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Peer Review File

The G4 Resolvase Dhx36 Modulates Cardiomyocyte Differentiation and Ventricular Conduction System Development

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have extensively revised their manuscript with addition of much new data. They have fully addressed all my ealrier points in their detailled rebuttal letter. This mansucript provides new insights into how gene expression is controlled by Dhx36 in the developing heart.

I have one further minor point: for the logic of the discussion on page 24 the authors should discuss and reference the known role of Nkx2-5, protein levels of which are Dhx36-dependent, in Purkinje network development.

Reviewer #2 (Remarks to the Author):

The authors have added a large amount of new data to revise this study, which tremendously improved its quality. In the current form, the conclusions are well apprehended and convincing to expand the understanding of Dhx36/RHAU/G4R in heart development, particularly, in cardiomyocyte differentiation and ventricular conduction system (VCS).

Reviewer #3 (Remarks to the Author):

The authors have made a genuine attempt to answer my prior comments. However, several issues still remain. First, because the Cre drivers used in the study function in all cardiomyocytes, no specific conclusions can be drawn about cell-autonomous phenotypes in the conduction system. For example, it remains possible that ventricular cardiomyocytes are dysfunctional, and the lack of a secreted factor indirectly influences VCS function. Second, the striking absence of Purkinje Fibers still appears inconsistent with the relatively mild alternation in QRS duration. In the absolute absence of the VCS, one would expect to see severe bundle branch block. Since Fig 4 only shows a zoomed in view, it is difficult to tell whether to expect RBBB or LBBB or both. An alternative possibility is that there Dhx36 directly regulates Cntn2 expression, in which case the use of an alternative marker is critical, although this is not consistent with the snmulti-ome data. Third, the authors shift away from Hcn4 as a mechanistic downstream target, but the new multi-ome data is largely descriptive and can only nominate potential targets. This needs to be followed up with immunostaining or in situ hybridization analysis. Furthermore, although the multi-ome data support the idea that the VCS is missing, there is no explanation for this observation. This missing

information seems critical to explain the histological (and multi-ome) phenotype, despite the fact that the EKG phenotype appears incongruent.

Point-by-point answer to reviewer comments:

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I have one further minor point: for the logic of the discussion on page 24 the authors should discuss and reference the known role of Nkx2-5, protein levels of which are Dhx36-dependent, in Purkinje network development.

ANSWER: We appreciate the reviewer's comments and are grateful for the very constructive feedback and suggestions that have significantly improved the quality and relevance of our manuscript. Regarding the minor point, we have now discussed this in the Discussion section (**second paragraph on page 25**) about the role of Nkx2-5 protein levels in Purkinje network development, adding two more references (**PMID: 15085192, PMID: 25053429**). Please also check response to the second point of Reviewer #3.

Reviewer #2 (Remarks to the Author):

The authors have added a large amount of new data to revise this study, which tremendously improved its quality. In the current form, the conclusions are well apprehended and convincing to expand the understanding of Dhx36/RHAU/G4R in heart development, particularly, in cardiomyocyte differentiation and ventricular conduction system (VCS).

ANSWER: We thank the reviewer for her/his positive comments and thoughtful suggestions and criticisms throughout the review process, which have substantially helped us to improve this work.

Reviewer #3 (Remarks to the Author):

The authors have made a genuine attempt to answer my prior comments. However, several issues still remain.

First, because the Cre drivers used in the study function in all cardiomyocytes, no specific conclusions can be drawn about cell-autonomous phenotypes in the conduction system. For example, it remains possible that ventricular cardiomyocytes are dysfunctional, and the lack of a secreted factor indirectly influences VCS function.

ANSWER: We appreciate the reviewer's feedback on our manuscript throughout the evaluation process.

While we agree that our data do not allow for firm conclusions about cell-autonomous phenotypes in the conduction system, they suggest that the Purkinje fiber network morphogenesis depends on the presence of Dhx36 in the surrounding ventricular cardiomyocytes during VCS development. We agree with the reviewer´s suggestion that secreted factors from surrounding cell types might influence VCS morphogenesis in our mutants. In this regard, we mentioned in the Discussion section that Netrin1 could serve as one such candidate; it is a secreted factor transcriptionally regulated by Dhx36 in working cardiomyocytes, which in turn regulates the Slit/Robo1 axis expressed in VCS cardiomyocytes. For Myh6^{Cre} and MCK^{Cre}, for which Cre activity occurs

later, *Dhx36* mutant mice show a much less defective VCS, suggesting that the already formed VCS may no longer depend on Dhx36 activity in the surrounding environment.

