91 APD=Action potential Duration
- 92 cSBRT=Cardiac stereotactic body radiation therapy
- 93 CV=Conduction velocity
- 94 Cx43=Connexin 43
- 95 FDHM=Full duration at half maximum
- 96 hiPSC-CMs=Human induced pluripotent stem cell-derived cardiomyocytes
- 97 ICD=Implantable cardioverter-defibrillator
- 98 NRVCs=Neonatal rat ventricular cardiomyocytes
- 99 Rx=Irradiation
- 100 TTP=Time-to-peak

- 102
- 103
- 104
- 105
- 106

107 **1. Introduction**

Scar-related ventricular arrhythmias are a significant cause of morbidity and mortality 108 in patients with impaired cardiac function and structural heart disease, often leading 109 to sudden cardiac death.¹ Cardiac stereotactic body radiotherapy (cSBRT) for the 110 treatment of ventricular tachycardia (VT) and ventricular fibrillation in patients with 111 arrhythmic events has been an emerging technology within the last years and has 112 shown promising effects in terms of immediate reduction of ventricular arrhythmias 113 and ICD-interventions.² The latency of expected reduction of VT burden after cSBRT 114 is of utmost importance due to the life-threatening character of ventricular arrhythmias. 115 The antiarrhythmic effects of cardiac irradiation are thought to include the induction of 116 scar homogenization and fibrosis. However, clinical studies consistently show that 117 irradiation has an early antiarrhythmic effect within days of treatment, i.e. before the 118 onset of fibrosis.^{2–5} The molecular processes responsible for this initial antiarrhythmic 119 120 effect have not yet been fully resolved.

The effect of immediate reduction of VT-burden was observed after 25Gy singlefraction photon irradiation (Rx).^{2,4} At present, there is a lack of information regarding the minimum radiation dosage required to achieve antiarrhythmic effects. Radiation dose is mainly derived from preclinical studies, proposing electrophysiologic effects at or above 24Gy due to the induction of fibrosis.^{4,6–8} This collectively suggests the potential for electrical remodeling independent of the onset of fibrosis.^{2,4}

127 VTs are typically a result of myocardial re-entry occurring in regions with 128 inhomogeneous scarring or at the edges of scars.⁹ This type of re-entry is made 129 possible due to the local slowing of electrical conduction caused by fibrosis as well as 130 a decrease in gap junction coupling.¹⁰

Little is known about the radiation-dependent effects at the molecular level of cardiac 131 electrophysiology; repeatedly, alterations of the cardiac conduction protein connexin 132 43 (Cx43) were described, but both up- and downregulation have been observed.^{11–15} 133 Recently an upregulation of Cx43 and the cardiac fast sodium channel Nav1.5 was 134 found six weeks after 25Gy whole heart irradiation in a mouse model with and without 135 myocardial infarction causing an increase in cardiac conduction velocity (CV) 136 reprogramming that was also achieved at lower Rx doses of 15 and 20Gy.¹⁶ After 137 25Gy whole heart irradiation in rats, dynamic changes in the cardiac proteome, 138 139 including proteins of the the cardiac conduction system were observed within one week.¹⁵ Significant functional electrocardiographic changes were also observed in 140 human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) exposed 141 to a single dose of X-rays of 20 to 50Gy.^{17,18} 142

In the present study, we evaluated the mechanisms underlying the immediate
antiarrhythmic effects of cSBRT. We hypothesized that radiation with less than 25Gy
is sufficient to induce antiarrhythmic electrical remodeling.

146

147

148

149

150

151

152

154 **2. Methods**

Full experimental procedures and any associated references are available asSupplemental Material.

157

158 **2.1 Ethics statement**

Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and issued by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1985), and the current version of the German Law on the Protection of Animals was followed. This study conforms to Directive 2010/63/EU of the European Parliament.

164

165 2.2 Statistics

Statistical analyses were conducted using GraphPad Prism 6.0 software and OriginPro 2022 software. Data are presented as box and whisker plots with confidence intervals (CI), and the number of experiments (n) is indicated in each section. We used the unpaired, two-tailed Student's t-test or Mann-Whitney U test for unequal variances and the paired Student's t-test for comparisons within experimental groups across different time points. Significance was denoted as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

173

174

176 **3. Results**

3.1 Irradiated NRVC monolayers showed higher conduction velocity, shortened action potential duration, and increased beating frequency

We studied how Rx affects CV and action potential duration (APD) in NRVCs. At 24h 179 after Rx, CV increased significantly (+30%, n=6; P<0.0001) (Fig. 1A). By 96h, CV was 180 1.6-fold higher than in controls (+165%, Ctrl *n*=5, Rx *n*=6, *P*=0.0014) (Fig. 1D). APD 181 did not differ significantly at 24h but shortened significantly by 96h (APD₅₀: -52%, Ctrl 182 183 *n*=5, Rx *n*=6; *P*<0.0001; APD₉₀: -50%, Ctrl *n*=5, Rx *n*=6; *P*<0.001) (Fig. 1E,F). Patchclamp measurements at 24h showed APD₉₀ shortening (-11%, *n*=27; *P*=0.026). No 184 significant apoptosis was observed at 24 or 96h after Rx (Fig. S1), and cardiac troponin 185 T (cTnt) mRNA expression remained stable (Fig S2). At 24h, cultures were mainly 186 cardiomyocytes with ~4% fibroblasts; fibroblast proliferation number increased by 96h 187 with no significant differences between irradiated and non-irradiated cultures (+28%, 188 n=5; P=0.469) (Fig. S3). Irradiated cardiomyocytes showed an increase in beating 189 frequency at 24h (+53%, *n*=6; *P*=0.002) and 96h (+59%, Ctrl *n*=8, Rx *n*=7; *P*<0.001) 190 post-Rx (Fig. 1G), with greater variability compared to controls. 191

192 **3.2 Ion channel remodeling after irradiation**

We next examined radiation-induced electrophysiological remodeling at the molecular level and analysed differences in the expression of ion channel genes involved in the ventricular action potential by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. At 24h after Rx, we found significant downregulation of the transcript levels of *Kcnq1/K*v7.1 (*n*=6, *P*=0.036) and *Kcnj8/K*ir6.1 (*n*=6, *P*=0.044) (Fig. 2A). Downregulation of *Kcnj8/*Kir6.1 transcript persisted at 96h after Rx (*n*=6, *P*<0.0001). In contrast, the transcript levels of *Cacna1c/*Cav1.2 (*n*=6, *P*=0.023) and

the potassium channels *Kcnd3*/K_v4.3 (*n*=6, *P*=0.0005), *Kcnh2*/K_v11.1 (*n*=6, *P*<0.0001), *Kcnq1*/K_v7.1 (*n*=6, *P*<0.0001), *Kcnk2*/K_{2P}2.1 (*n*=6, *P*=0.032), and *Kcnj2*/K_{ir}2.1 (*n*=6, *P*=0.0001) were significantly upregulated at 96h after Rx compared to those in control cultures. We found no differences in the expression of *Scn5a*/Na_v1.5, *Kcne1*/MinK, and *Kcnk3*/K_{2P}3.1. Western blotting of samples harvested at 96h after Rx confirmed the RT-qPCR results at the protein level (Fig. 2B, C).

