nature portfolio

Peer Review File

Dysregulation of N-terminal acetylation causes cardiac arrhythmia and cardiomyopathy

Corresponding Author: Professor Vassilios bezzerides

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have revised and improved the manuscript 'Dysregulation of N-terminal acetylation causes cardiac arrhythmia and cardiomyopathy' according to previous reviewer comments.
This manuscript presents important data on the impact of NAA10 and the NatA N-terminal acetyltransferase on human physiology, and more specifically how defective NAA10 causes cardiac rhythm disturbances.

Additional minor comments:

1. Line 25: Correct nomenclature for predicted protein change is p.(R4S) with the "p." outside the parenthesis.

2. Line 27: "Here we show that the NAA10R4S variant reduced enzymatic activity, decreased expression levels of NAA10/NAA15 proteins, (…)"

The authors have only showed decreased NAA10 expression levels in Fig. 3a. Remove NAA15 from this sentence (or include NAA15 blot in Fig. 3A showing decreased NAA15 levels),or re-write to "decreased NAA10-NAA15 complex formation" if this is what the authors mean to convey (as shown in Fig. 2c).

3. Figure 2c: Please include lysates next to immunoprecipitated samples in this panel.

4. Line 42-44: "N-terminal-acetyltransferase A (NatA), one of seven mammalian NATs (NatA, NatB, NatC, NatD, NatE, NatF and NatH) with distinct substrate specificities2 preferentially acetylates G, S, A, T, or V residues exposed at protein N-termini after iMet removal3."

NatA also Nt-acetylates Cys-starting N-termini after iMet cleavage (PMID: 38918375) so include 'C' here. There should also be a comma after "specificities" to separate the clauses in this sentence.

5. Line 59: "Recent large-scale exome sequencing projects of patients with congenital heart disease identified several de novo NAA15 variants17."
This study identified both inherited and de novo NAA15 variants.

6. Line 100: "In silico analysis of existing high-resolution structures of the NAA10-NAA15 complex22 (…)"
Should cite PMID: 29754825 since the PDB structure 6C9M was used.

7. Extended Data Fig. 2A "After administration of cyclohexamide to inhibit protein translation, the NAA10 protein was measured by a specific antibody and normalized to vinculin."
This sentence is repeated in the legend, please correct.

8. Extended Data Fig. 8 showing qPCR data: Figure is mislabelled as Extended Data Fig. 6, please correct to Fig. 8.

9. Line 303: “Consistent predictions and previous reports, the majority of Nt-acetylated proteins were substrates for NatA (Fig. 8a, b).”
Correct typo; “Consistent with”

10. Line 339: “While predominantly described in males, NAA10-related syndrome cases have been described in heterozygous females12,14.”
Please re-write. This statement is incorrect, there are more female than male cases described to date. For example, one recent study (PMID: 37130971) described 48 female cases and 8 male cases.

11. Several places in the manuscript: please change ‘mass spectroscopy’ to ‘mass spectrometry’ which is the correct term.

12. For all mass spectrometry data supporting Figure 8, please adjust supplemental data for better readability and understanding. Add tables to clearly show which proteins are detected as Nt-acetylated, N-terminal sequence etc. As it stands, it is difficult for the general reader to get an overview of the data and to verify the statements made in the text. Also remember to refer to each supplemental Figure and Table in the main text.

13. Line 304: “The remaining subset of identified proteins were predicted to be substrates for NatB based on the alpha-peptide sequence of either ME, or MD (Fig. 8c).”

Please double-check and verify that no NatC/E/F-type proteins were detected.

14. Line 310: “To determine if the NAA10-R4S variant impacts NatA enzymatic activity in cardiomyocytes, we normalized Nt-acetylated peptides by mass-spectroscopy to internal peptides for each identified protein and ranked them by the degree of Nt-acetylation (Fig. 8f). While not all the original set of Nt-acetylated proteins could be normalized, we demonstrated that 68% of NatA targets showed a reduction in Nt-acetylation (Fig. 8g). Proteins associated with nuclear envelope structure or transport including RAN and MATR3 and structural proteins including FHL1, SPTAN1, and CFL2 demonstrated decreased Nt-acetylation in NAA10R4S/Y-iPSC-CMs (Fig. 8f). These data support the hypothesis that the NAA10-R4S variant significantly impairs the function of NatA and reduces global Nt-acetylation in affected iPSC-derived CMs.”