Other potential secreted factor candidates known to regulate cardiac conduction system formation, such as neuregulin 1 (Nrg1; PMID: 12149465) and endothelin 1 (Edn1; PMID: 15759273), are secreted by endocardial cells. Indeed, in our sn-multiome data, we observed an increased population of endocardial endothelial cells expressing *Nrg1* and *Edn1* in our cKO mice, as well as a distinct population of fibroblasts expressing *Nrg1* (please see the **figure provided for the Reviewer**). Furthermore, our new *in situ* hybridization (ISH) experiments clearly show *Nrg1* upregulation in both E16.5 embryos and PD7 mutant hearts **(new Fig. 4c-g and Supplementary Fig. 2).** This expanded expression occurs in the mutant heart as a likely compensatory response to the defective morphogenesis of the VCS. We have now included and discussed these issues in the Results section of the revised manuscript (page 11, second paragraph). The multiomic data were not included in the previous version, which focused on the effects of *Dhx36* deletion in working cardiomyocytes (as previuosly requested by the reviewers); however, we are willing to include it as Supplementary Material. Nonetheless, we believe it would be redundant with the *Nrg1* ISH data that we have now incorporated in the revised version. We think that our conclusions are very strong because we have studied five different models of genetic inactivation of Dhx36 function: four cardiomyocyte-specific Cre drivers targeting different developmental stages, *Nkx2.5*^{Cre} or *Tnnt2*^{Cre} (embryonic cardiomyocytes), *Myh6*^{Cre} (fetal to adult cardiomyocytes), and *MCK*^{Cre} (late fetal to mature cardiomyocytes and skeletal muscle); and an inducible first heart field (FHF) and CCS-specific *Hcn4*^{CreERT2} driver (PMID: 23743334), to induce *Dhx36* deletion, which unexpectedly did not lead to a defective VCS phenotype or ECG defects (results summarized in the response to Reviewer 2 of our previous revised version). Investigating the potential cell autonomous and non-autonomous phenotypes and the concomitant secreted factors influencing Dhx36-dependent VCS development would indeed be of interest. However, we believe the Reviewer will agree that this would constitute a separate and substantial research project, far beyond the scope and timeframe of this study—such a project would require over two years of work and additional conditional KOs and inducible Cre drivers to generate clones of tagged mutant cells.

Second, the striking absence of Purkinje Fibers still appears inconsistent with the relatively mild alternation in QRS duration. In the absolute absence of the VCS, one would expect to see severe bundle branch block. Since Fig 4 only shows a zoomed in view, it is difficult to tell whether to expect RBBB or LBBB or both. An alternative possibility is that there Dhx36 directly regulates Cntn2 expression, in which case the use of an alternative marker is critical, although this is not consistent with the snmulti-ome data.

ANSWER: The reviewer's second concern questions the relatively mild alteration in the QRS interval given the striking absence of Purkinje fibers. In our opinion, shared by our cardiology collaborators and experts in arrhythmias in animal models and patients, we consider the electrical abnormalities of the mutants sufficiently severe and compatible with the VCS phenotype. Nevertheless, the scientific literature review on the roles of other genes implicated in VCS morphogenesis and their effects on the electrical phenotype revealed

several studies in KO mice demonstrating that mutations affecting CCS architecture lead to QRS interval changes similar to those observed in our *Dhx36* cKO mice (reviewed in PMID: 34436237).

The most extensively studied gene in this context is *Nkx2-5*, where mutations or deletions result in VCS hypoplasia or its near absence. These mutations induce a QRS interval increase from 12.9 ±0.3 ms in WT, to 15.7 ±0.7 ms in mutants, at 7 weeks of age (see their Table 1, PMID:15085192), which is milder than in our *Dhx36* cKO mice (Fig. 2). Additionally, *Nkx2-5* mutant mice develop progressive atrioventricular block, exacerbating with age, mirroring our mutant mice. Heterozygous *Nkx2-5* animals that survive past one year exhibit a greater QRS complex prolongation (from 12.5 ±0.3 ms in WT to 17 ±0.4 ms in mutants; see their Table 1: PMID:15085192). Similarly, a murine model of human congenital heart disease with a missense mutation in *Nkx2-5* showed progressive atrioventricular blocks and significant QRS interval prolongations by 17 months, though less pronounced than in our mice (see their Fig. 2: PMID: 26226998).