3.3 Irradiation improves intercellular coupling by upregulation of Cx43 and increased gap junction formation

To assess study effects of Rx on gap junctional coupling we assessed Cx43 208 expression (Fig. 3). At 24h after Rx, there were no significant changes in Cx43 mRNA 209 210 (P=0.316) (Fig. 3A) or protein levels (P=0.890) (Fig. 3B, C). However, by 96h, both Cx43 mRNA (P<0.0001) and protein levels were upregulated (P<0.0001) (Fig. 3A, C). 211 Immunostaining revealed a 45% increase in Cx43 expression at cell-to-cell contact 212 zones in irradiated cardiomyocytes (P<0.0001) (Fig. 3D), indicating increased gap 213 junction formation. Functional evaluation using fluorescence recovery after 214 photobleaching showed significantly faster fluorescence recovery in irradiated 215 cardiomyocytes (+24%, P=0.041), indicating enhanced functional gap junctions (Fig. 216 3E-G). 217

3.4 Irradiated neonatal rat ventricular cardiomyocytes showed altered calcium handling.

Calcium handling changes may affect CV and APD. Using IonOptix, we measured calcium transients (Fig. 4). At 24h, diastolic calcium increased in irradiated monolayers (+7%, P=0.0003) (Fig. 4B), together with a reduced time-to peak (TTP) and full duration at half maximum (FDHM) and time to peak (TTP; -26% and -23%,

respectively; *P*<0.0001) (Fig. 4B). At 96h, diastolic calcium remained elevated (+11%, *P*=0.0004), with further reductions in FDHM and TTP (-30% and -23%, respectively; *P*<0.0001) (Fig. 4D). Additionally, irradiated cardiomyocytes displayed increased calcium transient amplitudes and faster decay rates after 96h.

228 RT-qPCR and western blot analysis of genes involved in cardiac calcium handling 229 (Fig. 5) showed an increase in PLN mRNA at 24h after Rx (P=0.012), which further 230 increased by 96h (P<0.001), along with an increase in PLN protein (P=0.008). NCX 231 mRNA and protein levels rose at 96h post-Rx as well as RyR2 mRNA (P=0.005). No 232 significant changes were observed in CaMKII δ and CaMKII γ mRNA and protein levels 233 at 24 or 96h after Rx.

234 **3.5 Effects of irradiation on extracellular matrix**

Next, we investigated the effect of irradiation on extracellular matrix formation and conversion of fibroblasts to paracrine active myofibroblasts in mixed cultures of cardiomyocytes and fibroblasts. The fibroblast-to-myofibroblast switch is accompanied by increases in the expression of α -smooth muscle actin (α -SMA) and the production of extracellular matrix components, a key event in connective tissue remodeling. As shown by western blotting, Rx did not significantly increase α -SMA or vimentin protein levels (Fig. 6).

242

243

244

245

246

248 **4. Discussion**

We investigated cSBRT's antiarrhythmic effects in NRVC cultures. A single Rx-dose 249 250 of 20Gy led to an immediate rise in beat rate and CV, alongside significant APD shortening. We also observed changes in the expression of genes encoding ion 251 channel and calcium handling proteins, notably at 96h post-Rx. These findings support 252 253 the notion of an early antiarrhythmic impact preceding tissue damage. This study represents the first systematic exploration of cSBRT's immediate antiarrhythmic 254 effects, highlighting the role of ion channels, gap junctional coupling, and calcium 255 homeostasis in modifying cellular electrophysiology. 256

257 Irradiation affects electrophysiology in cardiomyocytes

In irradiated cardiomyocytes, there was a significant increase in beating frequency
compared to the unirradiated group, possibly due to increased diastolic calcium levels.
Mouse embryonic stem cells irradiated with 5Gy of γ-rays exhibited altered contractile
properties and reduced beating frequency after differentiation into cardiomyocytes.²⁰
This discrepancy may be explained by the use of different experimental models, but
could also suggest a dose-dependent effect.

Rx caused changes in the expression of cardiac ion channels directly affecting 264 membrane excitability. Rx induced downregulation of Kcnj8/Kir6.1 that persisted for 265 96h. Cacna1c/Cav1.2 and the potassium channels Kcnd3/Kv4.3, Kcnh2/Kv11.1, 266 Kcnk2/K2P2.1, and Kcnj2/Kir2.1 showed significant upregulation at 96h after Rx. 267 Kcnq1/K_v7.1 exhibited biphasic regulation with reduced expression at 24h and 268 increased expression after 96h. Collectively, the changes in repolarizing potassium 269 channel expression are suspected to shorten APD and to contribute to an increased 270 CV. Exposure of iPSC-CMs to 20Gy did not lead to significant upregulation of KCND3, 271

KCNH2, SCN5A, CACNA1C or GJA1 after 7 days in contrast to Rx doses of 25 or 272 30Gy.¹⁸ Interestingly, another research group reported a significant increase in Nav1.5 273 density six weeks after whole heart irradiation of mice with 20Gy.¹⁶ This disparity may 274 be explained by compensatory mechanisms or dynamic changes that occur following 275 irradiation, as indicated by large-scale proteomic analysis three and seven days after 276 2- and 25-Gy Rx.¹⁵ These alterations resulted in APD shortening at 24h after Rx. This 277 observation is consistent with our optical voltage mapping results, demonstrating 278 increased CV and consistent APD shortening in irradiated NRVC monolayers. 279

280 Irradiation improves impulse propagation and conduction velocity

Our data showed upregulation of Cx43 and improved gap junction coupling in NRVCs 281 at 96h after Rx, which can also in part explain the increased CV. Previous studies 282 have reported that Cx43 expression is very sensitive to ionizing radiation in various 283 cell lines.^{21–24} Our study adds to this observation and confirms the hypothesis that the 284 induced Cx43 proteins form functional channels. There are also contrary observations 285 in the field, whose cause has not been clarified. Kim et al. showed a non-significant 286 trend towards an increase in Cx43 mRNA following 20-Gy Rx of hiPSC-CMs.¹⁸ Cha et 287 al. even reported a reduction in Cx43 expression two and three weeks after irradiation 288 with 20 to 50Gy by immunostaining of adult rat hearts.¹⁷ The latter result is at odds 289 290 with the observation that cardiac-specific postnatal loss of Cx43 slowed ventricular CV and increased the susceptibility to spontaneous ventricular arrhythmias and sudden 291 cardiac death.²⁵ Moreover, other preclinical studeis also found increased cell-to-cell 292 conduction via Cx43 upregulation upon 25Gy irradiation.^{11–14} To our understanding, 293 the inconsistencies in the field may arise from differences between animal models, 294 type of radiation, and dose or timepoint of evaluation. Further studies are needed to 295 address these different observations. 296

Calcium homeostasis and calcium handling proteins are affected by single dose irradiation

Regarding calcium handling proteins, significant upregulation PLN, 299 of Cacna1c/Cav1.2, RYR2, and NCX mRNA and protein levels was detected after 300 exposure of NRVCs to 20Gy. Kim et al.¹⁵ reported an increase in RYR2 protein three 301 days after exposure of adult rats to 25Gy, which is consistent with our findings. As 302 mentioned previously, there are also reports indicating no significant changes in 303 CACNA1C expression following 20-Gy irradiation,¹⁸ a phenomenon that remains 304 unexplained. 305

