Quaking Inhibits Doxorubicin-Mediated Cardiotoxicity Through Regulation of Cardiac Circular RNA Expression

Shashi Kumar Gupta, Ankita Garg, Christian Bär, Shambhabi Chatterjee, Ariana Foinquinos, Hendrik Milting, Katrin Streckfuß-Bömeke, Jan Fiedler, Thomas Thum

Rationale: RBPs (RNA-binding proteins) have been described to be expressed and regulated in various organs including the heart. Little is known about the role of RBPs in heart failure induced by the chemotherapy drug doxorubicin and their interaction with circular RNAs.

Objective: We aimed to identify key RBPs involved in doxorubicin-mediated heart failure and to elucidate their function.

Methods and Results: Global transcriptome profiling from murine myocardium exposed to doxorubicin identified 5 differentially expressed RBPs. Expression of the RBP QKI1 (Quaking) in response to doxorubicin was strongly downregulated in rodent cardiomyocytes and human induced pluripotent stem cell–derived cardiomyocytes in vitro and in vivo in mice. Knockdown of QKI1 in primary cardiomyocytes increased apoptosis and atrophy after treatment with doxorubicin, whereas lentiviral mediated overexpression of QKI5 inhibited the doxorubicin-induced apoptosis in cardiomyocytes. In vivo, AAV9 (adeno-associated virus serotype 9)–mediated cardiac overexpression of QKI5 prevented cardiac apoptosis and cardiac atrophy induced by doxorubicin and improved cardiac function. Mechanistically, by lentiviral-based overexpression and CRISPR/Cas9-mediated silencing of QKI5, we identified regulated expression of specific circular RNAs derived from TTN (Titin), Fhod3 (Formin homology 2 domain containing 3), and Strn3 (Striatin, calmodulin-binding protein 3). Moreover, inhibition of TTN-derived circular RNA increased the susceptibility of cardiomyocytes to doxorubicin.

Conclusions: We here show that overexpression of QKI5 strongly attenuates the toxic effect of doxorubicin by regulating a set of circular RNAs. QKI5 is, thus, an interesting target molecule to combat doxorubicin-induced cardiotoxicity. (Circ Res. 2018;122:246-254. DOI: 10.1161/CIRCRESAHA.117.311335.)

Key Words: cardiotoxicity ■ doxorubicin ■ heart failure ■ noncoding RNA ■ RNA-binding protein

Cancer is a major public health concern worldwide, and its incidence is projected to rise because of an increasing age of the population. Chemotherapy drugs such as anthracyclines are often associated with cardiotoxicity leading to heart failure.1 In a retrospective analysis of 3 trials involving doxorubicin (an anthracycline class drug) treatment, 5%, 16%, and 26% of patients developed doxorubicin-mediated congestive heart failure at a cumulative dose of 400, 500, and 550 mg/m², respectively.2 Despite its toxic effects, doxorubicin is frequently used in the clinics because of unavailability of any superior therapy. Therefore, there is an immediate need for the development of therapeutic strategies aiming at the reduction of doxorubicin-related cardiotoxicity without compromising its therapeutic function.

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RNA, with the exception of tRNA and rRNA, not only was previously known to mainly serve as template for protein synthesis but also has regulatory function mainly through action of noncoding RNA species.3 Circular RNAs, a class of noncoding RNAs formed by back-splicing of exons, have recently been

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identified in eukaryotes with the help of deep sequencing and novel bioinformatics pipelines. Several reports demonstrate the presence of circular RNA molecules in various organs and cells including the heart. Several cardiac-specific genes like Titin and Ryanodine receptor 2 have been shown to form circular RNAs, a probable indication for a potential functional role. Recently, noncoding RNA (circular RNA) processing and function are dependent on RBPs (RNA-binding proteins).

Here, using a broad transcriptomic approach, we identified a RNA-binding protein known as Quaking to play an important role in doxorubicin-induced heart failure. Quaking was found to be downregulated in hearts exposed to doxorubicin. The Quaking isoform Qki5 was associated with expression of circular RNAs derived from Titin, Ryanodine receptor 2, and calmodulin-binding protein 3-derived circular RNAs in cardiomyocytes. AAV9 (adeno-associated virus serotype 9)-mediated overexpression of Qki5 attenuated doxorubicin-induced cardiomyocyte apoptosis and atrophy. Our work highlights potential use of RBPs as a novel therapy for doxorubicin-induced heart failure. Additionally, our work implies circular RNAs as mediators in doxorubicin-induced cardiotoxicity and paves way for future studies focusing on the role of circular RNAs in cardiovascular pathophysiology.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Acronym | Description |
|--------------|---------|-------------|
| AAV          | pLV     | adeno-associa ted virus plasmid Lentivirus |
| RBP          | QKI     | RNA-binding protein Quaking |
| TUNEL        |         | terminal deoxynucleotidyl transferase dUTP nick end labeling |

Statistics

All data were analyzed using GraphPad Prism software. Data are presented as mean±SEM, and an unpaired 2-tailed t test was performed to calculate significance between 2 groups, and 1-way ANOVA with post hoc Tukey test was used to calculate significance difference between 2 groups wherever required. Detailed method section can be found in the Online Data Supplement.

Results

Doxorubicin Downregulates Quaking Levels

We established an in vivo mouse model of doxorubicin-induced cardiotoxicity where we injected mice with doxorubicin (5 mg/kg) weekly for 5 consecutive weeks followed by scarification 1 week later (Online Figure IA). These mice showed clear signs of cardiac dysfunction.

Cancer survivors are prone to develop heart failure because of cardiotoxic side effects of chemotherapy drugs, like doxorubicin. Despite the presence of several RBPs in the heart, their involvement in doxorubicin-induced heart failure is not known. Here, using a broad transcriptomic approach, we identified a RBP Quaking to play an important role in doxorubicin-induced heart failure. Quaking was found to be downregulated in hearts exposed to doxorubicin. The Quaking isoform Qki5 was associated with expression of circular RNAs derived from Titin, Ryanodine receptor 2, and calmodulin-binding protein 3-derived circular RNAs in cardiomyocytes. AAV9 (adeno-associated virus serotype 9)-mediated overexpression of Qki5 attenuated doxorubicin-induced cardiomyocyte apoptosis and atrophy.

What New Information Does This Article Contribute?

- Treatment with doxorubicin is associated with reduced levels of RBP Quaking.
- Quaking expression was associated with expression of Titin (Titin)-, Ryanodine receptor 2 (Ryanodine receptor 2)-, Strn3 (Strn3)-, and Fhod3 (Fhod3)-derived circular RNAs in cardiomyocytes.
- AAV9 (adeno-associated virus serotype 9)-mediated overexpression of Qki5 attenuated doxorubicin-induced cardiomyocyte apoptosis, atrophy, and improved cardiac function.

What Is Known?

- Doxorubicin, a commonly used chemotherapy drug, induces myocardial apoptosis and atrophy leading to cardiac malfunction, which limit its effective use in many cancer patients.
- RBPs (RNA-binding proteins) are known to play a crucial role in cardiac disease and development.
- Quaking (Qki5) is an RBP that inhibits apoptosis induced by myocardial ischemia–reperfusion injury and regulates circular RNA formation during epithelial–mesenchymal transition.

Novelty and Significance

Our work highlights potential use of RBPs as a novel therapy for doxorubicin-induced heart failure. Additionally, our work implies circular RNAs as mediators in doxorubicin-induced cardiotoxicity and paves way for future studies focusing on the role of circular RNAs in cardiovascular pathophysiology.

Methods

The authors declare that all supporting data are available within the article (and its Online Data Supplement).

Animal Model

C57BL/6 N mice of 10 to 12 weeks of age were injected (intraperitoneally) with doxorubicin at a dose of 5 mg/kg once a week for consecutive 5 weeks. One week later after the last doxorubicin treatment, echocardiography data were recorded by an independent blinded researcher using the Vevo 2100 system (Fujifilm Visulasonics, Inc). Mice were euthanized, and hearts were harvested and processed for further molecular and cellular assays. Echocardiography data were analyzed using standard imaging protocols (M-mode and B-mode) for global cardiac volumes and functioning using the Vevostrain software (Fujifilm Visulasonics, Inc). Animal experiments were approved by the local authorities at Hannover Medical School and Niedersachsen Landesamt für Verbraucherschutz. AAV9 (adeno-associated virus serotype 9) was injected (intravenously) 1 week before first doxorubicin injection. Animal experiments were randomized and blinded with an internal number.