Please re-write to make it clear that SPTAN1 is not a NatA substrate (but a NatB substrate).

15. Line 318: “In addition to the identification of differentially Nt-acetylated proteins in our NAA10R4S/Y-iPSC-CMs, we sought to investigate the impact of NatA dysfunction on total protein expression. Quantitative mass-spectroscopy of total proteins isolated from WT and eNAA10R4S/Y-iPSC-CMs demonstrated differential expression of 84 proteins (Fig. 8h).”

Please make sure that these data are properly and clearly presented in supplemental and referred to.

16. Please assess all affected NatA substrates in NAA10 R4S cells (both specific proteins mentioned in the text, and the whole dataset with reduced Nt-acetylation) and their abundance (up, down, or no regulation) and discuss these data in the context of the N-degron pathways: whether or not proteins with less Nt-acetylation seems to be more or less stable (see for example: PMID: 35145090, PMID: 33523899, PMID: 37891180, PMID: 38519774, PMID: 39264755)

Reviewer #4

(Remarks to the Author)
The authors have submitted a revised manuscript with sufficient revisions. The authors adequately addressed all of my concerns and present an exciting study that will make a meaningful impact on the field.

Reviewer #5

(Remarks to the Author)
After evaluating Reviewer 3’S concerns, and as requested, my overall comment and evaluation of the rebuttal is that the authors have a made significant effort in addressing the concerns of Reviewer #3. My comments on the authors’ rebuttal on the two main concerns of the reviewer are listed below in blue font. Importantly, as noted in their rebuttal of Reviewer #3 concern1, the authors should reconstruct (and be less categorical) on the sentence on lack of QT prolongation and should refer to the relevant article provided therein (PMID 26256814). Otherwise, this rebuttal overall, is fine by me.

Important concerns of Reviewer #3 in the authors’ revision:

Concerns of reviewer #3’s (black font)
Authors’ rebuttal (red font)
My comment on adequacy of author’ rebuttal (blue font)

(Concern 1)
That the mechanistic link between calcium dysregulation and the APD and arrhythmic phenotype remains unexplored. This is critical to do so that a complete mechanistic picture can be formed and two phenotypes, one of APD alteration, and one of calcium overload, can be reconciled. For example, how does the elevated intracellular calcium lead to APD alterations (or does it?). Is this the cause of the arrhythmias?

