

# CAMK2D causes heart failure in mice with RBM20 cardiomyopathy

Corresponding Author: Dr Maarten van den Hoogenhof

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting story that provides new evidence that patients with dilated cardiomyopathy due to mutations in the RS domain of a splicing factor, RBM20 (~3% of DCM cases), lead to hyperactivation of CaMKII and that CaMKII is the fundamental cause of cardiomyopathy. Despite being a splicing factor, the authors find that an unknown circuit, activated by RBM20 knock out in mice, or by human RBM20 disease mutations in a knockin mouse and in iPSCs, leads to increased CaMKII expression. Intriguingly, the mechanism for increased CaMKII expression and activity appears to be splice variant agnostic. Furthermore, all of the CaMKII $\delta$  isoforms, in the absence of RBM20 or in the presence of its disease causing mutants, congregate in the nucleus in RBM20 disease models and knock outs by another untested mechanism. Like many good stories, this one raises more questions than it answers. Nevertheless, it would benefit from further work to understand the basic biology underlying, yet another, model where excessive CaMKII activity promotes myocardial dysfunction and arrhythmias.

Specific points:

1. The finding that the splicing function of RBM20 is immaterial to its pathological actions is interesting but, apparently, not unprecedented. Your studies, with some acknowledged limitations, make this point effectively. However, the key issue of how RBM20 mutants or knock out causes increased CaMKII message/protein/activity is not really addressed. The various mRNA analyses show that CaMKII is one of the more upregulated pathways but these studies do not shed light on how such a circuit operates. Is the level of CaMKII message/protein in the RBM20 knockout/knockin models affected by hesperadin treatment? Is the expression of other (non-CaMKII $\delta$ ) RBM20 targets increased similar to CaMKII $\delta$ ?
2. Similarly, RBM20 mutants/knock out cause mislocalization of various proteins. The reader is left wondering if some general principle is at play that explains how seemingly all CaMKII $\delta$  splice variants aggregate in the nucleus. Of course, a lot is known about CaMKII $\delta$  splice variants that have or lack an NLS (negatively regulated by phosphorylation) but there is no work to determine if these signals play a role. If there is not a conventional (e.g. NLS-mediated) mechanism for nuclear congregation of CaMKII $\delta$  isoforms what is happening?
3. The hesperidin studies provide translational credibility but, as the authors note in the Discussion, hesperidin is hardly a CaMKII selective inhibitor. It seems like a missed opportunity not to have tested the potential for interbreeding the CaMKII $\delta$  ko with the Rbm20-R636Q knock in mouse to confirm that hesperidin 'rescue' was due to on target actions.
4. The authors make the point that CaMKII is a specific mechanism for RBM20 cardiomyopathy and thus a novel example of a 'personalized' therapy. This framing is strained by the reality that excessive CaMKII activity is linked to multiple cardiomyopathy models and arrhythmias. This fact should be made clear in the Introduction and/or Discussion.

Reviewer #2

(Remarks to the Author)

Mutations in RBM20 cause an aggressive form of dilated cardiomyopathy (DCM) associated with a high risk of malignant ventricular arrhythmias. In this study, the authors investigated whether CAMK2D is the principal pathogenic effector in RBM20-related cardiomyopathy. Using Rbm20/Camk2d double knockout (DKO) mice, they report that loss of Camk2d protects against heart failure and sudden cardiac death. Rbm20-deficient hearts exhibit both aberrant splicing and overactivation of CAMK2D. Reintroduction of individual CAMK2D splice variants via AAV9 into DKO mice reinstated cardiac dysfunction, independent of splice variant, suggesting that CAMK2D overactivation—not missplicing—is the main disease driver. Furthermore, pharmacological inhibition of CAMK2D with hesperadin in Rbm20-R636Q knock-in mice rescued

cardiac function and ventricular remodeling. The authors conclude that CAMK2D overactivation is a central driver of RBM20-associated cardiomyopathy and propose that its inhibition could serve as a targeted therapeutic approach for DCM. This is an important, well-conducted, and clearly described study. However, I have several major concerns that should be addressed:

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#### Major Comments

##### 1. Insufficient Functional Assessment of Cardiac Protection

The conclusion that DKO mice are protected from cardiac dysfunction is currently under-supported. The authors rely heavily on echocardiography in anesthetized mice and present only a limited set of parameters (EF, E/E', LVIDd), with E/E' reported at just one time point. This is inadequate for a study drawing such critical conclusions. A comprehensive set of echocardiographic parameters should be provided at all three time points studied.

##### 2. Need for Load-Independent Hemodynamic Data

To substantiate the claim of protection against cardiac dysfunction, more robust functional assessments are required. Pressure-volume (PV) analysis should be performed to evaluate both systolic and diastolic function in a load-independent manner. Key parameters such as ESPVR slope (contractility) and EDPVR coefficient (diastolic stiffness) are essential for validating these conclusions.

##### 3. Apparent Contradiction in Figure 2E

The conclusion that DKO mice are protected appears inconsistent with the data presented in Figure 2E. This discrepancy should be clearly explained.

##### 4. Evidence of Diastolic Dysfunction in DKO Mice

The text states that atria are enlarged in DKO mice (line 144), which may suggest diastolic dysfunction. Are atrial tissue weights increased? Please provide this data. In addition, what is the EDPVR coefficient in DKO mice? This would help clarify whether diastolic function is truly preserved.

##### 5. Clarification on TTN missplicing and Functional Impact

The authors state that RBM20 cardiomyopathy is not a TTN missplicing disease (line 239). To convincingly support this, data should demonstrate that DKO mice have preserved diastolic function (via PV analysis and tissue mechanics), maintain a normal Frank-Starling mechanism, and exhibit unaltered exercise capacity.

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#### Minor Comments

##### 1. Sex of Mice

What is the sex of the mice used in this study? If both males and females were included, data should be analyzed and presented separately by sex.

##### 2. Missing Data Quantification

In several places, data quantification is lacking. For example, in Figure 3B.

##### 3. Statistics.

More detailed information is needed. For example was multiple testing correction performed in Figure 3C. Figure 4B bottom is described in the text as if significant changes are seen in R636Q mice upon Hesperadin treatment but no significance is shown on the Figure.

#### Reviewer #3

##### (Remarks to the Author)

This paper by van den Hoogenhof et al provides strong and convincing evidence for its interesting conclusion: that the heart failure in RBM20 cardiomyopathy is mediated by CaMKIIdelta. Interestingly, while RBM20 is a splice factor, the CaMKIIdelta effect does not appear to be caused by direct misregulation of CaMKIIdelta splicing, but by some form of splice-variant-independent overactivation. Notably, in turn, this mechanism then provides the opportunity for therapeutic intervention with pharmacology. Regarding the use of CaMKII inhibitors, the study exceeds the recently proposed standard to ensure CaMKII-specificity of the effect of the effects of pharmacological studies (see Brown and Bayer, 2024; which points out that the effect with one inhibitor should be backed up by at least one other inhibitor of a different class, whereas the previously typically demanded inclusion of an "inactive control compound" is much less useful). Although the current study uses only one single inhibitor, the back-up provided here is even better: CaMKIIdelta knockout and re-expression studies, which provides even more selectivity and specificity. However, while the experimental approach exceeds standards, the text should include better and more extensive description and discussion of the inhibitor used (especially since it is a somewhat unusual inhibitor that has not been used much in context of CaMKII previously).