Statistics

All data were analyzed using GraphPad Prism software. Data are presented as mean±SEM, and an unpaired 2-tailed t test was performed to calculate significance between 2 groups, and 1-way ANOVA with post hoc Tukey test was used to calculate significance difference between ≥2 groups wherever required. Detailed method section can be found in the Online Data Supplement.
of cardiac atrophy including reductions in heart weight to tibia length ratio, smaller cardiomyocyte cell sizes, and a significant decline in cardiac function as measured by echocardiography (Online Figure IB through ID; Online Table I). Electron microscopy confirmed myocardial damage as seen by less dense and destroyed myofibers in hearts of mice treated with doxorubicin (Online Figure IE). Global transcriptome profiling in cardiac tissue of doxorubicin-treated mice resulted in 113 differentially expressed mRNAs compared with vehicle control (Figure 1A; Online Table II). Because our aim was to identify RBPs involved in doxorubicin-induced cardiotoxicity, we compared the differentially expressed candidates with the complete list of mouse RBPs derived from the RBPDB (RNA-Binding Protein Database). Comparative analysis identified 5 RBPs among the dysregulated candidates. Cirbp, Mkm1, Rbm3, and Thumpd1 were found to be upregulated, whereas Qki was downregulated in response to doxorubicin. Qki was chosen for further studies because Qki was reported to inhibit cardiac apoptosis initiated by ischemia–reperfusion, and in addition, it is well known as a tumor suppressor gene. Therefore, we postulated that Qki overexpression would antagonize doxorubicin adverse effects on the myocardium and probably would not interfere with doxorubicin antitumor properties. First, we profiled protein levels of QKI in an organ panel of murine tissues and found abundant

Figure 1. Doxorubicin treatment reduced Qki (Quaking) expression. A, Heatmap showing differentially expressed mRNAs in mouse myocardium treated with vehicle control or doxorubicin (n=3 each). Qki protein (B) and mRNA (C) levels in the myocardium of mice that had received doxorubicin compared with vehicle control (B) n=10 vehicle and 15 doxorubicin and (C) n=9 vehicle and 9 doxorubicin). D, Expression of QKI in human failing heart compared with nonfailing heart (n=10 nonfailing and 20 failing). Doxorubicin (0.1 µmol/L) treatment for 72 h in HL-1 (E), neonatal rat cardiomyocytes (F), and human induced pluripotent stem cell–derived cardiomyocytes (G) significantly decreases Qki protein levels. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
expression of QKI in brain followed by the heart (Online Figure IIA). Furthermore, we investigated expression of Qki mRNA in various cardiac cell types and found Qki5 to be the most abundant isoform in the heart with highest expression in cardiomyocytes (Online Figure IIB). Similar to Qki5, Qki6 was also enriched in cardiomyocyte fraction, whereas Qki7 showed similar expression profiles in cardiomyocytes, fibroblasts, and endothelial cells (Online Figure II). Next, we validated our transcriptome approach and confirmed decreased protein and mRNA levels of all Quaking isoforms (Qki5, Qki6, and Qki7) in the myocardium after doxorubicin treatment (Figure 1B and 1C). Additionally, QKI mRNA was also found significantly downregulated in human failing compared with nonfailing hearts (Figure 1D). To study the significance of Qki on doxorubicin-induced cardiotoxicity and apoptosis, we treated HL-1, a mouse cardiomyocyte cell line, primary neonatal rat cardiomyocytes and human induced pluripotent stem cell–derived cardiomyocytes with doxorubicin. QKI levels were found to be significantly downregulated on treatment with doxorubicin in the HL-1 cardiomyocytes (Figure 1E), in primary rat cardiomyocytes (Figure 1F), and in human induced pluripotent stem cell–derived cardiomyocytes (Figure 1G). These results suggest an important role for quaking in cardiac pathophysiology.

**Quaking Exerts Protective Effects in Response to Doxorubicin**

To next test a potential functional role of Qki in cardiomyocytes, we used siRNAs to specifically knockdown Qki in primary rat cardiomyocytes (Online Figure IIIA). Qki silencing further induced apoptosis measured by an increased percentage of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive cardiomyocyte nuclei (Figure 2A and 2C) and led to increased cellular atrophy (Figure 2B and 2C) in the presence of doxorubicin. Furthermore, an increased caspase 3/7 activity was found in primary rat cardiomyocytes and H9C2 (rat myoblast cell line) cells after siRNA-mediated Qki inhibition compared with control in the presence of doxorubicin (Figure 2D: Online Figure IIIIB). Because Qki was decreased on doxorubicin treatment and further inhibition of Qki increased susceptibility toward doxorubicin, we hypothesized that overexpression of Qki may inhibit cardiomyocyte apoptosis induced by doxorubicin. To test this, we generated HL-1 cell lines with specific overexpression of quaking isoforms (Qki5, Qki6, and Qki7) that are present in mice (Figure 2E) and subjected them to doxorubicin treatment. Doxorubicin treatment strongly increased apoptosis as measured by caspase 3/7 activity in control (pLV [plasmid Lentivirus] empty) cells, whereas apoptosis was significantly attenuated with Qki5 overexpression, although little or no effects was seen with overexpression of Qki6 or Qki7, respectively (Figure 2F). Additionally, we also performed TUNEL staining in HL-1 cells and found a decrease in the Qki5-overexpressing cell lines on treatment with doxorubicin compared with controls (Figure 2G and 2H). In line, an improved survival in Qki5-overexpressing cells in response to doxorubicin was observed (Figure 2I). Similarly, apoptosis staining by annexin V and 7-AAD (7-aminoactinomycin D) showed a trend for an inhibitory effect of Qki5 on doxorubicin-induced apoptosis (Online Figure IIC and IID). Furthermore, AAV2 (adeno-associated virus serotype 2)-mediated Qki5 overexpression in vitro (Online Figure IIIA) also attenuated doxorubicin-induced cardiac atrophy in neonatal rat cardiomyocytes (Online Figure IIIE and IIII). These results indicated a protective effect of Qki5 in doxorubicin-induced cardiotoxicity.