Other genes implicated in CCS morphogenesis, such as *Etv1* and *Irx3*, present phenotypes similar to or milder than our *Dhx36* cKO mice. *Etv1* KO mice exhibit near-total absence of the His-Purkinje system (VCS), with a QRS interval increase from 10.6 ms in WT to 13.4 ms in KO mice aged 10-12 weeks (Fig. 2, PMID: 29967479). *Irx3* KO, with virtually non-existent VCS and progressive AV blocks (PMID: 26786475), show QRS intervals ranging from 10.4 ms in WT to 14.2 ms in 8-week-old mutant mice (Fig. 2, PMID: 21825130).

The transcription factors Tbx5 and Id2 also play roles in VCS formation, affecting QRS intervals in *Id2* KO (PMID: 15289437) and synergizing when co-deleted with *Tbx5* or *Nkx2-5* (PMID: 17604724). Furthermore, *Ncam-1* KO mice result in almost complete loss of Purkinje fibers, with a moderate (less than 20%) QRS interval increase (see their Fig. 3A; PMID: 34100064).

These studies in mice models collectively demonstrate that an increase in the QRS interval faithfully reflects the degree of VCS hypoplasia, aligning with the hypoplasia observed in our animal models. It seems evident that both mice and humans can generate alternative circuits to compensate for the total loss of a well-structured VCS, as otherwise they would succumb to sudden death much earlier than observed.

Regarding the difficulty in determining whether to expect RBBB or LBBB, or both, the Cntn2 whole-mount staining only exposes the left ventricle (PMID: 23526457). Due to the limited number of mice available, we have not been able to repeat these experiments. However, we have addressed this question by *in situ* hybridization (ISH) and have shown that the expression of the *Etv1*, *Slit2*, *Irx3*, and *Gja5* CCS markers is impaired in both left and right ventricles **(Fig. 4c-g and Supplementary Fig. 2).**

Third, the authors shift away from Hcn4 as a mechanistic downstream target, but the new multi-ome data is largely descriptive and can only nominate potential targets. This needs to be followed up with immunostaining or in situ hybridization analysis. Furthermore, although the multi-ome data support the idea that the VCS is missing, there is no explanation for this observation. This missing information seems critical to explain the histological (and multi-ome) phenotype, despite the fact that the EKG phenotype appears incongruent.

ANSWER: Following the reviewer´s suggestion, we performed ISH on hearts from E16.5 embryos and 7-day-old mice. We were able to detect weak *Hcn4* expression in WT PD7 hearts, but not in PD7 mutant hearts (**Fig. 4f**).

Furthermore, we extended our ISH analysis to other CCS markers, some of which had been previously suggested by the reviewer, such as *Etv1*, *Slit2*, *Irx3*, and *Gja5.* These experiments revealed defective gene expression, strongly suggesting impairment in the development of the cardiac conduction system in E16.5 mutant embryos, and the absence of the VCS in PD7 mutant hearts (Fig. 4c-g and Supplementary Fig. 2). In the embryonic mutant hearts, we observed reduced transcription of *Etv1, Gja5*, *Slit2, and Irx3,* relative to WT (**Supplementary Fig. 2a-f**). In PD7 WT hearts, we detected *Etv1*, *Gja5, Slit2,* and *Hcn4* transcription in the VCS, but not in the mutant hearts, suggesting a lack of VCS and supporting the results obtained with Cntn2 whole-mount and multiomic data (**Figs. 4b and 6).**

These set of experiments further supports that Dhx36 controls the development and morphogenesis of the CCS, providing information that aligns with the histological (and multiome) phenotype, as also suggested by the reviewer. We also believe that these data, despite evidencing severe defects in VCS architecture, are consistent with the EKG phenotype of our mutant mice (similar to that of other mutant mice of relevant genes implicated in VCS morphogenesis, as discussed above). Although our data strongly support an absence of the VCS, we lack complete experimental certainty. We have revised our initial statement throughout the text to now state that the VCS phenotype is hypoplastic rather than completely absent.

Finally, addressing the reviewer's concerns, we have confirmed the direct regulation of *Hcn4* and *Cntn2* transcription by Dhx36 with additional luciferase experiments. Despite initial technical difficulties due to the high GC content, we successfully cloned the promoters of *Hcn4* and *Cntn2*, which possess G4 structures (**see sheet II, Supplementary Table 9**). We now show that these promoters are transcriptionally active and respond to the resolution of G4 structures by Dhx36 (panels added to **new Fig. 9b**).