##### Specific comments.

1) It should be pointed out that the inhibitor used here (hesperadin) is an ATP-competitive inhibitor (i.e. one of three principally different classes of CaMKII inhibitors). This is important, as the different classes can have distinct additional effects on CaMKII (i.e. other than on enzymatic activity; as reviewed in Brown and Bayer, 2024).

2) There should be some discussion that hesperadin has an unusually high selectivity for the CaMKIIdelta isozyme (Zhang et al 2022; ref. 31; in this context, this reference should also be cited at the first mentioning in the results section in line 195, rather than in the next sentence that describes the in vivo dosing). However, additionally, it needs to be pointed out that hesperadin is not all that CaMKII-selective: In addition to inhibiting Aurora kinase B (as mentioned and cited; ref 31), it inhibited some six other kinases out of a panel of twenty five, i.e. almost 25% (Hauf et al 2003, JCB, PMID: PMC2172906; this needs to be cited).

3) The discussion contains a statement regarding CaMKII inhibitors that is highly misleading on several levels (line 263-265: "It comes with great promise that after 35 years work on CAMK2 and several attempts to develop a specific CAMK2 inhibitor, a first inhibitor is in a phase II clinical trial (clinicaltrials.gov, identifier NCT06005428) [34, 35]"). First (and least importantly),

the sentence seems to talk about selectivity rather than specificity. It also suggests that there have not been any selective (or specific) CaMKII inhibitors until now; this is far from correct (as reviewed, for example, in Brown and Bayer, 2024). The sentence also seems to imply that selectivity is directly connected with being in clinical trials. This is not the case. Instead, a successful drug has to be safe and effective; while selectivity may help with that, it does not necessarily do so. It is possible to be safe and effective despite hitting other targets. Vice versa, if hitting the intended target causes toxicity, it does not matter how selective the drug is. Finally, the sentence implies that this is the first CaMKII inhibitor in clinical trials, but this is not the case either. As it turns out, ruxolitinib is an ATP-competitive CaMKII inhibitor (Reyes Gaido et al., 2023, Sci Transl Med 15:eabq7839) and has been in the clinic for its better known role as JAK inhibitor.

4) Somewhat minor, but it might be helpful to include a specific statement that hesperadin should not be confused with hesperidin. This is because google searches of one compound will also show results for the other. Even worse, there is a review article (that focused on ref 31) that mentions hesperidin and even shows the structure for hesperidin, but otherwise talks about the effects described for hesperadin instead, such as being an inhibitor of Aurora kinase B and CaMKII.

Sincerely,  
K. Ulrich ("Ulli") Bayer

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)  
Thank you for your contribution

Please consider the following

The immunoblot data detecting CaMKII expression, included in the response document, after hesperadin shows a prominent doublet that is not present in the absence of hesperadin. The doublet may signify a PMT or another CaMKII splice variant or isoform. Please comment and, ideally, identify the new band. These data seem at odds with the contention that hesperadin has no action on CaMKII expression.

I think the revised statement - It must be noted, however, that CAMK2 activation is not a general 318 hallmark of all cardiac diseases; e.g. in a new mouse model with the human DCM-causing mutation 319 LMNA-K117fs we do not observe CAMK2 activation - should be amended; please substitute 'universal' for 'general'. Although the authors provide an interesting example of myocardial disease not improved by CaMKII inhibition, the broader/general reality is that excessive CaMKII activity is a feature of a remarkable preponderance of clinically-relevant acquired and genetic forms of myocardial diseases.

Reviewer #3

(Remarks to the Author)  
The authors have fully addressed my comments.

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Response to editors' comments:

Your manuscript "CAMK2D causes heart failure in RBM20 cardiomyopathy" has now been seen by 3 referees, whose comments are appended below. Although they find your work of potential interest, they have raised substantive concerns which in our view are sufficiently important to preclude publication of the work in Nature Cardiovascular Research, at least in its present form.

In general, we agree with the reviewers that the work would be of interest to the broad audience of Nature Cardiovascular Research. At the same time, the review process identified several opportunities to strengthen the work. We would be happy to look at a revised manuscript where all referees' concerns have been addressed experimentally, whenever needed (unless something similar has been accepted at Nature Cardiovascular Research or appeared elsewhere in the meantime).

We thank the editors and reviewers for their time and efforts and have now prepared our revised manuscript. We are grateful for the suggestions of the reviewers, and by conducting these experiments we could confirm many of our findings and this has substantially improved the manuscript.

At the reviewers' request, we have added additional echocardiographic data at multiple timepoints to substantiate our conclusions on the systolic and diastolic dysfunction we observe in *Rbm20* KO mice, and the rescue of these in the *Rbm20/Camk2d* DKO mice. We also analysed and presented these data separately by sex. Additionally, we have included PV-loop data, as well as isometric force measurements of single isolated skinned cardiomyocytes, to further show the functional effects of loss of CAMK2D on contractility and compliance in RBM20 cardiomyopathy. Together, these data show an increased compliance and a decreased systolic performance in *Rbm20* KO mouse hearts, while this systolic performance was prevented in the DKO mouse hearts. Moreover, we have added additional discussion on hesperadin, the CAMK2 inhibitor that we used in this study.

Reviewer #1 (Remarks to the Author):

This is an interesting story that provides new evidence that patients with dilated cardiomyopathy due to mutations in the RS domain of a splicing factor, RBM20 (~3% of DCM cases), lead to hyperactivation of CaMKII and that CaMKII is the fundamental cause of cardiomyopathy. Despite being a splicing factor, the authors find that an unknown circuit, activated by RBM20 knock out in mice, or by human RBM20 disease mutations in a knockin mouse and in iPSCs, leads to increased CaMKII expression. Intriguingly, the mechanism for increased CaMKII expression and activity appears to be splice variant agnostic. Furthermore, all of the CaMKII $\delta$  isoforms, in the absence of RBM20 or in the presence of its disease causing mutants, congregate in the nucleus in RBM20 disease models and knock outs by another untested mechanism. Like many good stories, this one raises more questions than it answers. Nevertheless, it would benefit from further work to understand the basic biology underlying, yet another, model where excessive CaMKII activity promotes myocardial dysfunction and arrhythmias.

We thank the reviewer for their time and their constructive comments.