**Quaking Regulates Cardiac Circular RNAs**

Previously, Qki was reported to regulate the formation of circular RNAs during epithelial to mesenchymal transition. Therefore, we decided to screen circular RNAs regulated by Qki in the heart to gain further mechanistic insights for the observed protective effect of Qki5. We selected a panel of highly expressed cardiac circular RNAs that we derived from previously published RNA-Seq data sets. An outline for our selection and filtering strategy is presented in Figure 3A. Details of the selected circular RNAs are presented in Online Table III. Selected circular RNAs were profiled in control (pLV empty) and pLV Qki5-overexpressing cell lines for their expression (Online Figure IVA). Additionally, we constructed a Qki knockdown HL-1 cell line with the CRISPR/Cas9 method (Online Figure IVB) and compared the expression of circular RNAs in 2 different knockdown cell lines (3+4 and 5+6 guide RNA combination) with the control (empty) cell line (Online Figure IVA). Candidate circular RNAs (Ttn [Titin] 105–111, Fhod3 [Foramin homology 2 domain containing 3], Strn3 2–7 [Striatin, calmodulin-binding protein 3], Arhgap32, Camsap1, Tic2 2–10, Gigyf, Slc8a1, and Hipk3 [Gene Name, exon number], which showed a reciprocal regulation on overexpression of Qki5 compared with CRISPR-mediated silencing of Qki were further investigated. We validated circular RNAs derived from the genes Tin, Fhod3, and Strn3 to be positively regulated with Qki expression, whereas Arhgap32 was negatively regulated (Figure 3B). Thus, these results demonstrate that Qki regulates the expression of cardiac circular RNAs that may serve as potential downstream mediators for the observed protective Qki5 effects. Next, we studied whether these circular RNAs would also be directly regulated by doxorubicin and indeed found that all of the circular RNAs were significantly downregulated with doxorubicin treatment in HL-1 cells (Figure 3C). Circular RNAs from Tin, Fhod3, and Strn3 were also downregulated in mouse hearts, which had doxorubicin-induced reductions in Qki levels, showing Qki as a likely upstream regulator (Figure 3D). Higher resistant to RNase R digestion before reverse transcription and polymerase chain reaction amplification confirmed that these candidates were indeed circular RNAs, whereas linear Hprt (hypoxanthine guanine phosphoribosyl transferase) was completely digested with RNase R treatment (Figure 3E). Furthermore, to confirm that these circular RNAs are downstream mediators of the Qki5 protective effect, we evaluated their expression levels in pLV Qki5-overexpressing cells compared with control (pLV empty) cells in the presence of doxorubicin. Similar to previous results, Tin 105–111, Fhod3, and Strn3 2–7 were downregulated in control (pLV empty) cells exposed to doxorubicin, whereas this reduction was totally absent in pLV Qki5-overexpressing cells (Figure 3F). We also checked the expression of these circular RNAs in Qki6 and Qki7 overexpression cell lines but only observed a modest increase confirming them to be specific targets of Qki5 (Online Figure IVC). Next, we inhibited Tin 105–111 circular RNA by a specific siRNA approach (Figure 3G) and
evaluated its effect on doxorubicin-induced apoptosis. SiRNA-mediated inhibition of Ttn 105–111 led to increased susceptibility to doxorubicin as evident by higher caspase activity (Figure 3H). Additionally, the knockdown of Ttn 105–111 in pLV Qki5-overexpressing cell line also resulted in an induced caspase activity (Figure 3I). On the contrary, lentiviral mediated overexpression of circular RNA Ttn 105–111 resulted in lower caspase 3/7 activity and increased survival as seen by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Online Figure IVD though IVF). These results suggest that circular RNAs, especially derived from the titin gene, are downstream mediators of Qki5-mediated protective effects.

**Quaking Protects Hearts From Doxorubicin-Induced Cardiotoxic Effects**

On the basis of our in vitro results, where we identified a beneficial effect of Qki5 overexpression, we speculated that in vivo overexpression of Qki5 could also reduce the doxorubicin-associated cardiotoxicity. To study the in vivo effects, we
injected adult mice with $2 \times 10^{12}$ AAV9 Qki5 viral particles or controls. QKI5 expression was successfully increased in the hearts with AAV9 Qki5 compared with controls (Online Figure VA). Contrary to our hypothesis, mice with AAV9 Qki5 showed deteriorated cardiac function evident by declined ejection fraction, increased systolic and diastolic volume, and thinner interventricular septum thickness (Online Figure VB through VF). Moreover, TUNEL staining revealed increased apoptotic cells in the myocardium of the AAV9 Qki5-treated mice contradicting the in vitro findings (Online Figure VG and VH). However, RNA-binding proteins could be located in nucleus or cytoplasm to serve different purposes. Qki5 has been shown to have nuclear localization signal and collectively transports other isoforms by heterodimerization.14 We hypothesized that probably alteration in localization of Qki could explain the contradicting results. Immunostaining of QKI in control mice hearts revealed that QKI is primarily located in the nucleus, whereas AAV9 Qki5 hearts showed QKI localized both in the cytoplasm and nucleus (Online Figure VI). To quantify a dependency between the amount of QKI overexpression and resulting subcellular...
localization, we transduced neonatal rat cardiomyocytes with AAV2 Qki5 at MOI (multiplicity of infection) of 5*10^3 (low dose) and 1*10^5 (high dose). QKI was localized mainly in the nucleus in the control and low-dose 5*10^3 transduced cardiomyocytes, whereas the high dose of 1*10^5 AAV resulted in mixed nuclear and cytoplasmic distribution as observed in the in vivo study (Online Figure VIA). Immunostaining of HL-1 cells revealed that QKI was primarily located in the nucleus in both pLV empty and Qki5 overexpression cells (Online Figure VIB). Thus, we speculate that higher overexpression of Qki5 results in additional cytoplasmic accumulation associated with more apoptosis. Therefore, along with overexpression of Qki5, its nuclear localization seems to be required to reverse the doxorubicin toxicity.

On the basis of these results, we next tested moderate doses of AAV9 viral particles (7.5*10^11) in the doxorubicin-induced cardiotoxicity model (Figure 4A). Here, QKI staining in hearts confirmed a preserved nuclear localization in AAV9 Qki5-treated mice (Online Figure VII). Doxorubicin led to similar decline in body weight in both the groups either with AAV9 control or AAV9 Qki5 (Figure 4B). Contrary to high dose (2*10^12) experiment, echocardiographic analyses of mice with moderate dose revealed preserved ejection fraction and interventricular septum thickness (D). Apoptosis measured by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining in AAV9 control and AAV9 Qki5 heart sections (E and F; n=5 each). Cardiomyocyte cell size measurement by Wheat germ agglutinin staining of heart (G and H; n=5 each). Bar=100 µm. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. a.u. indicates arbitrary unit; BW, body weight; EF, ejection fraction; and IVS s, interventricular septum thickness systole.
Discussion

RBP s that regulate the life cycle of RNA molecules are critical for cardiac biology. Here, we show that the RBP QKI, specifically the isoform Qki5, regulates cardiomyocyte apoptosis and atrophy induced by doxorubicin. The Qki5 isoform has nuclear localization signal and is known to be responsible for nuclear translocation of other isoforms by heterodimer formation. Previously, Pilote et al have reported that Qki7 can function as apoptotic inducer when located in the cytoplasm. We found that strong overexpression of Qki5 leads to both cytoplasmic and nuclear localization followed by massive apoptosis and cardiac dilatation. A possible explanation might be that too high expression of Qki5 favors homodimerization and discourages heterodimerization, thus leaving Qki6 and Qki7 to stay in cytoplasm initiating apoptosis. In strong contrast, a modest overexpression of Qki5 only resulted in nuclear localization and therapeutic effect in our doxorubicin-induced cardiotoxicity model. Thus, a narrow window exists for therapeutic utilization of Qki5 via viral based therapeutic strategies. An alternative strategy would be to increase the expression of Qki from its endogenous loci through the CRISPR-dCas9 technology, which could simultaneously increase the expression of all isoforms without disturbing their stoichiometry and could lead to nuclear localization to exert potential beneficial effects. Another approach could be to identify downstream beneficial effectors of Qki5 like circular RNAs and use them directly as therapeutic targets. Our findings confirm a central role of Quaking in the regulation of cardiac apoptosis and highlight its potential use as a therapeutic target with a narrow window, which could be improved via alternative strategies.

Deep sequencing and novel bioinformatics approaches led to the discovery of massive number of circular RNAs in the heart together with other organs. Despite their extensive presence, only few candidates like circular RNAs derived from FoxO3 and Cdr1 genes and HRCR were shown to regulate cardiac biology. Here, we report that circular RNAs derived from Tm, Fhod3, and Strn3 are regulated on doxorubicin treatment in the heart. Knockdown of a Tm-derived circular RNA increased the susceptibility to doxorubicin, elucidating the functional role of circular RNAs in cardiotoxicity. Additionally, we identified Quaking as a key regulator of these cardiac circular RNAs. Quaking was previously shown to regulate circular RNA expression during epithelial to mesenchymal transition, but its role in the heart remained unexplored.

Indeed, we here discovered Qki5 as a new target molecule to alleviate doxorubicin-induced cardiotoxic effects. Beneficial effects of a mild Qki5 overexpression were mediated through cardiomyocyte apoptosis inhibition, although we cannot rule out other effects on hypertrophy or contractility at this stage. Mechanistically, our study demonstrates circular RNAs to serve as crucial mediators involved in the observed antiapoptotic effects. Further studies are warranted to understand the role of circular RNAs and their role as effector molecules in cardiac cells and as potential therapeutic candidates in other cardiovascular disease models.

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Disclosures

T. Thum, J. Fiedler, and S.K. Gupta have filed and licensed patents about noncoding RNAs. T. Thum is founder of Cardior Pharmaceuticals GmbH. The other authors report no conflicts.