We believe that these experimental efforts have helped to clarify our findings and improve the manuscript, and we hope they satisfactorily address the reviewer’s concerns.

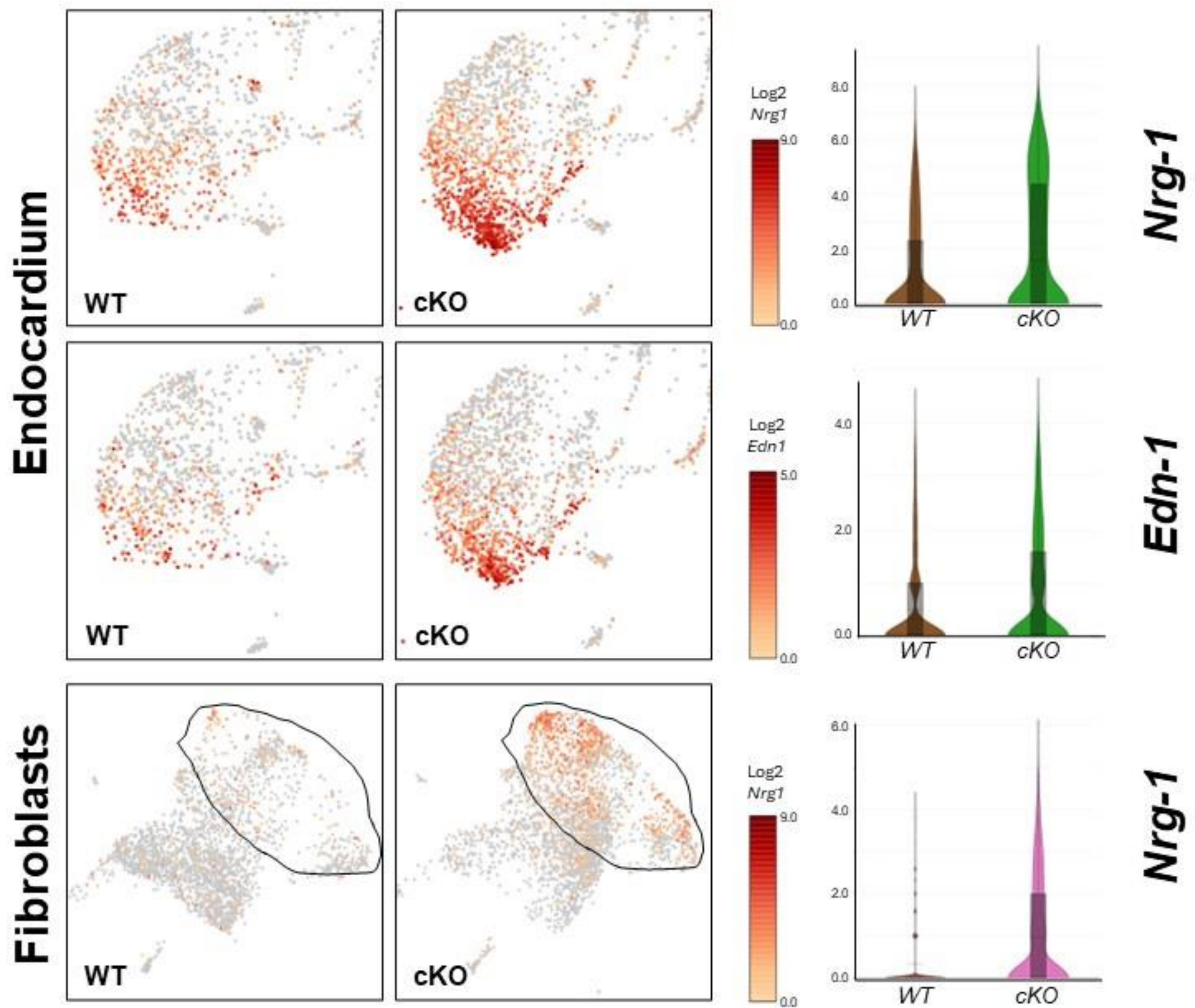

Figure for Reviewer #3: UMAP plots from multiomic analyses depict the RNA expression patterns of *Nrg1* and *Edn1* in both WT (left) and $Dhx36^{Tnnt2}$ (cKO) mutant hearts (right), focusing on the endocardial endothelial cells and fibroblasts. Right, violin plots of the corresponding expression.