At the functional level, a variety of changes in the calcium transient properties were 306 observed. We found persistently elevated radiation-dependent diastolic calcium 307 levels. To our understanding this increase might arise from radiation-induced oxidative 308 stress and reactive oxygen species (ROS) formation²⁶ leading to calcium leakage from 309 the sarcoplasmic reticulum, as proposed previously.²⁷ However, we did not measure 310 ROS formation in our model as this was not the scope of the present study. At 24 and 311 96h after Rx, we found faster kinetics of calcium transients as evidence by a reduction 312 of TTP and FDHM. At 96h after Rx, calcium transients also showed increased 313 amplitudes and faster decay rates. The increased amplitude may be associated with 314 315 the observed upregulation of *Cacna1c*/Cav1.2 and RyR2. The faster decay rate might be related to the increased abundance of NCX. In a comprehensive study²⁷, Sag et al. 316 assessed the acute (1h after irradiation) and chronic (one week after irradiation) 317 effects of 20-Gy mediastinal photon radiation on calcium handling in murine 318 cardiomyocytes. The investigation demonstrated an immediate elevation in calcium 319 transient amplitudes post-irradiation, as opposed to a sustained reduction in the 320 chronic phase. In contrast to our findings, the authors described an acute and chronic 321

irradiation-dependent activation of CaMKII due to increased oxidative stress.²⁷ In our 322 study, CaMKIIo and CaMKIIy levels were not affected by Rx. Also, while we found an 323 increase in SIc8a1 (NCX) expression and no change in Atp2a1 (SERCA) mRNA (Fig. 324 5) after Rx, in the study by Sag et al. NCX remained unchanged and SERCA2a 325 expression was decreased on week after Rx. We focused on the short-term effects up 326 to 96h post-Rx, leaving uncertainty regarding the normalization or exacerbation of 327 expression differences over time. However, both studies highlight the influence of 328 ionizing radiation on cardiac calcium handling, urging further research to grasp its 329 330 implications for antiarrhythmic effects.

331 Significance for antiarrhythmic therapy

In most heart diseases, re-entry mechanisms are based on remodeling processes 332 causing (local) conduction slowing.²⁸ Our data support early antiarrhythmic effects 333 through reprogramming of ion channels causing an increase in CV and APD 334 shortening in irradiated cardiomyocytes. These results are consistent with a study of 335 Anyukhovsky et al., which showed antiarrhythmic effects of improving the conduction 336 in slow conducting arrhythmogenic mouse tissue by Scn4a (Nav1.4) or Gjb1 (connexin 337 32) gene transfer.²⁹ Shortening of the effective refractory period of the targeted 338 myocardium could lead to enhanced local conduction³⁰ and consequently, the re-entry 339 340 phenomenon would decrease through an electrical homogenization. As a therapeutic approach, this could already demonstrate strong antiarrhythmic effects.^{31,32} The 341 described effects of abolishing inhomogeneities and delayed conduction properties 342 within the scar, partially achieved through altered ion channel expressions and 343 improved gap junctional coupling, may represent a potentially effective antiarrhythmic 344 concept in the treatment of ventricular tachycardias in patients with scar-related 345 mechanisms. These effects occur independently of the formation of fibrous scars, 346

suggesting that these electrical changes can already be effectively observed at lower
radiation doses. Further work in disease models will be essential to understand
whether the response of the diseased myocardium is similar to that of healthy tissue.

Although the antiarrhythmic mechanisms of Rx remain to be determined, several 350 studies showed early myocardial effects in a dose-dependent manner.^{15–18} A 351 persistent observation is that 20Gy is sufficient to achieve electrophysiological 352 remodeling, although it remains unclear if those effects are long-lasting as suggested 353 by our data. The currently used dose of 25Gy in patients was initially chosen to imitate 354 the effects seen after radiofrequency ablation. Currently, ~81% of the patients 355 receiving SBRT suffer from mild adverse effects^{2,4}, which highlights the importance of 356 improving its safety through dose reduction. The reported data give a mechanistic 357 insight into fast antiarrhythmic changes and underline the option for dose reduction 358 without loss of efficacy. Initial clinical reports support effective antiarrhythmic treatment 359 with a radiation dose even below 20Gy.33 360

361 **Potential limitations and future directions**

This study has several noteworthy limitations. We chose not to inhibit fibroblasts to 362 better mimic the extracellular environment, which could impact the results as 363 fibroblasts influence cardiomyocyte electrophysiology through heterocellular coupling. 364 Neonatal cardiomyocytes were selected for patch-clamp investigations, with the 365 present work focusing on a descriptive analysis of electrophysiological properties 366 within a maximum of 96h post-irradiation. Further studies in a disease model are 367 needed to enable the examination of adult cells over a longer observation period. Our 368 model does not replicate diseased hearts, which significantly affects the causal 369 interpretation of the results. Additionally, we used photon irradiation, but other 370

energies like protons or heavy ions may yield different effects and warrant
 investigation.^{34,35}

373

374 **5. Conclusion**

Our results indicate an association of acute irradiation effects and electrophysiological remodeling in cardiomyocytes. Changes of cellular ion channel expression, calcium homeostasis and cell-cell-propagation lead to electrophysiological changes. Although the final mechanism causing antiarrhythmic changes remains to be discovered, these results reveal new insight into acute antiarrhythmic effects of cSBRT therapy on cardiomyocytes.

381

382 6. Acknowledgement

383 We declare that no artificial intelligence (AI) was used in preparation of the present 384 manuscript.

We gratefully acknowledge the excellent technical assistance of Axel Schöffel, Teresa

Caspari, Patrizia Lo Vetere, Nicole Westerhorstmann and Miriam Baier.

388 7. References

- Zeppenfeld K, Tfelt-Hansen J, de Riva M, et al.: 2022 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: Developed by the task force for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death of the European Society of Cardiology (ESC) Endorsed by the Association for European Paediatric and Congenital Cardiology (AEPC). European Heart Journal 2022; 43:3997–4126.
- Kovacs B, Mayinger M, Schindler M, Steffel J, Andratschke N, Saguner AM:
 Stereotactic radioablation of ventricular arrhythmias in patients with structural
 heart disease A systematic review. Radiother Oncol 2021; 162:132–139.
- Ninni S, Gallot-Lavallée T, Klein C, et al.: Stereotactic Radioablation for
 Ventricular Tachycardia in the Setting of Electrical Storm. Circ Arrhythm
 Electrophysiol 2022; 15:e010955.
- 402 4. van der Ree MH, Blanck O, Limpens J, et al.: Cardiac radioablation—A 403 systematic review. Heart Rhythm Elsevier, 2020; 17:1381–1392.
- Robinson CG, Samson, Moore, et al.: Phase I/II Trial of Electrophysiology-Guided
 Noninvasive Cardiac Radioablation for Ventricular Tachycardia. Circulation
 American Heart Association, 2018; 139:313–321.
- 6. Blanck O, Bode F, Gebhard M, et al.: Dose-escalation study for cardiac radiosurgery in a porcine model. Int J Radiat Oncol Biol Phys 2014; 89:590–598.
- Chang JH, Cha M-J, Seo J-W, et al.: Feasibility study on stereotactic radiotherapy
 for total pulmonary vein isolation in a canine model. Sci Rep 2021; 11:12369.
- 8. Lehmann HI, Richter D, Prokesch H, et al.: Atrioventricular node ablation in
 Langendorff-perfused porcine hearts using carbon ion particle therapy: methods
 and an in vivo feasibility investigation for catheter-free ablation of cardiac
 arrhythmias. Circ Arrhythm Electrophysiol 2015; 8:429–438.
- 415 9. de Chillou C, Lacroix D, Klug D, et al.: Isthmus characteristics of reentrant
 416 ventricular tachycardia after myocardial infarction. Circulation 2002; 105:726–
 417 731.
- 418 10. de Bakker JM, van Capelle FJ, Janse MJ, et al.: Slow conduction in the infarcted
 419 human heart. "Zigzag" course of activation. Circulation 1993; 88:915–926.
- Amino M, Yoshioka K, Tanabe T, et al.: Heavy ion radiation up-regulates Cx43
 and ameliorates arrhythmogenic substrates in hearts after myocardial infarction.
 Cardiovasc Res Oxford Academic, 2006; 72:412–421.
- Amino M, Yoshioka K, Fujibayashi D, et al.: Year-long upregulation of connexin43
 in rabbit hearts by heavy ion irradiation. Am J Physiol Heart Circ Physiol 2010;
 298:H1014-1021.