Specific points:

1. The finding that the splicing function of RBM20 is immaterial to its pathological actions is interesting but, apparently, not unprecedented. Your studies, with some acknowledged limitations, make this point effectively. However, the key issue of how RBM20 mutants or knock out causes increased CaMKII message/protein/activity is not really addressed. The various mRNA analyses show that CaMKII is one of the more upregulated pathways but these studies do not shed light on how such a circuit operates. Is the level of CaMKII message/protein in the RBM20 knockout/knockin models affected by hesperadin treatment? Is the expression of other (non-CaMKII $\delta$ ) RBM20 targets increased similar to CaMKII $\delta$ ?

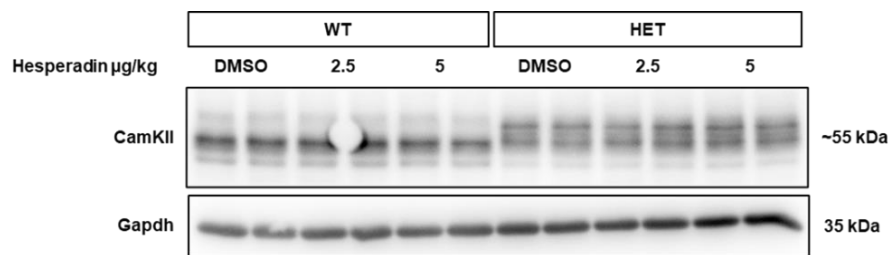
To investigate how CAMK2D is activated, we tried immunoblotting of phosphorylated and oxidized CAMK2D, but we could not confirm specificity of these antibodies as they also showed a signal in CAMK2 KO hearts (PMID: 25124496).

Hesperadin treatment does not alter CAMK2D protein expression, nor does it alter protein expression of other RBM20 targets, but inhibits CAMK2D activity and subsequently phosphorylation of its targets. We have performed immunoblotting of CAMK2 (see reviewer figure 1) and plotted the protein expression of RBM20 and its targets (including CAMK2D) from our proteomic data, and have added this information to the manuscript (Page 10, lines 237-239) and in Supplemental Figure 9.

*"Rbm20 mRNA expression nor RBM20 protein expression or expression of RBM20 targets was affected by hesperadin treatment (Figure 4D, Supplemental Figure 9)."*

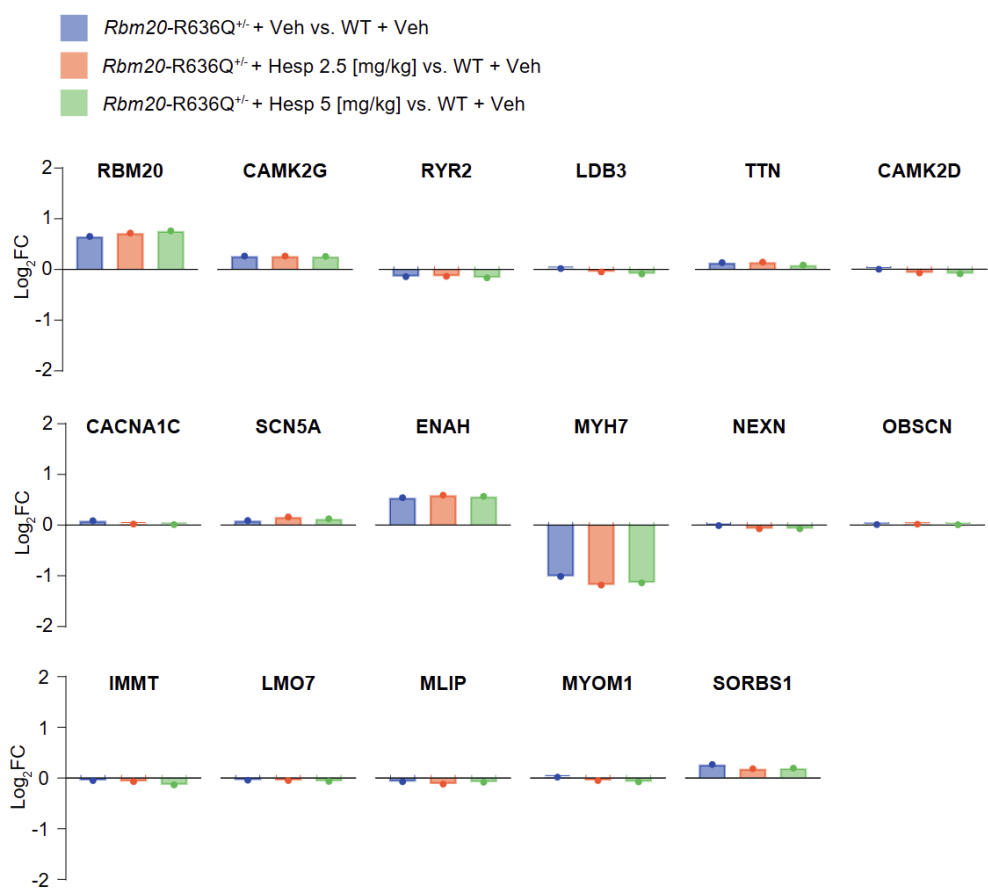
We have not yet identified how CAMK2D is activated in RBM20 cardiomyopathy, but this is currently investigated in our lab. The focus of the current manuscript is to analyse the functional role and contribution of CAMK2D, as well as the translational potential to inhibit CAMK2, in RBM20 cardiomyopathy.

Reviewer Figure 1:



Western blot of CAMK2 in hesperadin treated mouse hearts.

Supplemental Figure 9. Hesperadin treatment does not affect protein expression of RBM20 or RBM20 splicing targets.



Log<sub>2</sub>FC of protein expression from proteomic data of *Rbm20*-R636Q<sup>+/-</sup> mouse hearts with vehicle treatment (blue bars), with 2.5 mg/kg hesperadin treatment (red bars), or with 5 mg/kg hesperadin treatment (green bars) versus wildtype mouse hearts with vehicle treatment.

2. Similarly, RBM20 mutants/knock out cause mislocalization of various proteins. The reader is left wondering if some general principle is at play that explains how seemingly all CaMKIId splice variants aggregate in the nucleus. Of course, a lot is known about CaMKIId splice variants that have or lack an NLS (negatively regulated by phosphorylation) but there is no work to determine if these signals play a role. If there is not a conventional (e.g. NLS-mediated) mechanism for nuclear congregation of CaMKIId isoforms what is happening?

When single splice variants are overexpressed, each splice variant shows characteristic localization which is based on the presence or absence of the NLS in exon 14 (see Reviewer Figure 2). Thus, the increased nuclear localization of endogenous CAMK2D in RBM20 cardiomyopathy seems surprising given that the splice variant that includes the NLS (CAMK2D-B) is even underrepresented (see main figure 1). A discrepancy between overexpressed and endogenous CAMK2D splice variants has also been reported by Mishra et al. (PMID 21998325). Mishra et al. also found endogenous CAMK2D splice variants overrepresented in the nuclear fraction, independent from the inclusion of an NLS.