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**This study involves testing of therapeutic or diagnostic agent in animal models:**

Yes

**Study Design**

| Item                                                                 | Yes/No |
|----------------------------------------------------------------------|--------|
| The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. |        |
| An overall study timeline is provided.                                |        |
| The protocol was prospectively written.                               |        |
| The primary and secondary endpoints are specified.                   |        |
| For primary endpoints, a description is provided as to how the type I error multiplicity issue was addressed (e.g., correction for multiple comparisons was or was not used and why). (Note: correction for multiple comparisons is not necessary if the study was exploratory or hypothesis-generating in nature). | N/A    |
| A description of the control group is provided including whether it matched the treated groups. |        |

**Inclusion and Exclusion criteria**

| Item                                                                 | Yes/No |
|----------------------------------------------------------------------|--------|
| Inclusion and exclusion criteria for enrollment into the study were defined and are reported in the manuscript. | N/A    |
| These criteria were set *a priori* (before commencing the study).   | N/A    |

**Randomization**

| Item                                                                 | Yes/No |
|----------------------------------------------------------------------|--------|
| Animals were randomly assigned to the experimental groups. If random assignment was not used, adequate explanation has been provided. |        |
| Type and methods of randomization have been described.               | No     |
| Allocation concealment was used.                                     | No     |
| Methods used for allocation concealment have been reported.         | No     |

**Blinding**

| Item                                                                 | Yes/No |
|----------------------------------------------------------------------|--------|
| Blinding procedures with regard to masking of group/treatment assignment from the experimenter were used and are described. The rationale for nonblinding of the experimenter has been provided, if such was not performed. |        |
| Blinding procedures with regard to masking of group assignment during outcome assessment were used and are described. |        |
| If blinding was not performed, the rationale for nonblinding of the person(s) analyzing outcome has been provided. | N/A    |

**Sample size and power calculations**

| Item                                                                 | Yes/No |
|----------------------------------------------------------------------|--------|
| Formal sample size and power calculations were conducted before commencing the study based on *a priori* determined outcome(s) and treatment effect(s), and the data are reported. |        |
| If formal sample size and power calculation was not conducted, a rationale has been provided. | N/A    |

**Data Reporting**
Baseline characteristics (species, sex, age, strain, chow, bedding, and source) of animals are reported.

The number of animals in each group that were randomized, tested, and excluded and that died is reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided for all experimental groups.

Baseline data on assessed outcome(s) for all experimental groups are reported.

Details on important adverse events and death of animals during the course of the experiment are reported for all experimental groups.

Numeric data on outcomes are provided in the text or in a tabular format in the main article or as supplementary tables, in addition to the figures.

To the extent possible, data are reported as dot plots as opposed to bar graphs, especially for small sample size groups.

In the online Supplemental Material, methods are described in sufficient detail to enable full replication of the study.

**Statistical methods**

The statistical methods used for each data set are described.

For each statistical test, the effect size with its standard error and $P$ value is presented. Authors are encouraged to provide 95% confidence intervals for important comparisons.

Central tendency and dispersion of the data are examined, particularly for small data sets.

Nonparametric tests are used for data that are not normally distributed.

Two-sided $P$ values are used.

In studies that are not exploratory or hypothesis-generating in nature, corrections for multiple hypotheses testing and multiple comparisons are performed.

In "negative" studies or null findings, the probability of a type II error is reported.

**Experimental details, ethics, and funding statements**

Details on experimentation including formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring are described.

Both male and female animals have been used. If not, the reason/justification is provided.

Statements on approval by ethics boards and ethical conduct of studies are provided.

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Supplemental Material

Quaking inhibits doxorubicin-mediated cardiotoxicity through regulation of cardiac circular RNA expression

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Supplementary Methods

Cell culture & Reagents

HL-1 cells were cultured in Claycomb medium together with 10% FCS, norepinephrine, L-Glutamine and penicillin/streptomycin as per manufacturer’s protocol. H9C2 cells were cultured in DMEM with 10% FBS and penicillin/streptomycin. Neonatal rat cardiomyocytes were isolated from one to three day old rat babies as previously described. Human iPS derived cardiomyocytes were differentiated at the lab of Dr. Katrin Streckfuss-Bömeke as previously described. All the cells were treated with 0.1 μM Doxorubicin (Sigma-Aldrich) either for forty eight or seventy two hours. siRNA against Quaking (#rn.Ri.Qki.13.2 Integrated DNA Technologies) and custom designed circular RNA Ttn 105-111 (Qiagen) were transfected with Lipofectamine 2000 (Life Technologies) in OptiMEM medium.

Si Circular Ttn 105-111 – 5’ GUUCAUGAAGGUGUCUAUTT3’

Cardiac cell Fractionation

Cardiac cell fractionation was performed as described previously.

CRISPR Cas9-mediated knockdown of Quaking

Three guide RNAs were designed at exon 1 and exon 2 of the Qki mouse gene using http://crispr.mit.edu/ webpage and synthesized at EuroFins MWG. Guide RNAs were phosphorylated and annealed by incubation at 37°C for thirty minutes and then ramping 5°C every minute starting from 95°C until 25°C. Annealed oligos were cloned in LentiCRISPRv2 plasmid with BsmBI restriction digestion. Guide RNAs in LentiCRISPRv2, together with PAX2 and MD2.G plasmids were transfected in HEK293FT cells for lentivirus production. Cell supernatant was harvested and concentrated with Lenti-X Concentrator (ClonTech) and viral particles were collected next day. HL-1 cells were transduced with viral particles from two different guide RNAs in combination (1+2, 3+4 and 5+6). Three days later transduced cells were selected with puromycin. Knockdown of Qki was confirmed by western blot. The following sequences were used for guide RNAs

Exon 1-

Guide RNA 1 5’CACCGGGATCTTCAACCACCTCGAG 3’

Guide RNA 3 5’CACCGCAACCACCTCGAGCGGCTGC 3’

Guide RNA 5 5’CACCGCGTCCAGCAGCCGCTCGAGG 3’

Exon 2-

Guide RNA 2 5’CACCGACAATGGTCCCACCACGCAGGC 3’
Guide RNA 4 5’CACCGGCAGAATTGCCTGACGCGGT 3’

Guide RNA 6 5’CACCGTGCAGAATTGCCTGACGCGG 3’

**Caspase Assay**

Caspase assay was performed with Caspase-Glo 3/7 kit (Promega) as per the manufacturer instructions. Briefly, cells were treated with 0.1μM Doxorubicin in normal medium with no FCS for forty eight hours. Next caspase assay reagent provided with kit was added in equal amount to the medium and incubated for thirty minutes at room temperature. Luminescence reading was measured at HT Synergy (Biotek) plate reader.

**MTT Assay**

MTT assay was performed using Cell proliferation kit I (Roche) as per the manufacturer instruction. Briefly, HL-1 cells were seeded in 96 well plate and next day treated with 0.1μM Doxorubicin in normal medium without FCS for forty eight hours. Next, 10μL MTT reagent was added and incubated for four hours followed by addition of dissolving reagent and incubated overnight. Next day absorbance was measured at 580nm and 690nm at HT Synergy (Biotek) plate reader.

**Annexin V & 7-AAD staining**

Flow Celllect Annexin Red kit (Millipore) was used to stain apoptotic cells as per the manufacturer instruction. Briefly, cells were seeded in 24 well plate and next day treated with 0.1μM Doxorubicin in normal medium without FCS for forty eight hours. Then cells were trypsinized and harvested and stained with Annexin-V for 15 minutes at 37°C in 1X assay buffer. Annexin-V stained cells were centrifuged and washed with 1X assay buffer. Next cells were incubated with 7-AAD for 5 minutes and acquired on Guava (Millipore) flow cytometer. Data was analyzed with FLOWJO software.