- Viczenczova C, Szeiffova Bacova B, Egan Benova T, et al.: Myocardial connexin43 and PKC signalling are involved in adaptation of the heart to irradiationinduced injury: Implication of miR-1 and miR-21. Gen Physiol Biophys 2016;
 35:215–222.
- 430 14. Viczenczova C, Kura B, Egan Benova T, et al.: Irradiation-Induced Cardiac
 431 Connexin-43 and miR-21 Responses Are Hampered by Treatment with
 432 Atorvastatin and Aspirin. International Journal of Molecular Sciences
 433 Multidisciplinary Digital Publishing Institute, 2018; 19:1128.
- Kim BH, Jung JW, Han D, Cha M-J, Chang JH: One-Week Dynamic Changes in
 Cardiac Proteomes After Cardiac Radioablation in Experimental Rat Model. Front
 Cardiovasc Med 2022; 9:898222.
- 437 16. Zhang DM, Navara R, Yin T, et al.: Cardiac radiotherapy induces electrical
 438 conduction reprogramming in the absence of transmural fibrosis. Nat Commun
 439 2021; 12:5558.
- 17. Cha M-J, Seo J-W, Kim HJ, et al.: Early Changes in Rat Heart After High-Dose
 Irradiation: Implications for Antiarrhythmic Effects of Cardiac Radioablation. J Am
 Heart Assoc 2021; 10:e019072.
- 18. Kim JS, Choi SW, Park Y-G, et al.: Impact of High-Dose Irradiation on Human
 iPSC-Derived Cardiomyocytes Using Multi-Electrode Arrays: Implications for the
 Antiarrhythmic Effects of Cardiac Radioablation. Int J Mol Sci 2021; 23:351.
- 446 19. Grune T, Ott C, Häseli S, Höhn A, Jung T: The "MYOCYTER" Convert cellular
 447 and cardiac contractions into numbers with ImageJ. Sci Rep Nature Publishing
 448 Group, 2019; 9:15112.
- Rebuzzini P, Fassina L, Mulas F, et al.: Mouse embryonic stem cells irradiated
 with γ-rays differentiate into cardiomyocytes but with altered contractile
 properties. Mutat Res 2013; 756:37–45.
- Sottas V, Wahl C-M, Trache MC, et al.: Improving electrical properties of iPSC cardiomyocytes by enhancing Cx43 expression. Journal of Molecular and Cellular
 Cardiology 2018; 120:31–41.
- 455 22. Glover D, Little JB, Lavin MF, Gueven N: Low dose ionizing radiation-induced
 456 activation of connexin 43 expression. International Journal of Radiation Biology
 457 Taylor & Francis, 2003; 79:955–964.
- Banaz-Yaşar F, Tischka R, Iliakis G, Winterhager E, Gellhaus A: Cell Line
 Specific Modulation of Connexin43 Expression after Exposure to Ionizing
 Radiation. Cell Communication & Adhesion Taylor & Francis, 2005; 12:249–259.
- 461 24. Azzam EI, de Toledo SM, Little JB: Expression of CONNEXIN43 is highly
 462 sensitive to ionizing radiation and other environmental stresses. Cancer Res
 463 2003; 63:7128–7135.

- 464 25. Gutstein DE, Morley GE, Tamaddon H, et al.: Conduction slowing and sudden
 465 arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ
 466 Res 2001; 88:333–339.
- 467 26. Kim W, Lee S, Seo D, et al.: Cellular Stress Responses in Radiotherapy. Cells
 468 Multidisciplinary Digital Publishing Institute, 2019; 8:1105.
- 469 27. Sag CM, Wolff HA, Neumann K, et al.: Ionizing radiation regulates cardiac Ca
 470 handling via increased ROS and activated CaMKII. Basic Res Cardiol 2013;
 471 108:385.
- 472 28. Holmes JW, Laksman Z, Gepstein L: Making better scar: Emerging approaches
 473 for modifying mechanical and electrical properties following infarction and
 474 ablation. Progress in Biophysics and Molecular Biology 2016; 120:134–148.
- 475 29. Anyukhovsky EP, Sosunov EA, Kryukova YN, et al.: Expression of skeletal
 476 muscle sodium channel (Nav1.4) or connexin32 prevents reperfusion arrhythmias
 477 in murine heart. Cardiovasc Res 2011; 89:41–50.
- 478 30. Roell W, Lewalter T, Sasse P, et al.: Engraftment of connexin 43-expressing cells 479 prevents post-infarct arrhythmia. Nature 2007; 450:819–824.
- 480 31. Greener ID, Sasano T, Wan X, et al.: Connexin43 gene transfer reduces
 481 ventricular tachycardia susceptibility after myocardial infarction. J Am Coll Cardiol
 482 2012; 60:1103–1110.
- 483 32. Lau DH, Clausen C, Sosunov EA, et al.: Epicardial border zone overexpression
 484 of skeletal muscle sodium channel SkM1 normalizes activation, preserves
 485 conduction, and suppresses ventricular arrhythmia: an in silico, in vivo, in vitro
 486 study. Circulation 2009; 119:19–27.
- 487 33. Huang S-H, Wu Y-W, Shueng P-W, et al.: Case report: Stereotactic body radiation
 488 therapy with 12 Gy for silencing refractory ventricular tachycardia. Front
 489 Cardiovasc Med 2022; 9:973105.
- 490 34. Takami M, Hara T, Okimoto T, et al.: Electrophysiological and Pathological Impact
 491 of Medium-Dose External Carbon Ion and Proton Beam Radiation on the Left
 492 Ventricle in an Animal Model. J Am Heart Assoc 2021; 10:e019687.
- Rapp F, Simoniello P, Wiedemann J, et al.: Biological Cardiac Tissue Effects of
 High-Energy Heavy Ions Investigation for Myocardial Ablation. Scientific
 Reports Nature Publishing Group, 2019; 9:5000.

- **8. Figures**
- 499 Figure 1













533 9. Figure legends

Graphical abstract: Overview of methods and major findings affecting the cellular
 electrophysiology 96h after 20Gy photon irradiation.

Fig.1: Irradiated NRVC monolayers showed significantly increased conduction 536 velocity and increased beating frequency. A-F: Optical voltage mapping 537 experiments of control and irradiated monolayers. (A, D) Conduction velocity (CV), (B, 538 E) action potential duration at 50% repolarisation (APD₅₀), and (C, F) action potential 539 duration at 90% repolarization (APD₉₀) of control (Ctrl) and irradiated monolayers 24h 540 (A-C) or 96h (D-F) after irradiation (Rx) (Ctrl, n=5 and 6, resepctively; Rx, n=6) (G-H: 541 Video Analysis of beating frequency using the Macro Myocyter for ImageJ with (G) 542 representative contraction curves of a control and an irradiated cardiomyocyte 96h 543 after Rx and (H) analysis of beating frequency before Rx (Ctrl *n*=6; Rx, *n*=8), 24h after 544 Rx (n=6, each) and 96h after Rx (Ctrl n=8; Rx, n=7). Data are shown as box and 545 whisker plots. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). 546

Fig.2: Radiation-induced ion channel remodeling 24 and 96h after Rx. (A) mRNA transcripts of ion channels of the ventricular action potential 24 and 96h after Rx (n=6, each). Representative western blots of ion channels 96 h after Rx compared to Ctrl and corresponding analysis of optical density are shown (n=6, each; B and C). Data are represented as box and whisker plots. (*p<0.05, **p<0.01, ***p<0.001).