Overexpression of single cardiac splice variants *in vitro* results in an imbalanced stoichiometry in relation to the endogenous splice variants. Thus, it is likely that, in an endogenous setting, CAMK2D splice variant-independent mechanisms contribute relatively more to the localization of the enzyme.

One possibility is the presence of the NLS of other CAMK2 genes. For instance, in skeletal muscle it has been shown by Ulli Bayer that alpha KAP (which is driven by an intronic promotor in the CAMK2A gene, and includes the association domain of CAMK2A as well as an NLS) can act as a scaffold protein for CAMK2, and can direct CAMK2 to the nucleus (PMID: 9755160 and PMID: 12470297). Alternatively, PTMs or other regulatory mechanisms might affect localization, but this area is currently underexplored and deserves future studies.

Reviewer Figure 2 contained unpublished immunohistochemistry data showing subcellular localization of CAMK2D splice variants in different cell types, and was shared confidentially with the reviewers. Therefore, this data has been removed from the Transparent Review File.

3. The hesperidin studies provide translational credibility but, as the authors note in the Discussion, hesperidin is hardly a CaMKII selective inhibitor. It seems like a missed opportunity not to have tested the potential for interbreeding the CaMKII $\delta$  ko with the Rbm20-R636Q knock in mouse to confirm that hesperidin 'rescue' was due to on target actions.

Indeed, it would be an elegant experiment to cross *Camk2d* KO to the *Rbm20*-R636Q KI mouse line. Unfortunately, the *Rbm20*-R636Q KI and the *Camk2d* KO mouse line come from (and are in) different institutions, which is why we have not been able to cross them yet. However, as we are interested to push the translational angle, we preferred a pharmacological and therapeutic approach to convince pharmaceutical companies to initiate tailored therapies with CaMKII inhibitors.

The answer to this reviewer's question additionally included data from CAMK2 inhibition with a novel and more selective CAMK2 inhibitor in *Rbm20*-R636Q KI mice to address the reviewer's concern, but this data is currently unpublished and was shared confidentially with the reviewers. Therefore, this data has been removed from the Transparent Review File. It is of note, however, that the treatment of *Rbm20*-R636Q KI mice with this more selective CAMK2 inhibitor reproduced the effect seen with hesperidin treatment in the same mouse model.

4. The authors make the point that CaMKII is a specific mechanism for RBM20 cardiomyopathy and thus a novel example of a 'personalized' therapy. This framing is strained by the reality that excessive CaMKII activity is linked to multiple cardiomyopathy models and arrhythmias. This fact should be made clear in the Introduction and/or Discussion.

We agree with the reviewer that increased CAMK2 activity is not specific to RBM20 cardiomyopathy. CAMK2 activation is seen in many heart diseases, but not all. To clarify this, we added data derived from another (new) cardiomyopathy mouse model with the human DCM-causing mutation LMNA-



K117fs, where we do not observe CAMK2 activation, in Supplemental Figure 5. This shows that CAMK2 activation is not a general hallmark of all cardiac diseases. Moreover, activation (as seen in other models) does not necessarily mean causality. In our lab, we used *Camk2* KO mice in different disease models. These data are in preparation for further manuscripts. As the most alarming example, we found that increased CAMK2 activity in HCM is protective in the neonatal phase, since early *Camk2* deletion in HCM mice increases mortality. Deletion of *Camk2* in adult HCM shows a neutral outcome. In Takotsubo cardiomyopathy, CAMK2 activation is also protective since it inhibits Calcineurin signalling (which promotes Takotsubo cardiomyopathy) (PMID: 39195924). In ischemia/reperfusion injury-induced remodelling or in pressure overload, CaMKII activation is causal for contractile dysfunction (PMID: 25193973 and PMID: 25124496). These observations show that CAMK2D activation is not always detrimental, and in fact sometimes protective, in heart disease. Therefore, it is important to do studies as reported here as we believe this is an essential step towards tailored therapies. In fact, RBM20 cardiomyopathy is the first genetic cardiomyopathy now proven to be driven by CAMK2. So far, we have not identified another genetic cardiomyopathy with a similar outcome by CAMK2 inhibition.

Nevertheless, we have changed the wording throughout the text, and added discussion of CAMK2 activation in different forms of cardiac disease.

The new text reads (Page 8, lines 192-195):

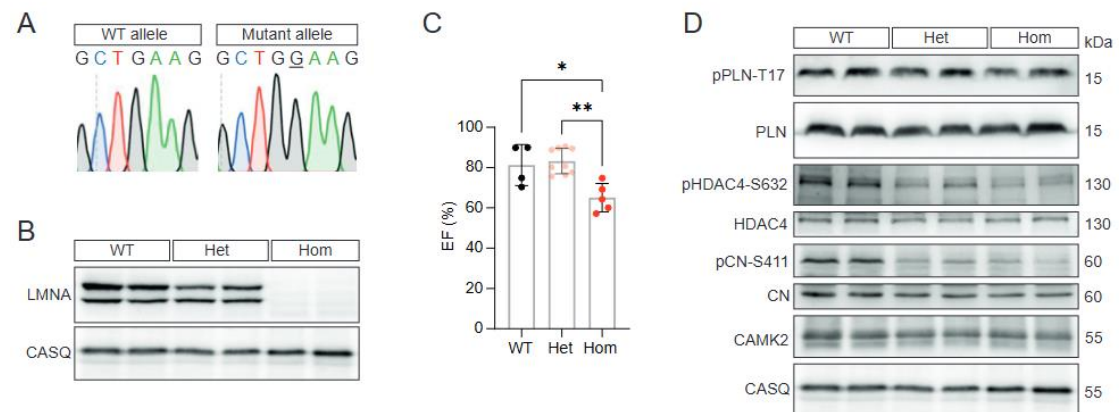
*"Even though CAMK2 activity has been implicated in many forms of cardiac dysfunction, we explored whether CAMK2 activation is a general hallmark of heart disease, and investigated CAMK2 activity in a new mouse model with the human DCM-causing LMNA-K117fs mutation, where we did not observe increased CAMK2 activity (Supp Figure 5)."*

and (Page 13, lines 316-321):

*"In addition, CAMK2 activation contributes to multiple forms of heart disease, e.g. pressure-induced heart failure, post-infarction remodelling, as well as atrial/ventricular arrhythmia [16]. It must be noted, however, that CAMK2 activation is not a general hallmark of all cardiac diseases; e.g. in a new mouse model with the human DCM-causing mutation LMNA-K117fs we do not observe CAMK2 activation. Our data suggest that CAMK2D inhibition can now be tested as a cause-directed DCM therapy for RBM20 cardiomyopathy."*

New Supplemental Figure 5:

Supplemental Figure 5. Mouse model with LMNA-K117fs mutation.