**TUNEL Staining**

Cells were fixed with 4% paraformaldehyde for twenty minutes at room temperature and then permeabilized with ice-cold 0.1% Triton-X-100 in PBS for two minutes at room temperature. Next cells were incubated with enzyme labelling solution provided with In Situ cell death detection kit (Roche) for one hour at 37°C. For negative staining, enzyme was not added to the labelling solution. Cells were then washed and incubated with Dapi for 15 minutes. In case of neonatal rat cardiomyocytes, cells were stained with sarcomeric alpha-actinin (Sigma-Aldrich #A7811) after Tunel staining, in order to facilitate specific counting of Tunel positive cardiomyocytes. Images were taken with Nikon Eclipse Ti microscope and images were analyzed with NIS Elements. For analysis in each case, ten different images were analyzed from different regions and average value was taken. Three data points representing three individual experiments are shown.

Similarly, cryosections of heart were also processed for staining. Numerous images covering complete heart section was taken and Tunel positive nuclei were counted in each field. Average value for each heart was calculated.
**Cell size measurement**

Primary neonatal rat cardiomyocytes were seeded in 48 well plates, fixed with paraformaldehyde, permeabilized with 0.1% Triton-X-100 and stained with sarcomeric alpha-actinin (Sigma-Aldrich #A7811) and dapi. Images were taken with Nikon Eclipse Ti microscope and images were analyzed with NIS Elements. For analysis in each case, ten different images were analyzed from different regions and average value was taken. Three data points representing three individual experiments are shown.

Paraffin embedded or cryo preserved heart sections were stained with Alexa Fluor 488 labelled wheat germ agglutinin (Invitrogen) and dapi. Images were taken with Nikon Eclipse Ti microscope and images were analyzed with NIS Elements. For each heart six-ten different images were taken from different regions of the heart section and nearly two hundred to six hundred cells (depending upon the availability) area were measured and average value was taken for each heart.

**Immunostaining**

Neonatal rat cardiomyocytes or HL-1 cells were fixed with 4% paraformaldehyde for ten minutes and permeabilized with 0.1% Triton-X-100 for 10 minutes. Blocking was done for thirty minutes with donkey serum and then incubated with QKI antibody (Sigma-Aldrich) (1:100) at 4°C overnight. Next day, cells were incubated with secondary antibody Anti-Rabbit-Alexa 594/488 (Invitrogen) and dapi for 30 minutes. Images were taken with Nikon Eclipse Ti microscope and images were analyzed with NIS Elements.

Similarly, cryosections of hearts were also stained for QKI.

**Electron microscopy**

All tissues were immersion-fixed in 150 mM HEPES, pH 7.35, containing 1.5% formaldehyde and 1.5% glutaraldehyde. Embedding and section preparation was done as before 4 at Institute of Functional and Applied Anatomy, Hannover Medical School. Images were at Transmission Electron Microscopy Morgagni 268 located in Central Electron Microscopy Facility, Hannover Medical School.

**RNA Isolation & PCR**

RNA isolation from cell culture and heart tissue was done using Trifast (Peqlab) as per the manufacturer’s instructions. Isolated RNA (500ng-1000ng) was reversed transcribed with random primer using iScript Select cDNA synthesis kit (Biorad). Real-Time quantitative PCR was done with iQ SYBR Green mix (Biorad) on C1000 Touch Thermocycler (Biorad) using specific primer pairs listed in Supplementary Table 4. For amplification of circular RNAs, divergent primers were used while normal linear transcripts were amplified by convergent primers as usual. For RNase R resistant assay RNA was incubated with RNase R (Biozym Scientific) at 37°C for 10 minutes and heat activated at 95°C for three minutes. RNA was then reverse transcribed and amplified by specific PCR primers as mentioned before.
Circular RNAs screening were performed in pLV Empty, pLV Qki5, Cri Empty, Cri Qki 3+4 and Cri Qki 5+6 cell lines in triplicates. Validation was performed as n=3 individual experiments with three replicates each time.

Lentiviral overexpression cell lines

Qki5, Qki6 and Qki7 cDNA were synthesized as gene string from Thermo Fisher Scientific and cloned in pLV lentiviral backbone. Lentiviral overexpression of Qki5, Qki6 and Qki7 in HL-1 cells was mediated by transduction of lentivirus from pLV plasmid carrying cDNA sequence of Qki5, Qki6, Qki7 and empty control. pLV plasmid were provided by Prof. Axel Sambach, Hannover Medical School. For lentiviral production, HEK293T were transfected with pLV plasmid together with helper plasmids (vsvg, gagpol, rev and NovB2) using CaCl2. Medium was changed after eight hours of transfection. Viral supernatant was collected forty eight to seventy two hours later. HL-1 cells were transduced with lentivirus and after three days puromycin selection was initiated to remove non-transduced cells. After puromycin selection, overexpression was confirmed by western blot.

Similarly, exonic sequence of Ttn 105-111 circular RNA was synthesized as gene string from Thermo Fisher Scientific and cloned in circular RNA forming plasmid pCDNA3.1(+), ZKSCAN1 MCS exon vector (Addgene) with EcoRV and SacII. Then the insert together with repeat element responsible for circularization was released with digestion using KpnI and XhoI and cloned into pLV (lentiviral) plasmid. Virus production and permanent cell line were made similar to Qki.

Western Blotting

Cell pellet were lysed in 1X Cell lysis buffer (Cell Signaling) and isolated protein was measured by Bradford (Biorad) for quantification. 15 – 30 μg of protein was loaded for each sample on SDS-polyacrylamide gel to resolve the protein. Proteins were transferred from SDS PAGE gel to polyvinylidene fluoride membrane in Mini PROTEAN Tetra cell (Biorad). Specific proteins were identified by following antibodies: Quaking (Sigma #HPA019123) and Vinculin (Sigma #V9131). HRP conjugated secondary antibody (Cell Signalling) was used for detection of bands. Band intensity was calculated by Image J software.

Human Cardiac tissue samples

Human patient data was provided by Prof. Hendrik Milting. Heart tissue samples were obtained at Herz- und Diabeteszentrum NRW, Ruhr-Universität Bochum, Bad Oeynhausen. Patients gave informed consent for the use of their explanted hearts (ethical committee by the Medical Faculty of the Ruhr-University Bochum, suboffice Bad Oeynhausen). Failing group included ten patients each with ischemic cardiomyopathy and dilated cardiomyopathy, while non-failing included ten healthy donor hearts. Details of the patients are provided in Supplementary Table 4.

mRNA profiling

Microarray was performed on n=3 samples from each vehicle control and doxorubicin. The Microarray utilized represents a refined version of the Whole Mouse Genome Oligo Microarray 4x44K v2 (Design ID 026655, Agilent Technologies), called ‘048306On1M’ (Design ID 066423) developed at the Research Core Unit Transcriptomics (RCUT) of Hannover Medical School. Microarray design was created at Agilent’s eArray portal using a 1x1M design format for mRNA.
expression as template. All non-control probes of design ID 026655 have been selected to be printed four times within a region comprising a total of 181560 Features (170 columns x 1068 rows). Four of such regions were placed within one 1M region giving rise to four microarray fields per slide to be hybridized individually (Customer Specified Feature Layout). Control probes required for proper Feature Extraction software operation were determined and placed automatically by eArray using recommended default settings.

250ng of total RNA were used for synthesis of aminoallyl-UTP-modified (aaUTP) cRNA with the ‘Quick Amp Labeling kit, no dye’ (#5190-0447, Agilent Technologies) according to the manufacturer’s recommendations, except that reaction volumes were quartered and contained NTP-mix was exchanged by NTP Set (ATP, CTP, GTP, UTP) and aminoallyl-UTP (Fermentas, Thermo Scientific; order numbers R1091, R0481, respectively). Final NTP concentrations used for in-vitro transcription were 2.5mM (ATP, CTP, GTP) and 1.25mM (UTP, aaUTP). The labeling of aaUTP-cRNA was performed by use of Alexa Fluor 555 Reactive Dye (#A32756; LifeTechnologies) as described in the Amino Allyl MessageAmp™ II Kit Manual (#AM1753; Life Technologies) except that reaction volumes were quartered.