Fig.3: Irradiation increased cardiac cell-to-cell coupling. (A) RT-qPCRs 24 and 96h after Rx (n=12, each). Western blot results 24 (B) and 96h after Rx (C) (n=6, each). (D) Quantification of the Cx43-area occupied relative to the membrane contact area 96h after Rx (Ctrl, n=113; Rx n=115). (E) Representative immunofluorescent stainings of Cx43 (red), cTnT (green) and DAPI (blue). A white box indicates the

⁵⁵⁷ location of Cx43 at the membrane contact area between neighbouring cells (scale ⁵⁵⁸ bar=25µm). (E) Representative FRAP experiments with control and irradiated ⁵⁵⁹ monolayers 96h after Rx (SB=25µm). (F) Average time courses of FRAP and (G) ⁵⁶⁰ analysis of percent recovery 96h after Rx (Ctrl, n=23; Rx, n=15). Data are shown as ⁵⁶¹ box and whisker plots. (*p<0.05, ***p<0.001, ****p<0.0001).

Fig.4: Calcium transient analysis 24 and 96h after Rx. (A) and (G): Representative traces of control (black) and irradiated monolayers (red) 24 (A) and 96h after Rx (G). Analysis of diastolic calcium, peak amplitude, time-to-peak (TTP), full duration at half maximum (FDHM) and decay 24h after Rx (Ctrl, n=31; Rx, n=37). Analysis of diastolic calcium, peak amplitude, TTP, FDHM and decay 96h after Rx (Ctrl, n=39; Rx, n=52). Data are shown as box and whisker plots. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Fig.5: RT-qPCR and western blotting results of calcium handling proteins. (A) Changes in the mRNA transcripts of RYR2, SERCA (Atp2a1), NCX (Slc8a1), PLN and CaMKIId and CaMKIIy at 24 and at 96h after Rx (n=6, each). (B) Western blotting of NCX and PLN 96h after Rx (n=6, each). (C) Western blotting of CaMKII δ and CaMKII γ 96 h after Rx (n=6, each). Data are shown as box and whisker plots. (*p<0.05, **p<0.01, ***p<0.001).

Fig.6: Irradiation did not affect structural features of cardiac fibroblasts in mixed cardiomyocyte and fibroblast cultures. Western blotting of α -SMA and vimentin (Vim) 96h after Rx with respective blots shown and analysis of optical density (n=6, each). Data are shown as box and whisker plots relative to Ctrl.

579

1 Supplemental Material

Cardiac stereotactic body radiotherapy to treat malignant ventricular arrhythmias directly affects the cardiomyocyte electrophysiology

4

Authors: Christine Mages, MD ^{a,b,c,d#}, Heike Gampp ^{a,b#}, Ann-Kathrin Rahm, MD ^{a,b,c,d},
Juline Hackbarth ^{a,b}, Julia Pfeiffer ^{a,b}, Finn Petersenn ^{a,b}, Xenia Kramp ^{a,b}, Fatemeh
Kermani ^e, Juan Zhang, PhD ^f, Daniel Pijnappels, PhD ^f, Antoine A. F de Vries, PhD ^f,
Katharina Seidensaal, MD ^{g,h}, Bernhard Rhein, PhD ^{g,h}, Jürgen Debus, MD ^{g,h}, Nina
D. Ullrich, PhD ^{c,e,i,}, Norbert Frey, MD ^{a,b,c,d}, Dierk Thomas, MD ^{a,b,c}, Patrick Lugenbiel,
MD ^{a,b,c*}

11

¹² ^aDepartment of Cardiology, University Hospital Heidelberg, Heidelberg, Germany;

^bHeidelberg Center for Heart Rhythm Disorders (HCR), University Hospital
 Heidelberg, Heidelberg, Germany;

¹⁵ ^cGerman Centre for Cardiovascular Research (DZHK), Partner site
 ¹⁶ Heidelberg/Mannheim, University of Heidelberg, Heidelberg, Germany;

¹⁷ ^dInformatics4Life Consortium (Institute for Informatics Heidelberg), Heidelberg,
 ¹⁸ Germany;

^eDivision of Cardiovascular Physiology, Institute of Physiology and Pathophysiology,
 Heidelberg, Germany;

fDepartment of Cardiology, Leiden University Medical Center, Leiden, the Netherlands

^gDepartment of Radiation Oncology, Heidelberg University Hospital, Heidelberg,
 Germany;

^hHeidelberg Institute of Radiation Oncology (HIRO), Heidelberg, Germany;

²⁶ ⁱDepartment of Physiology, University of Bern, Switzerland

27

28 # authors contributed equally

*Address for correspondence: Patrick Lugenbiel, MD, FEHRA; Department of 30 Cardiology, Heidelberg University, Im Neuenheimer Feld 410, 69120 Heidelberg, 31 Germany: Tel.: ++496221 568855; Fax: ++49 32 6221 565514; E-Mail: patrick.lugenbiel@med.uni-heidelberg.de 33

- 34 Supplemental Methods
- 35

36 Isolation of Neonatal Rat Ventricular Cardiomyocytes (NRVCs) and Cell Culture

Primary cardiomyocytes were isolated from the hearts of 1- to 3-day old neonatal 37 Wistar rats. Animal housing conditions and experimental procedures were performed 38 in compliance with the German Law for the Protection and Use of Laboratory Animals. 39 Rats were decapitated and the hearts dissected and washed in ice-cold Hank's 40 balanced salt solution. Atria and large vessels were removed, ventricles were cut into 41 small pieces and digested using DNAse II (Sigma-Aldrich, St. Louis, MO) and trypsin 42 (Thermo Fisher Scientific, Waltham, MA), performing eight 10-min digestion steps at 43 37°C. The supernatant was collected in fetal bovine serum (FBS) after each step, 44 pooled and centrifuged to separate the cells from remaining pieces of tissue. The cells 45 were then resuspended in Dulbecco's modified Eagle medium (DMEM/F-12; 11039, 46 Thermo Fisher Scientific) supplemented with 10% FBS (10270, Thermo Fisher 47 Scientific), 1% penicillin/streptomycin (15140122, Thermo Fisher Scientific) and 1x L-48 glutamine (25030024, Thermo Fisher Scientific). A two-layer Percoll gradient was 49 used to separate non-cardiomyocytes from cardiomyocytes. Collected cells were 50 passed through a cell strainer (40µm, BD Falcon, Franklin Lakes, NJ) and then seeded 51 52 into wells of 6-well cell culture plates (Thermo Fisher Scientific) coated with 0.02% gelatine (Thermo Fisher Scientific). For fluorescence recovery after photobleaching 53 (FRAP), patch clamp and calcium transient experiments, cells were seeded on glass-54

bottom dishes (Mattek, Ashland, OR) coated with 0.02% gelatine. The NRVCs were
cultured in supplemented DMEM/F-12 as mentioned above at 37°C in 95% humidified
air with 5% CO2. NRVCs showed spontaneous contraction after 24h in culture, at
which time point the culture medium was replaced with fresh supplemented DMEM/F12. NRVCs were cultured for a total of 6 days.