A. Sanger sequencing of wildtype and mutant LMNA allele. B. Western blot of LMNA in wildtype, heterozygous, and homozygous LMNA-K117fs mouse hearts. C. Ejection fraction at 3 weeks of age. D. Western blots of CAMK2D phosphorylation targets.

Reviewer #2 (Remarks to the Author):

Mutations in RBM20 cause an aggressive form of dilated cardiomyopathy (DCM) associated with a high risk of malignant ventricular arrhythmias. In this study, the authors investigated whether CAMK2D is the principal pathogenic effector in RBM20-related cardiomyopathy. Using Rbm20/Camk2d double knockout (DKO) mice, they report that loss of Camk2d protects against heart failure and sudden cardiac death. Rbm20-deficient hearts exhibit both aberrant splicing and overactivation of CAMK2D. Reintroduction of individual CAMK2D splice variants via AAV9 into DKO mice reinstated cardiac dysfunction, independent of splice variant, suggesting that CAMK2D overactivation—not mis-splicing—is the main disease driver. Furthermore, pharmacological inhibition of CAMK2D with hesperadin in Rbm20-R636Q knock-in mice rescued cardiac function and ventricular remodeling. The authors conclude that CAMK2D overactivation is a central driver of RBM20-associated cardiomyopathy and propose that its inhibition could serve as a targeted therapeutic approach for DCM. This is an important, well-conducted, and clearly described study. However, I have several major concerns that should be addressed:

**We thank the reviewer for their time and their constructive comments.**

Major Comments

**1. Insufficient Functional Assessment of Cardiac Protection**

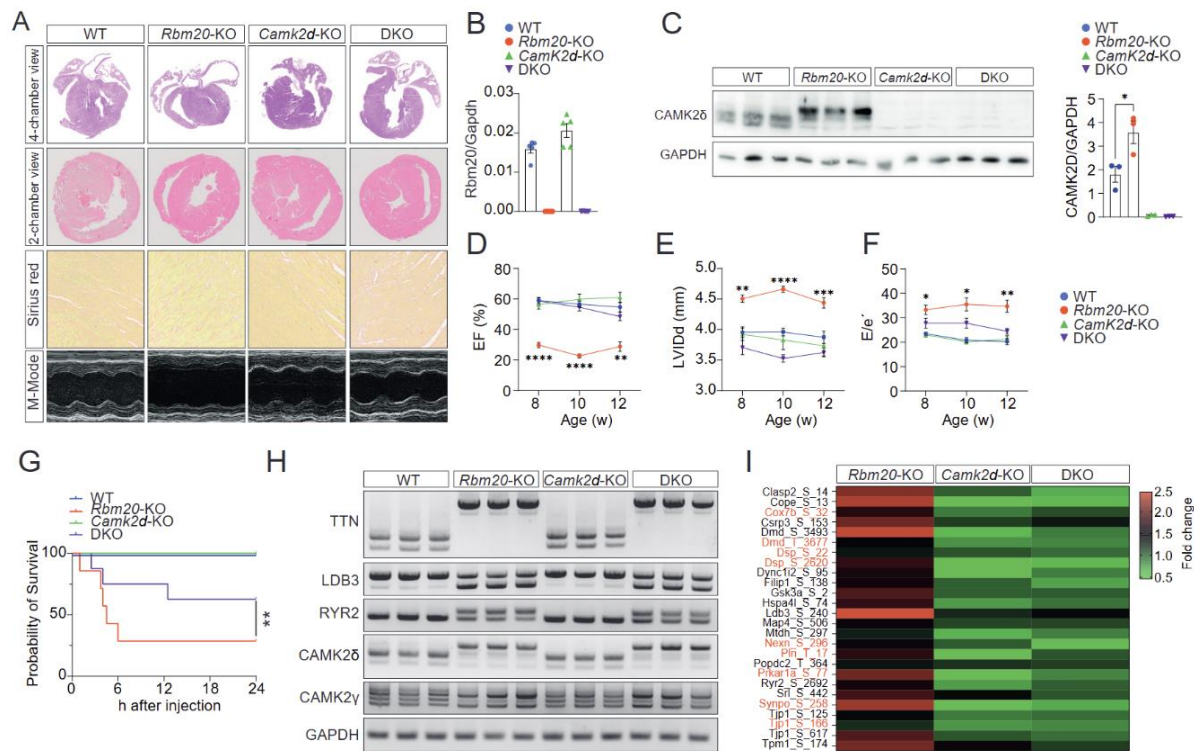
The conclusion that DKO mice are protected from cardiac dysfunction is currently under-supported. The authors rely heavily on echocardiography in anesthetized mice and present only a limited set of parameters (EF, E/E', LVIDd), with E/E' reported at just one time point. This is inadequate for a study drawing such critical conclusions. A comprehensive set of echocardiographic parameters should be provided at all three time points studied.

**We agree with the reviewer that our conclusion that DKO mice are protected against cardiac dysfunction can be better supported. Therefore, we have added another cohort of mice where we have investigated systolic and diastolic function at different time points. We have updated our main Figure 2 with the new systolic and diastolic echo data, and moved the previous cohort to**

Supplemental Figure 2. In this new cohort, we could confirm complete rescue of ejection fraction, a normalization of the E/e' ratio, and a decrease of LVIDd, at all three timepoints measured. Additional echocardiographic parameters can be found in Supplemental Table 1.

In addition, we now provide PV-loop analysis as well as single isolated skinned cardiomyocyte force measurements to substantiate our claims that CAMK2D inhibition in RBM20 cardiomyopathy protects against cardiac dysfunction (see below).

### Updated Figure 2:



A. H&E and Picrosirius Red staining of hearts of WT, *Rbm20* KO, *Camk2d* KO, and *Rbm20/Camk2d* DKO mice. B. qPCR of *Rbm20*. C. Western blotting of CAMK2D in left ventricular tissue. D-F. Cardiac function measured by echocardiography. EF = ejection fraction, LVIDd = left ventricular diameter at diastole. Significance was tested using one-way ANOVA with Tukey's multiple comparisons test. G. Survival after arrhythmia induction. Significance was tested using a Log-rank (Mantel-Cox) test. H. RT-PCR of RBM20 splicing targets. I. Heatmap of differentially phosphorylated proteins that are increased in the *Rbm20* KO, and normalized in the DKO. Proteins in red are known/predicted CAMK2 phosphorylation targets.