Prior to the reverse transcription reaction, 0.5μl of a 1:1000 dilution of Agilent’s ‘One-Color spike-in Kit stock solution’ (#5188-5282, Agilent Technologies) were added to each 250ng of total RNA sample.

cRNA fragmentation, hybridization and washing step s were carried-out as recommended in the ‘One-Color Microarray-Based Gene Expression Analysis Protocol V5.7’, except that 800ng of each fluorescently labeled cRNA population were used for hybridization.

Slides were scanned on the Agilent Micro Array Scanner G2565CA (pixel resolution 3 μm, bit depth 20). Data extraction was performed with the ‘Feature Extraction Software V10.7.3.1’ using the extraction protocol file ‘GE1_107_Sep09.xml’, except that ‘Multiplicative detrending’ algorithm was inactivated.

Extracted signal intensities were filtered with cut-off of 2000 to remove low expressed mRNAs and global normalization was performed. Candidates with significant fold change of ≥ 1.5 were selected and heatmap was generated with Clustvis online tool.

**Adeno-associated virus production**

HEK 293T cells were transfected with AAV-Empty or AAV-Qki5 plasmid together with pDG (a kind gift from Prof. Roger Hajjar, Mount Siani Hospital, New York) (pDG2 for AAV2 and pDG9 for AAV9) with polyethyleneimine. Medium was changed next day and cells were left for seventy two hours. Supernatant was collected and mixed with 40% Polyethylene Glycol 8000 solution for an hour and later incubated overnight at 4°C. Cells were harvested and lysed in presence of benzonase (Novagen). Supernatant was centrifuged at 2800xg for 15 minutes to collect precipitated virus and mixed with the cell lysate. Cell lysate was ultracentrifuged on iodixanol (OptiPrep, Progen) gradient in ultracentrifuge (Beckman Coulter) at 63000 rpm for one hour in Ti 70 rotor. AAVs were extracted from 40% fraction and concentrated using Amicon Ultra 15 (Millipore) columns. AAvs were titrated with PCR primer amplifying CMV (Cytomegalovirus) promoter.
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Online Figure I. Doxorubicin-induced cardiotoxicity mouse model. (A) Schematic representation of the in vivo animal model, where doxorubicin was injected weekly at 5mg/kg dose for five consecutive weeks. Body weight (B), heart weight to tibia length (C) and cardiomyocyte cell size (D) in animals which received doxorubicin compared to control (n=9 each). (E) Destroyed and less dense myofibres in doxorubicin treated mice were visualized by electron microscopy. *p≤0.05, **p≤0.01, ****p≤0.0001, a.u. arbitrary unit, Scale bar represents 2000nm
Online Figure II. (A) Expression of QKI in different mouse organs, (n=1). (B) Qki isoforms expression level in different cardiac cell types (n=3 each).
Online Figure III. Quaking modulation inhibits doxorubicin mediated cardiotoxicity. (A) AAV2 mediated overexpression of QKI5 and siRNA mediated inhibition of QKI in neonatal rat cardiomyocytes. (B) Caspase 3/7 activity in H9C2 cells transfected with siRNA against Qki or control in presence or absence of doxorubicin. (C-D) Annexin-V and 7-AAD staining of pLV Empty and pLV Qki5 overexpressing cells, in response to doxorubicin. (E-F) Cell size measurements of cardiomyocytes transduced with AAV2 Qki5 or control vectors in the presence of doxorubicin. **p≤0.01, ***p≤0.001, a.u. arbitrary units.
Online Figure IV. Screening of circular RNAs regulated by Quaking. Expression of circular RNAs selected after filtering in pLV Qki5, Cri 3+4 and Cri 5+6 HL-1 cell lines normalized to empty cell lines (A). Western blot showing CRISPR-mediated knockdown of Quaking (B). (C) Expression levels of circular RNAs derived from Ttn, Fhod3 and Strn3 in pLV Qki6 and Qki7 overexpressing cells. (D) Real-time PCR confirming overexpression of circular RNA Ttn 105-111 in HL-1 cells (E). Caspase3/7 activity in Ttn 105-111 overexpressing HL-1 cells compared to control upon doxorubicin treatment. (F) Cellular survival seen by MTT assay in Ttn 105-111 overexpressing HL-1 cells compared to pLV Empty controls. *p≤0.05, RLU – Relative Luminescence Unit, CRi - Crispr
Online Figure V. Higher overexpression of Qki5 leads to pronounced cytoplasmic localization and causes adverse effects in the heart. (A) Western blot experiments show expression of QKI in myocardium of mice injected with AAV9-Qki5 compared to control. (B-F) Cardiac function measured by echocardiography in AAV9-Qki5 mice compared to control. (G-H) Higher number of apoptotic cells visualized by TUNEL staining in AAV9-Qki5 treated myocardium. (I) Immunostaining of QKI in heart shows nuclear localization in control while AAV9-Qki5 mice show distribution in cytoplasm and nucleus both. Scale Bar represents 100 μM. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, EF –
Ejection Fraction, Vol d – Volume diastole, Vol s – Volume systole, IVS s – Interventricular septum systole, IVS d – Interventricular Septum diastole
Online Figure VI. High amount of Qki5 leads to cytoplasmic localization of QKI. (A) Transduction of neonatal rat cardiomyocytes with high dose (MOI-1*10⁵) causes redistribution of QKI in cytoplasm and nucleus, while low dose (MOI-5*10³) did not. (B) Quaking immunostaining in pLV empty and pLV Qki5 overexpression cell lines shows nuclear localization of QKI. Scale Bar represents 100μM.
Online Figure VII. QKI staining in mice treated with 7.5*10^{11} AAV9 Qki5 viral particles showed nuclear localization in heart similar to control hearts. Scale Bar represents 100μM.

|                | Vehicle n=5 (Mean±SEM) | Doxorubicin n=4 (Mean±SEM) | Significance  |
|----------------|------------------------|----------------------------|---------------|
| EF %           | 69.31 ± 1.534          | 47.98 ± 1.897              | p< 0.0001     |
| FS%            | 38.38 ± 1.170          | 23.84 ± 1.077              | p< 0.0001     |
| Vol s (μL)     | 19.61 ± 2.238          | 38.55 ± 4.255              | p=0.0041      |
| Vol d (μL)     | 63.25 ± 4.943          | 73.56 ± 5.491              | p=0.2057      |
| LVPW s (mm)    | 1.159 ± 0.0918         | 0.8602 ± 0.0673            | p= 0.0414     |
| LVPW d (mm)    | 0.8086±0.0762          | 0.6812 ± 0.0554            | p=0.2400      |
| IVS s (mm)     | 1.534 ± 0.0987         | 1.078 ± 0.0451             | p=0.0064      |
| IVS d (mm)     | 0.8391±0.0487          | 0.7277 ± 0.0211            | p= 0.0975     |