60

61 **Photon Irradiation**

NRVCs were subjected to 20Gy single-fraction photon irradiation on day 2 of cell
culture using a biological cabinet X-ray irradiator X-RAD 320 (Precision X-Ray, North
Branford, CT) at 320 keV and a dose rate of 500 cGy/min.

65

66 **Optical Voltage Mapping**

To assess the dynamics of action potential propagation in NRVC monolayers, optical 67 mapping experiments were conducted. Using a fast and highly sensitive camera 68 system MICAM Ultima - L (SciMedia USA, Costa Mesa, CA) and BrainVision Analyzer 69 70 version 16.04.20 software (Brainvision, Tokyo, Japan), the propagation of electrical action potential in the cell cultures was recorded, visualized and analyzed. Cells were 71 seeded in gelatin-coated 6-well plates at a density of 10⁶ cells/well. The monolayers 72 were incubated with 8µM of the voltage-sensitive fluorescent dye di-4-ANEPPS 73 (D1199, Thermo Fisher Scientific) in DMEM/F-12 for 10 min in an incubator at 37°C, 74 5% CO₂ and 95% humidified air. Then, fresh DMEM/F-12 medium was applied, and 75 the cells were placed on a constant heating plate at 37°C for the duration of the 76 experiments. 77

Action potential light ($\lambda ex = 525 \pm 25$ nm) was emitted from a halogen arc lamp and 78 delivered to the monolayers via epi-illumination. The fluorescent emission light (λ em 79 >590nm) was first passed through a diverging lens (1x Plan-Apo, WD=61.5mm; Leica, 80 Wetzlar, Germany) and then a dichroic mirror and eventually focused on a 100×100 81 pixels complementary metal oxide semiconductor camera (MiCAM05-Ultima, 82 SciMedia) through a 2x converging lens. The cells were electrically stimulated for the 83 84 experiments at a frequency of 1Hz with an epoxy resin-coated bipolar platinum electrode (pulse strength 8V, pulse duration 10ms). The electrode was connected to 85 a stimulus generator STG 2004 (Multi Channel Systems, Reutlingen, Germany) 86 controlled by the software MC Stimulus II (v3.5.0, Multi-Channel Systems). The signals 87 were acquired at a spatial resolution of 205 µm/pixel. The velocity of propagation and 88 action potential characteristics were recorded during electrical stimulation at 1Hz. 89 Signals were averaged with those from the 8 nearest pixels to minimize noise 90 artefacts. The conduction velocity was analyzed, and action potential duration (APD) 91 was calculated at 50% (APD₅₀) and 90% (APD₉₀) of repolarization. Statistical analysis 92 was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, 93 CA). 94

95 Video Analysis of Beat Frequency

Beat frequency in recorded videos of cardiomyocytes was measured using the recently developed macro Myocyter (version 1.0), an analytical software tool for the ImageJ (version 1.52b) image processing software.¹ By scaling the time-dependent changes in pixel intensity in successive video images of recorded cardiomyocytes, Myocyter allowed visualization of cellular contractility. Spontaneous contractions of neonatal cardiomyocytes were recorded using an iPhone XR (Apple, Cupertino, CA)

connected to the eyepiece of a Leica DMi1 microscope via a camera adapter (Bresser,
 Rhede, Germany). Video recordings were done at 60 frames/s for 10-15s. Data
 extraction with Myocyter was performed according to the developer's instructions and
 beat frequency was analyzed using ImageJ 1.50i.²

106

107 RNA Isolation and Reverse Transcription Quantitative Real-Time PCR (RT-108 gPCR)

RNA isolation and RT-qPCR were performed as published elsewhere.³ Briefly, RNA 109 was isolated using QIAZol Lysis Reagent (Qiagen, Hilden, Germany) according to the 110 manufacturer's instructions. RNA concentration was determined using a NanoDrop 111 2000/2000c spectrophotometer (Thermo Fisher Scientific) at a wavelength of λ =260 112 nm. The isolated RNA (3µg) was converted into DNA using the Maxima First Strand 113 cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the 114 manufacturer's instructions. RT-qPCR was performed using the StepOnePlus PCR 115 System (Thermo Fisher Scientific) and TaqMan Gene Expression Assay primers 116 (Applied Biosystems, Foster City, CA; see Supplemental Table 1). Normalization was 117 done using primers and probes for the housekeeping enzyme glyceraldehyde-3-118 phosphate dehydrogenase (GAPDH) using delta-delta-Ct method. All RT-qPCR 119 reactions were performed in triplicate or a higher number of replicates, and a non-120 template control and dilution series were included on each plate for quantification. 121 Data are expressed as the average of triplicates. All measurements were adjusted 122 using a standard probe, and quantification was corrected for the amplification 123 efficiency derived from the standard curves. 124

125

126 Protein Isolation and Western Blot Analysis

Proteins were extracted from NRVC and protein immunodetection was performed by 127 sodium dodecyl sulfate (SDS)-polyacrylamide (PAA) gel electrophoresis and western 128 blotting as previously reported.³ Proteins were extracted in 20mM Tris-HCl, 0.5% 129 Nonidet P-40, 0.5% sodium deoxycholate, 150mM NaCl, 1 mM EDTA, 1mM Na₃VO₄, 130 1mM NaF and inhibitors of proteases (cOmplete, Mini Protease Inhibitor Cocktail; 131 Roche Applied Science, Indianapolis, IN). The resulting samples were centrifuged at 132 4° C for 30 min and $14,000 \times q$, and the protein concentration in the supernatants was 133 determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific). Equal 134 amounts of total protein were separated on SDS- 7.5-15% PAA gels and transferred 135 to nitrocellulose membranes for 2h at 200mV (Amersham Protran 0.45 NC; GE 136 Healthcare Life Sciences, Freiburg, Germany). After blocking in PBST containing 5% 137 non-fat milk for 2h at room temperature (RT), membranes were incubated overnight 138 with the primary antibodies listed in Supplemental Table 2. Next, the membranes were 139 incubated with matching horseradish peroxidase (HRP)-conjugated goat anti-rabbit 140 secondary antibody (ab6802, Abcam, Cambridge, United Kingdom), HRP- conjugated 141 goat anti-mouse IgG (H+L) secondary antibody (1031-05, Southern Biotech, 142 Birmingham, AL) or HRP-conjugated goat anti-mouse IgG1 cross-adsorbed 143 secondary antibody (A10551, Thermo Fisher Scientific,). Signals were developed with 144 the enhanced chemiluminescence detection reagent (ECL Western blotting Reagents; 145 GE Healthcare, Buckinghamshire, United Kingdom). GAPDH was used as an internal 146 control. Quantification of optical density was performed with ImageJ 1.50i software. 147