## 2. Need for Load-Independent Hemodynamic Data

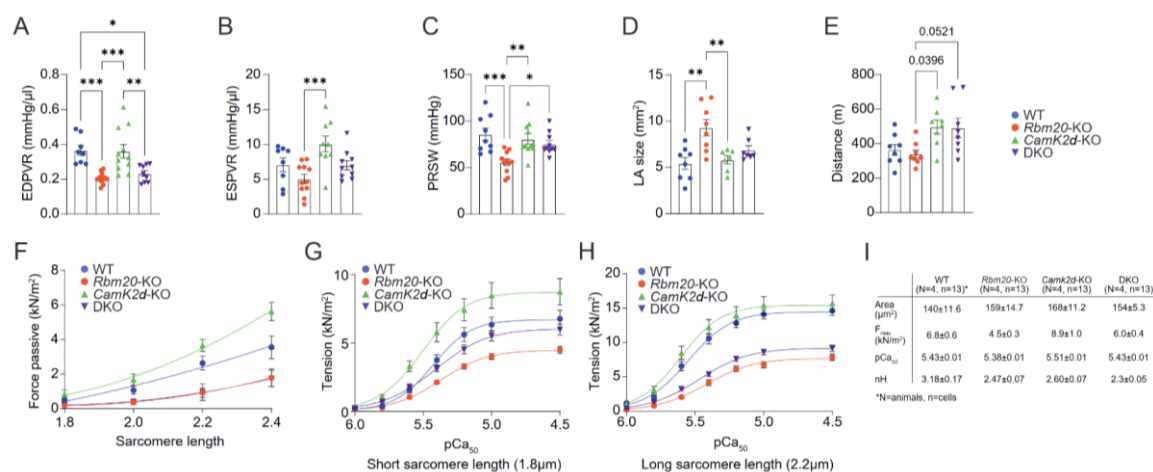
To substantiate the claim of protection against cardiac dysfunction, more robust functional assessments are required. Pressure-volume (PV) analysis should be performed to evaluate both systolic and diastolic function in a load-independent manner. Key parameters such as ESPVR slope (contractility) and EDPVR coefficient (diastolic stiffness) are essential for validating these conclusions.

We have performed PV-loops to evaluate systolic and diastolic function, which has been added to the manuscript in Supplemental Figure 3. With regards to diastolic function, we have observed a decrease in the EDPVR coefficient in the *Rbm20* KO mouse hearts (and the DKO mouse hearts), which is counter-intuitive when looking at the increased E/e' ratio. However, loss of RBM20-dependent splicing

of TTN leads to a much more compliant TTN splice variant, which would explain the increased compliance of the ventricle. This has also been observed by others (see for example PMID: 34851694, reference 12 in the manuscript). We hypothesize that the increased E/e' ratio is secondary to the systolic dysfunction, potentially due to ventricular and atrial dilation and regurgitation in the *Rbm20* KO mice. Passive force measurements of single isolated skinned cardiomyocytes support an increased compliance at the cellular level of *Rbm20* KO and DKO cardiomyocytes,

We have also evaluated systolic performance by evaluating the ESPVR slope and the PRSW (preload-recruitable stroke work), and found a decrease in PRSW in the *Rbm20* KO mouse hearts, which was attenuated in the DKO mouse hearts. PRSW is a relatively load- and size-independent index that reflects preload reserve (Frank–Starling mechanism), and therefore provides a robust measure of chamber contractility. Because ESPVR is not entirely load independent and is influenced by cavity size and geometry, we also normalised the ESPVR slope to end-diastolic volume and obtained similar results to the non-normalised ESPVR. Collectively, these results confirmed that contractility after loss of RBM20 is decreased, and that this is prevented by the loss of CAMK2D. We have substantiated these data by performing force measurements on single skinned cardiomyocytes from these mice, which similarly showed increased compliance (decreased passive force development) in the *Rbm20* KO and DKO cardiomyocytes. We also observed decreased force development (a contractility surrogate) in *Rbm20* KO cardiomyocytes (decreased pCa50 and Fmax), which was alleviated in DKO cardiomyocytes, especially at sarcomere lengths of 1.8  $\mu$ m, which lies within the *in vivo* operating range in mice: ~1.90  $\mu$ m in diastole to ~1.68  $\mu$ m in systole (PMID: 26712849, reference 31 in the manuscript). With the addition of these data, we believe that we show compelling evidence that genetic inhibition of CAMK2D protects against cardiac dysfunction by restoring contractility.

Supplemental Figure 3. Functional measurements of Rbm20/Camk2d DKO mouse hearts/cardiomyocytes.



### 3. Apparent Contradiction in Figure 2E

The conclusion that DKO mice are protected appears inconsistent with the data presented in Figure 2E. This discrepancy should be clearly explained.

We agree that the sentence was not clear, and have amended the text accordingly. The new text reads (Page 8, lines 178-181):

*"To investigate susceptibility to lethal cardiac arrhythmias, we induced arrhythmias by injection of epinephrine and caffeine, and found that while 70% Rbm20 KO mice died within 24 hours, only 37.5% of DKO mice died, indicating that DKO mice were partially protected against sudden cardiac death after arrhythmia induction (Figure 2G)."*

#### 4. Evidence of Diastolic Dysfunction in DKO Mice

The text states that atria are enlarged in DKO mice (line 144), which may suggest diastolic dysfunction. Are atrial tissue weights increased? Please provide this data. In addition, what is the EDPVR coefficient in DKO mice? This would help clarify whether diastolic function is truly preserved.

We have measured atrial size using echocardiography (Supplemental Figure 3, see above), and observe a significant increase in atrial size in the *Rbm20* KO mouse hearts, while this was blunted in the DKO mouse hearts. The EDPVR coefficient is decreased in both *Rbm20* KO and DKO hearts, indicative of increased compliance of the ventricle. We hypothesize that the increased E/e' ratio in the *Rbm20* KO hearts is secondary to the systolic dysfunction, potentially due to ventricular and atrial dilation.

#### 5. Clarification on TTN mis-splicing and Functional Impact

The authors state that RBM20 cardiomyopathy is not a TTN mis-splicing disease (line 239). To convincingly support this, data should demonstrate that DKO mice have preserved diastolic function (via PV analysis and tissue mechanics), maintain a normal Frank-Starling mechanism, and exhibit unaltered exercise capacity.

We agree with the reviewer that this was overstated. The point we were making here is not that TTN mis-splicing does not have an effect on the (function of the) heart, but rather that TTN mis-splicing is tolerated in RBM20 cardiomyopathy if you inhibit CAMK2D.

In general, the fact that truncating *RBM20* mutations in patients and complete loss of RBM20 in KO models lead to DCM and arrhythmia argues that mis-splicing of RBM20-targets contribute to the disease phenotype. However, we suggest here that mis-splicing leads secondarily to CAMK2D activation, which in turn leads to the disease phenotype. We have added this to the manuscript and the new text now reads (Page 12, lines 276-277):

*"This shows that RBM20 cardiomyopathy is not only caused by mis-splicing of RBM20 targets, including TTN, but to a large extent by CAMK2D activation."*

The PV-loop analysis and isometric force measurements of skinned cardiomyocytes further show that compliance of the ventricles is increased in both *Rbm20* KO and DKO mouse hearts, at the tissue and cell level. Exercise tolerance is increased in *Camk2d* KO and DKO mice (Supplemental Figure 3).