Online Table I. Cardiac functions of Vehicle- and Doxorubicin- treated mice measured by echocardiography.
| Downregulated Genes | Upregulated Genes |
|---------------------|-------------------|
| Acta1               | Adamtsl4          |
| Actg1               | Alas2             |
| Actg1               | Amyl              |
| Ankrd23             | Artl              |
| Ano10               | Bpgm              |
| Arl6ip5             | Btg3              |
| B2m                 | Camta1            |
| Bcl6                | Cdk5rap3          |
| Bst2                | Cirbp             |
| C1qc                | Cpt1a             |
| Cend1               | Ctgf              |
| Cd93                | Dbp               |
| Col1a2              | Eif4ebp1          |
| Col3a1              | ENSMUST00000040914|
| Col4a1              | Gadp10            |
| Cotl1               | Hba-a1            |
| Csf1r               | Hbb-b2            |
| Eef1e1              | Hbb-bt            |
| Emp1                | Irl2bp2           |
| ENSMUST00000099042  | Itripp            |
| ENSMUST00000099046  | Ivns1abp          |
| ENSMUST00000099050  | Lrcc10            |
| ENSMUST00000099683  | Mcl1              |
| ENSMUST00000099684  | Mkl1              |
| ENSMUST0000173809   | Mnt               |
| ENSMUST0000177969   | Nppa              |
| Fabp5               | Nr1d2             |
| Fam131a             | Nr4a1             |
| Fn1                 | Pde1c             |
| G0s2                | Ptgsd             |
| Gm1987              | Rbm3              |
| H2-Eb1              | Rit1              |
| H2-K1               | Scgb1c1           |
| H2-K2               | Serpinb6b         |
| Hfe2                | Slec25a37          |
| HQ258995            | Smim4             |
| Hsph1               | Tef               |
| Ifi27               | Thumpd1           |
| Ifit122             | Timp4             |
| Igsf1               | Tns2              |
| Igtp                | Tspan4            |
| Irgm2               | Ttn               |
| Lyz1                | Usp2              |
| Mal                 |                   |
| Marcks              |                   |
| NAP111417-1         |                   |
Online Table II. List of mRNAs differentially regulated in doxorubicin treated hearts compared to vehicle control.
| chr  | start    | end      | strand | index | GeneSymbol | Annotation                  | nExons | length |
|------|----------|----------|--------|-------|------------|----------------------------|--------|--------|
| chr1 | 43141698 | 43153254 | -      | mmuCirc_000119 | Fhl2 | CTO:ANNOTATED:CDS:5UTR | 2      | 354    |
| chr1 | 75497008 | 75514282 | -      | mmuCirc_002066 | Obsl1 | ITO:ALT_ACCEPTOR:CDS:5UTR | 1      | 17275  |
| chr1 | 87364104 | 87380008 | +      | mmuCirc_002098 | Gigyf2 | CTO:ANNOTATED:CDS | 5      | 494    |
| chr1 | 87880368 | 87881939 | +      | mmuCirc_000305 | Dgkd | CTO:ANNOTATED:CDS | 2      | 192    |
| chr10| 12436281 | 12455564 | -      | mmuCirc_000119 | Umr | CTO:ANNOTATED:CDS | 5      | 725    |
| chr11| 6313788  | 6317080  | +      | mmuCirc_001237 | Ogdh | CTO:ANNOTATED:CDS | 3      | 386    |
| chr11| 59055377 | 59061107 | -      | mmuCirc_001406 | Obscn | CTO:ANNOTATED:CDS | 3      | 792    |
| chr11| 59055604 | 59061107 | -      | mmuCirc_001409 | Obscn | CTO:ANNOTATED:CDS | 2      | 528    |
| chr11| 59055961 | 59061687 | -      | mmuCirc_001410 | Obscn | CTO:ANNOTATED:CDS | 2      | 528    |
| chr11| 65218529 | 65233184 | +      | mmuCirc_001466 | Myocd | CTO:ANNOTATED:CDS | 4      | 360    |
| chr12| 51654799 | 51661713 | -      | mmuCirc_001998 | Stm3 | CTO:ANNOTATED:CDS | 6      | 703    |
| chr12| 51655402 | 51661713 | -      | mmuCirc_002000 | Stm3 | CTO:ANNOTATED:CDS | 3      | 260    |
| chr13| 11680966 | 11688013 | -      | mmuCirc_002359 | Ryr2 | CTO:ANNOTATED:CDS | 4      | 427    |
| chr13| 35815909 | 35816575 | +      | mmuCirc_002451 | Cdy1 | CTO:ANNOTATED:CDS | 1      | 667    |
| chr13| 103884143| 103889285| -      | mmuCirc_002718 | Erbb2Ip | CTO:ANNOTATED:CDS:5UTR | 3      | 364    |
| chr15| 88811297 | 88812159 | -      | mmuCirc_003512 | Alg12 | CTO:ANNOTATED:CDS | 2      | 328    |
| chr16| 11116795 | 11128703 | -      | mmuCirc_003625 | Txndc11 | CTO:ANNOTATED:CDS | 3      | 445    |
| chr16| 32950292 | 32961744 | +      | mmuCirc_003757 | Lrce3 | CTO:ANNOTATED:CDS | 5      | 625    |
| chr16| 33423121 | 33434974 | +      | mmuCirc_003769 | Zfp148 | CTO:ANNOTATED:CDS:5UTR | 3      | 567    |
| chr16| 58424561 | 58433463 | +      | mmuCirc_003842 | Dcbld2 | CTO:ANNOTATED:CDS | 2      | 366    |
| chr16| 94383912 | 94403368 | +      | mmuCirc_003895 | Ttc3 | CTO:ANNOTATED:CDS:5UTR | 10     | 911    |
| chr16| 6137211 | 6139156 | +      | mmuCirc_003928 | Tulp4 | CTO:ANNOTATED:CDS:5UTR | 1      | 1946   |
| chr17| 10238899 | 10282983 | +      | mmuCirc_003949 | Qk | CTO:ANNOTATED:CDS | 3      | 404    |
| chr17| 34715638 | 34797443 | +      | mmuCirc_004045 | Tnxb | ITO:ALT_ACCEPTOR:CDS:3UTR | 1      | 81761  |
| chr17| 34716301 | 34798563 | +      | mmuCirc_004046 | Tnxb | ITO:ALT_ACCEPTOR:CDS:3UTR | 1      | 82263  |
| chr18| 81647809 | 81649638 | -      | mmuCirc_004295 | Slc8a1 | CTO:ANNOTATED:CDS:5UTR | 1      | 1830   |
| chr18| 5633204 | 5705243 | +      | mmuCirc_004341 | Zeb1 | CTO:ALT_ACCEPTOR:CDS | 1      | 72040  |
| chr18| 25026662 | 25028169 | +      | mmuCirc_004438 | Phoxd3 | CTO:ANNOTATED:CDS | 3      | 558    |
| chr18| 35610871 | 35613502 | +      | mmuCirc_004508 | Papi2 | CTO:ANNOTATED:CDS:5UTR | 2      | 344    |
| chr2 | 23537328 | 23545579 | +      | mmuCirc_005197 | Spo1l | CTO:ANNOTATED:CDS:5UTR | 7      | 869    |
| chr2 | 25965663 | 25966948 | -      | mmuCirc_005227 | Camsap1 | CTO:ANNOTATED:CDS | 2      | 425    |
| chr2 | 70770004 | 70786995 | +      | mmuCirc_005427 | Tlk1 | CTO:ANNOTATED:CDS | 2      | 191    |
| chr2 | 76857904 | 76881839 | -      | mmuCirc_005504 | Tn | CTO:ANNOTATED:CDS | 34     | 6213   |
| chr2 | 76857904 | 76884197 | -      | mmuCirc_005505 | Tn | CTO:ANNOTATED:CDS | 35     | 6501   |
| chr2 | 76863274 | 76868085 | -      | mmuCirc_005516 | Tn | CTO:ANNOTATED:CDS | 7      | 780    |
| chr2 | 104470749| 104471847| -      | mmuCirc_005592 | Hipk3 | CTO:ANNOTATED:CDS:5UTR | 1      | 1099   |
| chr2 | 131511984| 131516443| +      | mmuCirc_005746 | Smox | CTO:ANNOTATED:CDS:5UTR | 2      | 461    |
| chr2 | 142739523| 142740991| -      | mmuCirc_005775 | Kir16b | CTO:ANNOTATED:CDS | 2      | 172    |
| chr3 | 51308050 | 51326035 | -      | mmuCirc_006048 | Elf2 | CTO:ANNOTATED:CDS:5UTR | 3      | 484    |
| chr5 | 65565989 | 65594548 | +      | mmuCirc_007206 | Ube2k | CTO:ANNOTATED:CDS | 5      | 465    |
| chr5 | 118593333| 118593570| +      | mmuCirc_007425 | Med13l | CTO:ANNOTATED:CDS | 1      | 238    |
| chr6 | 47576536 | 47577667 | -      | mmuCirc_007816 | Ezh2 | CTO:ANNOTATED:CDS:5UTR | 2      | 253    |
| Chromosome | Start | End | Gene Name | CTO Type | Description | Length |
|------------|-------|-----|-----------|----------|-------------|--------|
| chr6       | 119052601 | 119057515 | Cacna1c | ANNOTATED:CDS | 2 | 428 |
| chr7       | 99935162  | 99955540 | Rnf169   | ANNOTATED:CDS | 3 | 340 |
| chr7       | 121047838 | 121057399 | Mettl9   | ANNOTATED:CDS | 3 | 586 |
| chr8       | 11212664  | 11241198 | Col4a1   | ALT_DONOR:ALT_ACCEPTOR:CDS | 31 | 3111 |
| chr8       | 45759053  | 45775703 | Sorbs2   | ANNOTATED:CDS | 6 | 714 |
| chr8       | 79118175  | 79136663 | Zfp827   | ANNOTATED:CDS | 3 | 402 |
| chr8       | 84771784  | 84772315 | Nfix     | ANNOTATED:CDS | 1 | 532 |
| chr8       | 95365314  | 95366422 | Mmp15    | ANNOTATED:CDS | 2 | 278 |
| chr8       | 123382527 | 12338443 | Tcf25    | ANNOTATED:CDS | 2 | 194 |
| chr9       | 14571623  | 14575439 | Amot1    | ANNOTATED:CDS | 3 | 524 |
| chr9       | 22887581  | 22887996 | Bbs9     | ALT_DONOR:CDS:3UTR | 2 | 243 |
| chr9       | 32246447  | 32250787 | Arhgap32 | ANNOTATED:CDS | 5 | 679 |
| chr9       | 96591950  | 96611499 | Rasa2    | ANNOTATED:CDS | 4 | 394 |
| chr9       | 96602713  | 96611499 | Rasa2    | ANNOTATED:CDS | 3 | 317 |