148

150 **FRAP**

FRAP was assessed as an indicator of gap junctional coupling efficiency. 151 Photobleaching and imaging were performed on an Olympus FluoView confocal laser 152 scanning microscopy (Olympus Corporation, Tokyo, Japan) using a 60x water 153 immersion objective (1.2 NA). NRVC monolayers seeded on glass-bottom dishes were 154 incubated for 20 min in the dark with 500µl of 0.5µM calcein-AM (Thermo Fisher 155 Scientific) in Tyrode's solution. Calcein-AM was converted to green fluorescent calcein 156 by intracellular esterases. After de-esterification, cells were washed with prewarmed 157 Tyrode's solution for 10 min. Afterwards, the dye was no longer able to diffuse through 158 the plasma membrane and could only leave cells through gap junctions. One 159 cardiomyocyte within a monolayer was bleached with a laser power of 50% at 10 160 µs/pixel for 5s. Calcein diffusion from neighbouring cells into the bleached cell was 161 measured over time and recovery of fluorescence was recorded in 50 images taken 162 every 10s with a laser power of 0.5% at 2µs/pixel. Analysis was done in ImageJ to plot 163 the time course of fluorescence recovery. After subtraction of background 164 fluorescence and bleaching area, the plot was normalized to the fluorescence intensity 165 before bleaching. Parameters were analysed using OriginLab software (OriginLab 166 Corporation, Northampton, MA) and ImageJ 1.50i software. 167

168 Immunofluorescence Analysis

NRVCs on 20×20mm glass coverslips were washed with cold phosphate-buffered saline (PBS) for three times, fixed with 4% paraformaldehyde solution in PBS (Roti-Histofix, Carl Roth, Karlsruhe, Germany) for 10 min at RT. Cells were permeabilized with PBS containing 0.1% Triton X-100 (Merck, Darmstadt, Germany) for 3 min, followed by a blocking step with 5% Gibco normal goat serum (Thermo Fisher

Scientific) and 0.1% Triton X-100 in PBS for 2h. Cells were incubated overnight with 174 the following primary antibodies: rabbit polyclonal to connexin 43 (Cx43), mouse 175 monoclonal to cardiac troponin T (cTnT), rabbit polyclonal to cTnT and mouse 176 monoclonal to vimentin. Next, cells were incubated with matching secondary 177 antibodies conjugated to Alexa Fluor dyes with different excitation-emission spectra 178 for 2h at RT and protected from light, using Alexa Fluor 568-conjugated donkey-anti-179 180 rabbit IgG (H+L) (A11057, diluted 1:1000; Thermo Fisher Scientific) or Alexa Fluor 488-conjugated goat-anti-mouse IgG (H+L) (A32723; diluted 1:1000, Thermo Fisher 181 182 Scientific). All antibodies were diluted in blocking buffer. Cells were mounted with Fluoroshield (4`,6-diamidino-2-phenylindol (Sigma-Aldrich containing DAPI 183 dihydrochloride (Abcam) for nuclear staining. Laser-scanning confocal imaging was 184 used to estimate the expression of Cx43. Images were taken on a Leica SP8 confocal 185 microscope with a 63x oil immersion objective and analyzed using the ImageJ 1.50i 186 software. All immunofluorescence images were taken at the same light intensity and 187 photodetector setting to ensure a fair comparison. Cell surface Cx43 levels were 188 expressed as the ratio of the area of Cx43 plaque to the intercellular border area. 189

190 Calcium Transient Measurements

Calcium transients of NRVCs were measured with the calcium- sensitive fluorescent dye fura-2-AM (Thermo Fisher Scientific). NRVCs seeded on glass-bottom dishes were loaded with 1.5µM of fura-2-AM in Tyrode's solution and incubated for 20 min, followed by washing with Tyrode's solution and waiting 10 min for de-esterification. Cells were continuously perfused with pre-heated Tyrode's solution containing Probenecid (100µM; Sigma Aldrich) to prevent secretion of fura-2. Calcium transients were recorded using an IonOptix system (IonOptix, Dublin, Ireland). NRVCs were

exposed to light from a xenon lamp passing through fast-switching filters of 340nm 198 and 380nm to determine the ratio of bound and unbound calcium ions in the cells. 199 Fluorescence emission light was collected at 510nm. Data are presented as fura-2 200 ratio (F340/F380nm) and collected using the IonWizard software developed by 201 IonOptix. Three representative calcium transients were analysed per recording using 202 OriginPro software. Assessed parameters comprised diastolic calcium level, 203 204 amplitude, time-to-peak, full duration at half maximum and decay. Decay of calcium transients was calculated using an exponential decay function. 205

206

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red of 209 Roche Applied Science according to the manufacturer's protocol. Cells on glass 210 coverslips were washed three times with PBS and then fixed with 4% buffered 211 paraformaldehyde for 10 min. After another washing step, the cells were 212 permeabilized by incubation with 0.1% Triton- X100 in PBS for 2 min at 4°C. The 213 positive control consisted of permeabilized cells treated for 10 min at RT with 214 recombinant DNase I (3U/ml in 50 mM Tris-HCI (pH 7.5); Roche, Mannheim, 215 Germany), 1mg/ml bovine serum albumin) to induce double-strand breaks. Next, 5µl 216 Enzyme Solution and 45µl Label Solution were mixed and added to each coverslip. 217 Instead of the reaction mix, 50µl Label Solution was added to the negative control 218 219 sample. After incubation for 1h in a humidified dark chamber at 37°C, the coverslips were washing three times with PBS covered with antifade containing For 220 immunofluorescent stainings evaluating the proportion of cardiomyocytes and 221

fibroblasts in culture, imaging was performed with an inverted Axio Observer Z1 microscope and an Axiocam 506 camera (Carl Zeiss) Cells were labelled with rabbit polyclonal anti-troponin T antibodies to identify (cardiomyocytes and mouse monoclonal anti-vimentin antibodies to mark cardiac fibroblasts. Secondary antibodies were Alexa Fluor 568-conjugated donkey-anti-rabbit (A11057, diluted 1:1000; Thermo Fisher Scientific) and Alexa Fluor 488-conjugated goat-anti-mouse (A32723; diluted 1:1000, Thermo Fisher).

Immunofluorescence analysis of cell culture was performed with an inverted Axio 229 Observer Z1 microscope (Carl Zeiss, Jena, Germany) and an Axiocam 506 camera 230 (Carl Zeiss). TUNEL-positive cells were counted manually, and ImageJ 1.41 software 231 was used to calculate the total cell number in 3x3 tiles. Excitation wavelengths in the 232 range of 520-560nm (maximum 540nm; green) were used as well as detection 233 wavelengths in the range of 570-620nm (maximum 580nm, red). The percentage of 234 TUNEL-positive cells was calculated by dividing TUNEL-positive cells by total cell 235 count. 236

237 Patch Clamp Recordings of Action Potentials

For action potential recordings, NRVCs were cultured on 35mm glass-bottom dishes 238 at single cell density. Measurements were performed in whole-cell patch-clamp 239 configuration using a HEKA EPC-10 patch clamp amplifier (HEKA Instruments, 240 Holliston, MA) connected to an inverted Olympus IX81 FluoView1000confocal laser 241 scanning microscope (Olympus, Tokyo, Japan) as previously described.² Data were 242 acquired using PatchMaster (HEKA Instruments). Glass pipettes were pulled from 243 borosilicate glass capillaries (GB150-8P; Science Products Hofheim am Taunus, 244 Germany) using a DMZ Universal Puller (Zeitz Instruments, Martinsried, Germany) to 245

achieve pipette resistances of 1.5 - 2.5MΩ. The intracellular solution consisted of 120 mM KCl, 10mM HEPES, 5mM MgCl₂, 5 mM EGTA, 2.5mM Na₂-ATP, and the pH was adjusted to 7.2 using KOH. The external solution contained normal Tyrode (140mM NaCl, 6mM KCl, 1.8mM CaCl₂, 1.1mM MgCl₂, 10mM D-glucose, 10mM HEPES) and the pH was adjusted to 7.4 using NaOH. To determine the action potential stimulation threshold, a series of stepwise increasing stimulation currents (ranging from 100pA to 500nA with a step size of 100pA) was delivered. The first current resulting in an amplitude above the threshold was used to trigger action potentials. Action potential parameters were analyzed using OriginPro software.