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#### Minor Comments

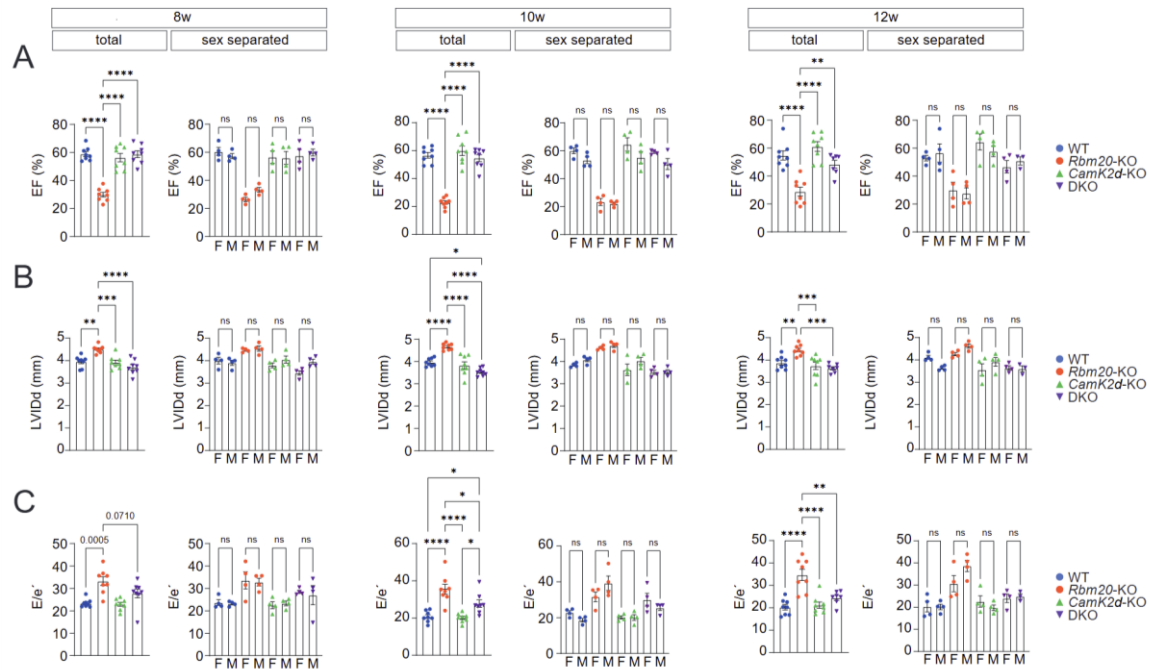
##### 1. Sex of Mice

What is the sex of the mice used in this study? If both males and females were included, data should be analyzed and presented separately by sex.

The first cohort of DKO mice was done with only male mice, but in our new cohort we have included both male and female mice. The data has also been analysed and presented separately by sex, and we

have seen no differences between sexes, at any timepoint (Supplemental Figure 1). This is in line with our previous observations that mouse models of RBM20 cardiomyopathy do not present with sex differences (PMID: 36367695).

### Supplemental Figure 1. Echocardiography of *Rbm20/Camk2d* DKO mice.



A. Ejection fraction (EF) as measured by echocardiography at 8, 10, 12 weeks of age. B. Left ventricular internal diameter at diastole (LVIDd) as measured by echocardiography at 8, 10, 12 weeks of age. C. E/e' ratio Left graph (total) is all mice together, right graph is sex separated. Significance was tested with a one-way ANOVA with Tukey's multiple comparisons test.

## 2. Missing Data Quantification

In several places, data quantification is lacking. For example, in Figure 3B.

We have added quantifications of Figure 2C and Figure 3B, which have been added to the respective figures.

## 3. Statistics.

More detailed information is needed. For example was multiple testing correction performed in Figure 3C.

We indeed used multiple testing correction (Tukey's multiple comparisons test) and have added this information to the figure legends.

Figure 4B bottom is described in the text as if significant changes are seen in R636Q mice upon Hesperadin treatment but no significance is shown on the Figure.

We agree with the reviewer that this text was misleading, as the decrease in diastolic volume is not significant, and we have changed the text accordingly.

The new text reads (Page 10, lines 234-236):



*"In addition, we observed a trend in reduced diastolic volume in the Rbm20-R636Q KI mouse hearts after treatment, suggesting beneficial effects on structural remodelling (Figure 4B)."*

Reviewer #3 (Remarks to the Author):

This paper by van den Hoogenhof et al provides strong and convincing evidence for its interesting conclusion: that the heart failure in RBM20 cardiomyopathy is mediated by CaMKIIdelta. Interestingly, while RBM20 is a splice factor, the CaMKIIdelta effect does not appear to be caused by direct misregulation of CaMKIIdelta splicing, but by some form of splice-variant-independent overactivation. Notably, in turn, this mechanism then provides the opportunity for therapeutic intervention with pharmacology. Regarding the use of CaMKII inhibitors, the study exceeds the recently proposed standard to ensure CaMKII-specificity of the effect of the effects of pharmacological studies (see Brown and Bayer, 2024; which points out that the effect with one inhibitor should be backed up by at least one other inhibitor of a different class, whereas the previously typically demanded inclusion of an "inactive control compound" is much less useful). Although the current study uses only one single inhibitor, the back-up provided here is even better: CaMKIIdelta knockout and re-expression studies, which provides even more selectivity and specificity. However, while the experimental approach exceeds standards, the text should include better and more extensive description and discussion of the inhibitor used (especially since it is a somewhat unusual inhibitor that has not been used much in context of CaMKII previously).

*We thank the reviewer for their time and their constructive comments.*

Specific comments.

1) It should be pointed out that the inhibitor used here (hesperadin) is an ATP-competitive inhibitor (i.e. one of three principally different classes of CaMKII inhibitors). This is important, as the different classes can have distinct additional effects on CaMKII (i.e. other than on enzymatic activity; as reviewed in Brown and Bayer, 2024).

*We have added this information in the text.*

2) There should be some discussion that hesperadin has an unusually high selectivity for the CaMKIIdelta isozyme (Zhang et al 2022; ref. 31; in this context, this reference should also be cited at the first mentioning in the results section in line 195, rather than in the next sentence that describes the in vivo dosing). However, additionally, it needs to be pointed out that hesperadin is not all that CaMKII-selective: In addition to inhibiting Aurora kinase B (as mentioned and cited; ref 31), it inhibited some six other kinases out of a panel of twenty five, i.e. almost 25% (Hauf et al 2003, JCB, PMCID: PMC2172906; this needs to be cited).