**Online Table III.** Detailed list of circular RNAs selected for screening after our filtering criteria mentioned in the Figure 3A.
| Gene name     | Fwd primer 5' | Rev primer 5' |
|--------------|--------------|--------------|
| Circ_Ttn 104-110 | AAGAGGGCTACGATGAGGG | GTACAGTTCCGCGTGTGCTTC |
| Circ_Ttn 84-118 | CAAAGAAACCTGCTCCCGAAG | ATCGGTGCTGTTCCAGTGAC |
| Circ_Slc8a1 | TCTGGAGCTGAGGAGAATGT | TTGGTGAGGAGACTTAATCG |
| Circ_Hipk3 | CATCGTACCTCAAACCGAGA | ACAACACCGCTTGCTCTAC |
| Circ_Ryr2 | CCTCTCTGACACTGGAGGACAT | CCTTGGCCTCTGACGTGTCAT |
| Circ_Nfix | CTCGAACCCCGGCTCCTGTTG | GGTCTGAGGGTGGAGGAGA |
| Circ_Zfp827 | ACCCTCAATTTAAGAATCTGC | ATGTTCCGGCTGGGTGATAT |
| Circ_Rnfl69 | CCAGATGAGCTCAAGACCTTC | CTTCTCAAGGCACTCATACCTC |
| Circ_Med13l | TCTGGAGCTCGAGGAAATGT | GAAACTTAAACAGGAGGTGCTT |
| Circ_Qki | GAGGAGGGCTACGAGGAGAAGG | AGGCAGAGGTGGAGTAGGGT |
| Circ_Camsap | AGGGATATTCTGGTGGGGAGATGA | GTGTCAAGGTGTAAGCCCAATCC |
| Circ_Spopl | GCCAGATGAGCTCAAGACCTTC | TGGGATATACCGAATACATTCT |
| Circ_Camsap | CACCTCCAATTCAAAAGATCTGC | ATGTTCCGGCTGGGTGATAT |
| Circ_Lrch3 | GAAACTTAAACAGGAGGTGCTT |
| Circ_Ogdh | GAGATGCTGATCTGGACTCCT | GCCGAGCATCGTGCAGCTTC |
| Circ_Strm3 2-7 | GTCCTCTGACACTGGAGGACAT | CTTCTCAAGGCACTCATACCTC |
| Circ_Cdyl | AGGGAGGGCTACGAGGAGAAGG | AGGCAGAGGTGGAGTAGGGT |
| Circ_Tusp4 | TCTGGAGCTGAGGAGAATGT | TTGGTGAGGAGACTTAATCG |
| Circ_Elf2 | GAGATGCTGATCTGGACTCCT | GCCGAGCATCGTGCAGCTTC |
| Circ_Ube2k | GAGGAGGGCTACGAGGAGAAGG | AGGCAGAGGTGGAGTAGGGT |
| Circ_Mett9 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Rasa2 | GAGATGCTGATCTGGACTCCT | GCCGAGCATCGTGCAGCTTC |
| Circ_Fhl2 | AGGGATATTCTGGTGGGGAGATGA | GTGTCAAGGTGTAAGCCCAATCC |
| Circ_Giggyf | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Dgkd | GAGGAGGGCTACGAGGAGAAGG | AGGCAGAGGTGGAGTAGGGT |
| Circ_Utrn | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Myocd | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Erbb2ip | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Alg12 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Debld2 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Ttc3 2-10 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Fhod3 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Paip2 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Kif16b | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Ezh2 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Ttc3 2-11 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Sorb2 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Tc25 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Bbs9 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Arhgap32 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Txndc1 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Caca1 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Circ_Strm3 2-4 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Mmu_Qki 5 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Mmu_Qki 6 | GCCAGGTCACCTCGGATT | ATGTTCCGGCTGGGTGATAT |
| Gene     | Fwd primer 5’       | Rev primer 5’       |
|----------|---------------------|---------------------|
| Mmu_Qki  | GGGCCTGAAGCTGGGTTAAT | CATCCAGCAATGCAATGGG3’ |
| Hsa_Qki  | GCAAAATAGGGCAAGGCCC3’ | GCGTCTCTGTAGGCGGCCATT3’ |
| Mmu_Hprt | GCGTCGTGATTAGCGATGAT3’ | TCCTTCATGACATCTCGAGCA3’ |

**Online Table IV.** List of primers used for real-time PCR
| Pat.Nr. | Disease | OP     | Gender | age | EF | LVEDD |
|--------|---------|--------|--------|-----|----|-------|
| 1      | ICM     | LVAD-IP| m      | 59  | 15 | 60    |
| 2      | ICM     | LVAD-IP| m      | 45  | 20 | 62    |
| 3      | ICM     | LVAD-IP| m      | 48  | 10 | 71    |
| 4      | ICM     | LVAD-IP| m      | 62  | 17 | 65    |
| 5      | ICM     | LVAD-IP| m      | 62  | 14 | 81    |
| 6      | ICM     | LVAD-IP| m      | 45  | 20 | 69    |
| 7      | ICM     | LVAD-IP| m      | 73  | 20 | 69    |
| 8      | ICM     | LVAD-IP| m      | 56  | 10 | n.v.  |
| 9      | ICM     | LVAD-IP| m      | 35  | n.v.| 65    |
| 10     | ICM     | LVAD-IP| m      | 47  | 26 | 52    |
| 11     | DCM     | LVAD-IP| m      | 44  | 10 | 85    |
| 12     | DCM     | LVAD-IP| m      | 46  | 19 | 66    |
| 13     | DCM     | LVAD-IP| m      | 58  | 20 | 78    |
| 14     | DCM     | BVAD-IP| m      | 38  | 10 | n.v.  |
| 15     | DCM     | LVAD-IP| m      | 50  | 15 | n.v.  |
| 16     | DCM     | LVAD-IP| m      | 62  | 10 | 90    |
| 17     | DCM     | LVAD-IP| m      | 62  | 24 | 92    |
| 18     | DCM     | LVAD-IP| m      | 40  | 10 | 62    |
| 19     | DCM     | LVAD-IP| m      | 73  | 35 | 42    |
| 20     | DCM     | LVAD-IP| w      | 58  | 15 | 62    |
| 1      | NF      | Spender| w      | 54  |    |       |
| 2      | NF      | Spender| m      | 54  |    |       |
| 3      | NF      | Spender| m      | 43  |    |       |
| 4      | NF      | Spender| m      | 61  |    |       |
| 5      | NF      | Spender| m      | 50  |    |       |
| 6      | NF      | Spender| w      | 55  |    |       |
| 7      | NF      | Spender| m      | 28  |    |       |
| 8      | NF      | Spender| w      | 55  |    |       |
| 9      | NF      | Spender| m      | 52  |    |       |
| 10     | NF      | Spender| m      | 40  |    |       |

**Online Table V.** Human patient details.