Supplemental Figure 1: Irradiation did not impact apoptosis rate in NRVCs. Representative fluorescence stainings of TUNEL assays (scale bar, 100 μ m) at (A) 24h and (C) 96h after radiation (Rx). Red nuclear fluorescence reflects endonucleolytic DNA degradation and apoptosis. Mean apoptosis rates at (B) 24h (*n*=3, each) and (D) 96h after Rx (*n*=3, each). TUNEL (Texas Red, TxR)-positive cells are expressed in relation to the total number of cells. Data are shown as box and whisker plots. Ctrl, control.







Supplemental Figure 3: NRVC to cardiac fibroblast ratio at 24h and 96h after irradiation. Representative stainings of cardiomyocytes (red, cTNT) and fibroblasts (green, vimentin) (A) 24h and (C) 96h after irradiation (Rx). Analysis of the ratio of cells in culture (B) 24h after Rx (Ctrl: n=5, Rx: n=6) and (D) 96h after Rx (n=5, each). Data are shown as box and whisker plots. Ctrl, control.



Supplemental Figure 4: Action potential characteristics of control and irradiated
 cardiomyocytes 24h after Rx. (A): Representative traces of ventricular action
 potentials of control cardiomyocytes (black) and irradiated cardiomyocytes (red) at 24h

- after Rx. (B): Analysis of resting membrane potential (RMP), action potential amplitude
- (APA) and action potential duration at 20%, 50%, 70% and 90% repolarization (APD₂₀,
- APD₅₀, APD₇₀, APD₉₀), n=27, each. Data are shown as box and whisker plots.
- 296 (*p<0.05).
- 297
- 298 **Supplemental Table 1**: PCR primers sorted alphabetically

	ThermoFisher	
	Catalogue	
Primer	Number	Chromosome Location
	Rn00667869_m	Chr.12: 13715843 - 13718813 on Build
rActb	1	Rnor_6.0
	Rn01508014_m	Chr.1: 197855912 - 197875038 on Build
rAtp2a1	1	Rnor_6.0
	Rn00709287_m	Chr.4: 150639043 - 151269159 on Build
rCacna1c	1	Rnor_6.0
	Rn00560913_m	Chr.2: 230900907 - 231130336 on Build
rCamkIIδ	1	Rnor_6.0
	Rn01537033_m	
rCamkIIγ	1	Chr.15: 3936714 - 3995740 on Build Rnor_6.0
		Chr.4: 157676396 - 157680271 on Build
rGapdh	Rn01775763_g1	Rnor_6.0
		Chr.20: 37876589 - 37889097 on Build
rGja1	Rn06415115_s1	Rnor_6.0

	Rn04339184_m	Chr.2: 207923775 - 208140727 on Build
rKcnd3	1	Rnor_6.0
	Rn01767120_m	Chr.11: 32498260 - 32508420 on Build
rKcne1	1	Rnor_6.0
	Rn01442522_m	
rKcnh2	1	Chr.4: 7355161 - 7387243 on Build Rnor_6.0
		Chr.10: 47282208 - 47343501 on Build
rKcnj12	Rn02533449_s1	Rnor_6.0
		Chr.10: 99429265 - 99442520 on Build
rKcnj2	Rn00568808_s1	Rnor_6.0
	Rn01492857_m	Chr.4: 176783287 - 176789143 on Build
rKcnj8	1	Rnor_6.0
	Rn00597042_m	Chr.13: 107690111 - 107886476 on Build
rKcnk2	1	Rnor_6.0
	Rn04223042_m	
rKcnk3	1	Chr.6: 27154274 - 27190209 on Build Rnor_6.0
	Rn00583376_m	Chr.1: 216293087 - 216630339 on Build
rKcnq1	1	Rnor_6.0
	Rn01434045_m	Chr.20: 34633157 - 34642904 on Build
rPIn	1	Rnor_6.0
	Rn01470303_m	Chr.17: 65533998 - 65955606 on Build
rRyr2	1	Rnor_6.0
	Rn00565502_m	Chr.8: 128169191 - 128266639 on Build
rScn5a	1	Rnor_6.0

	Rn04338914_m	
rSlc8a1	1	Chr.6: 4245582 - 4564263 on Build Rnor_6.0
	Rn00562059_m	Chr.13: 52662973 - 52680992 on Build
rTnnt2	1	Rnor_6.0

Supplemental Table 2: Western blotting antibodies sorted alphabetically.

Primary Antibody	Class	Host	Dilution	Company
			.0	Invitrogen,
Anti-alpha-smooth-muscle-		mous		Thermo Fisher
actin (14-9760-82)	monoclonal	е	1:1000	Scientific
Anti-beta actin antibody				
(ab8227)	polyclonal	rabbit	1:3000	Abcam
Anti-CaMKII delta antibody	0			
(ab181052)	polyclonal	rabbit	1:1000	Abcam
Anti-CaMKII gamma antibody				
(ab262701)	polyclonal	rabbit	1:1000	Abcam
				Invitrogen,
Anti-Cav1.2 (CACNA1C)		mous		Thermo Fisher
antibody (MA5-27717)	monoclonal	е	1:1000	Scientific
Anti-Cx43 antibody				
(ab11370)	polyclonal	rabbit	1:3000	Abcam
Anti-GAPDH antibody				
(ab181602)	polyclonal	rabbit	1:10000	Abcam

Anti-KCNK2/TREK-1				
antibody (APC-047)	polyclonal	rabbit	1:1000	Abcam
Anti-Kir2.1 (KCNJ2) antibody				
(APC-026)	polyclonal	rabbit	1:1000	Abcam
Anti-Kir6.1 (KCNJ8) antibody		mous		
(ab241996)	monoclonal	е	1:1000	Abcam
Anti-Kv11.1 (KCNH2)			C	
antibody (APC-062)	polyclonal	rabbit	1:1000	Abcam
Anti-Kv4.3 (KCND3) antibody			0	
(APC-017)	polyclonal	rabbit	1:1000	Abcam
		0		Invitrogen,
Anti-Kv7.1 (KCNQ1) antibody	Q	mous		Thermo Fisher
(MA5-27676)	monoclonal	е	1:1000	Scientific
Anti-Na _v 1.5 (SCN5A) (493-				
511) antibody (ASC-005)	polyclonal	rabbit	1:500	Almone Labs
Anti-NCX (SLC8A1) antibody				
(ASC-005)	polyclonal	rabbit	1:1000	Alomone Labs
				Invitrogen,
Anti-Phospholamban (PLN)		mous		Thermo Fisher
(MA3-922)	monoclonal	е	1:1000	Scientific
Anti- Vimentin (VIM) antibody		mous		
(ab8069)	monoclonal	е	1:1000	Abcam

303 Supplemental references

- 1. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MA,
- 305 https://imagej.net/ij/, 1997-2018.
- 2. Grune T, Ott C, Häseli S, Höhn A, Jung T. The "MYOCYTER" Convert cellular
- and cardiac contractions into numbers with ImageJ. Sci Rep. 2019;9:15112.
- 308 3. Syren P, Rahm A-K, Schweizer PA, et al. Histone deacetylase 2-dependent
- ventricular electrical remodeling in a porcine model of early heart failure. Life Sci.
- 310 2021;281:119769.

Journal Pre-pri