*We agree with the reviewer that it is important to point out that Hesperadin is not CAMK2-selective, and that our study, in that regard, can only be used as a proof-of-principle. We have added this to the text, and have added this reference.*

*The new text reads (Page 13, lines 300-310):*

*"The use of hesperadin to inhibit CAMK2D in patients may not be favourable because even though it inhibits CAMK2D more than other CAMK2 isozymes (20-200 fold more selective), it also inhibits other kinases such as Aurora kinase B, AMPK, Lck, MKK1, MAPKAP-K1, CHK1, and PHK [33, 36]. The current study, however, serves as a first proof of principle study that now calls for validation with a selective*

*CAMK2 inhibitor. After 35 years work on CAMK2 and the development of multiple CAMK2 inhibitors (see for review [37]), there has not been a CAMK2-selective inhibitor that made it to clinical practice. The only CAMK2 inhibitor that is currently used in clinical practice is Ruxolitinib, but this is for its better known role as a JAK inhibitor in patients with myelofibrosis [37-39]. Therefore, it comes with great promise that a first specific and selective CAMK2 inhibitor is currently in a phase II clinical trial (clinicaltrials.gov, identifier NCT06005428) [40, 41]."*

3) The discussion contains a statement regarding CaMKII inhibitors that is highly misleading on several levels (line 263-265: "It comes with great promise that after 35 years work on CAMK2 and several attempts to develop a specific CAMK2 inhibitor, a first inhibitor is in a phase II clinical trial (clinicaltrials.gov, identifier NCT06005428) [34, 35]"). First (and least importantly), the sentence seems to talk about selectivity rather than specificity. It also suggests that there have not been any selective (or specific) CaMKII inhibitors until now; this is far from correct (as reviewed, for example, in Brown and Bayer, 2024). The sentence also seems to imply that selectivity is directly connected with being in clinical trials. This is not the case. Instead, a successful drug has to be safe and effective; while selectivity may help with that, it does not necessarily do so. It is possible to be safe and effective despite hitting other targets. Vice versa, if hitting the intended target causes toxicity, it does not matter how selective the drug is. Finally, the sentence implies that this is the first CaMKII inhibitor in clinical trials, but this is not the case either. As it turns out, ruxolitinib is an ATP-competitive CaMKII inhibitor (Reyes Gaido et al., 2023, Sci Transl Med 15:eabq7839) and has been in the clinic for its better known role as JAK inhibitor.

We agree with the reviewer that this statement was somewhat misleading, and we have amended the statement to properly reflect the status and advantages of CAMK2D inhibition in patients.

The new text reads (Page 13, lines 300-310):

*"The use of hesperadin to inhibit CAMK2D in patients may not be favourable because even though it inhibits CAMK2D more than other CAMK2 isozymes (20-200 fold more selective), it also inhibits other kinases such as Aurora kinase B, AMPK, Lck, MKK1, MAPKAP-K1, CHK1, and PHK [33, 36]. The current study, however, serves as a first proof of principle study that now calls for validation with a selective CAMK2 inhibitor. After 35 years work on CAMK2 and the development of multiple CAMK2 inhibitors (see for review [37]), there has not been a CAMK2-selective inhibitor that made it to clinical practice. The only CAMK2 inhibitor that is currently used in clinical practice is Ruxolitinib, but this is for its better known role as a JAK inhibitor in patients with myelofibrosis [37-39]. Therefore, it comes with great promise that a first specific and selective CAMK2 inhibitor is currently in a phase II clinical trial (clinicaltrials.gov, identifier NCT06005428) [40, 41]."*

4) Somewhat minor, but it might be helpful to include a specific statement that hesperAdin should not be confused with hesperIdin. This is because google searches of one compound will also show results for the other. Even worse, there is a review article (that focused on ref 31) that mentions hesperidin and even shows the structure for hesperidin, but otherwise talks about the effects described for hesperadin instead, such as being an inhibitor of Aurora kinase B and CaMKII.

We agree with the reviewer and have added a statement in the text (Page 10, line 228). As a side note, not only google shows results of hesperidin when looking for hesperadin, PubMed also prefers to show studies of hesperidin when looking for hesperadin, due to the larger amounts of studies on the first compound.



## Response to reviewers

### Reviewer #1:

Remarks to the Author:

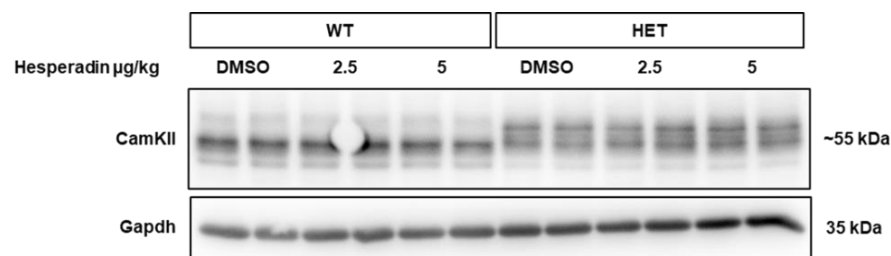
Thank you for your contribution

Please consider the following

The immunoblot data detecting CaMKII expression, included in the response document, after hesperadin shows a prominent doublet that is not present in the absence of hesperadin. The doublet may signify a PMT or another CaMKII splice variant or isoform. Please comment and, ideally, identify the new band. These data seem at odds with the contention that hesperadin has no action on CaMKII expression.

We thank the reviewer for their time to evaluate our revised manuscript. In this instance, it seems that the reviewer has misread the immunoblot. The immunoblot in question is provided below. The doublet is not dependent on hesperadin treatment, but rather on the genotype of the mice. In the heterozygous Rbm20-R636Q KI mice (HET), we observe missplicing of CAMK2D, and the doublet seen in these mice represent CAMK2D-4 and CAMK2D-A. This is in line with main Figure 1 of the manuscript, and with published literature (see for example PMID: 29650543 or PMID: 22466703). Hesperadin treatment does not seem to have an effect on CAMK2D expression, as seen in Reviewer Figure 1 and Extended Data Figure 9.

### Reviewer Figure 1:



Western blot of CAMK2 in hesperadin treated mouse hearts.

I think the revised statement - It must be noted, however, that CAMK2 activation is not a general 318 hallmark of all cardiac diseases; e.g. in a new mouse model with the human DCM-causing mutation 319 LMNA-K117fs we do not observe CAMK2 activation - should be amended; please substitute 'universal' for 'general'. Although the authors provide an interesting example of myocardial disease not improved by CaMKII inhibition, the broader/general reality is that excessive CaMKII activity is a feature of a remarkable preponderance of clinically-relevant acquired and genetic forms of myocardial diseases.

We have substituted 'universal' for 'general'.

### Reviewer #2:

None

### Reviewer #3:

Remarks to the Author:

The authors have fully addressed my comments.