(Authors' response)
Based on our data and the literature, elevated diastolic calcium levels do not contribute to AD prolongation. This is supported by the observation that there is no QT prolongation in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), despite clear evidence of increased calcium leak through the RyR2 channel and elevated diastolic calcium levels (PMC6750809 and PMC10311407).
My Comment:
The authors (Yoshinaga et al) should be less categorical on the comment on elevated diastolic calcium and APD prolongation given changes in, and elevation of intracellular calcium can in some instances lead to changes in APD and arrhythmogenesis.
Importantly, I disagree with a statement above regarding a lack of QT prolongation in patients with CPVT because it is not strictly correct! Although for the most part, QT interval in CPVT patients is typically normal, it has been reported however, that some CPVT patients with pathogenic RyR2 mutations have significant QT prolongation and, in a few such cases, may have been misdiagnosed as concealed long QT syndrome (LQTS), given that both diseases (LQTS as CPVT) are associated with syncope or sudden death following stress or swings in emotional states (PMID: 26256814) Interestingly, for the treatment of such CPVT patients showing QT prolongation, the normalization with flecainide perhaps may be relevant and supportive of authors' contention on the role of ion channel dysfunction in APD prolongation underlying the arrhythmogenesis they investigated.
Our data strongly supports that APD prolongation is caused by the combination of increased late INa and decreased IKs which are both canonical mechanisms of APD prolongation and increased risk of arrhythmogenesis, as in long QT syndrome.
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For the most part, I agree with the rebuttal above)
Elevated intracellular calcium levels are frequently associated with heart failure and HCM but not directly to APD prolongation We demonstrated that diastolic calcium levels were elevated secondary to impaired Ca reuptake and extrusion as observed in heart failure. We did not see the evidence of Ca2+ leak from SR, which is known to be the major cause of arrhythmogenesis in RyR2-based disorders such as CPVT. We propose that arrhythmia is largely caused by ion channel dysfuncton(Nav1.5 and Kv7.1). Our data supports the hypothesis that elevated diastolic Ca2+ is caused by dysregulation of Ca2+ reuptake and Ca2+ extrusion, as observed in heart failure. Therefore, while there is dysregulation of Ca2+ signaling, our data supports that APD prolongation secondary to ion channel dysregulation is the primary mechanism for arrhythmogenesis in our models. Indeed, we only observed early after depolarizations (EADs) which are associated with APD prolongation and long QT syndrome as opposed to delayed after depolarizations (DADs) which are associated with increased calcium leak during our extension electrophysiologic analyses. We have included this important observation in the discussion and its implications for the mechanism of arrhythmia in NAA10 dysfunction.
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(Concern 2) The authors do put forward some new experiments showing differential protein expression levels of Nav1.5 and KCNQ1 (with a marked increase in Nav1.5) at the membrane. While the Nav1.5 data is most interesting; it is unclear why expression of Kv7.1 at the membrane was not 'assed' in iPSC-CMs (as it was with Nav1.5) and why an over expressed HEK293 cell model was used? Taking a step back, the link between NAA10 function and ion channel trafficking alterations remains unexplored and would seem to be a critical mechanistic link.
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My Comment:
I agree with authors' rebuttal in the statement above regarding difficulty and the frustration in detecting and adequately quantifying KCNQ in iPSC-CMs. Data presented in Fig 4 albeit with some limitations, are suggestive of defective trafficking as proposed by the authors.

Reviewer #6

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In the manuscript titled 'Dysregulation of N-terminal acetylation causes cardiac arrhythmia and cardiomyopathy' the authors have adequately responded to my comments, and we the corrections, additions and edits have strengthened the quality of the manuscript.

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POINT-BY-POINT RESPONSES

Below are our point-by-point responses to the reviewers' comments. We have included the reviewer comments verbatim with our responses in *italicized green*, to distinguish them from the previous comments. We have included the corrections that are now in the manuscript with the corresponding line numbers as appropriate. Corrections in the revised manuscript are in red for increased visibility.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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Additional minor comments:

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>> Thank you for pointing this out. We corrected it. "(p.R4S)" → "p.(R4S)"

2. Line 27: "Here we show that the NAA10R4S variant reduced enzymatic activity, decreased expression levels of NAA10/NAA15 proteins, (…)"

The authors have only showed decreased NAA10 expression levels in Fig. 3a. Remove NAA15 from this sentence (or include NAA15 blot in Fig. 3A showing decreased NAA15 levels),or re-write to "decreased NAA10-NAA15 complex formation" if this is what the authors mean to convey (as shown in Fig. 2c).
>> Thank you for pointing this out. We re-wrote this part following your advice. "decreased expression levels of NAA10/NAA15 proteins" → "decreased NAA10-NAA15 complex formation"

3. Figure 2c: Please include lysates next to immunoprecipitated samples in this panel.

4. Line 42-44: "N-terminal-acetyltransferase A (NatA), one of seven mammalian NATs (NatA, NatB, NatC, NatD, NatE, NatF and NatH) with distinct substrate specificities2 preferentially acetylates G, S, A, T, or V residues exposed at protein N-termini after iMet removal3."

NatA also Nt-acetylates Cys-starting N-termini after iMet cleavage (PMID: 38918375) so include 'C' here.
There should also be a comma after "specificities" to separate the clauses in this sentence.
>> Thank you very much for letting us know the updated novel information. This is very interesting. We added the reference.

5. Line 59: "Recent large-scale exome sequencing projects of patients with congenital heart disease identified several de novo NAA15 variants17."
This study identified both inherited and de novo NAA15 variants.
>> Thank you for pointing this out. We corrected as follows: "several de novo NAA15 variants" → "several inherited and de novo NAA15 variants"

6. Line 100: “In silico analysis of existing high-resolution structures of the NAA10-NAA15 complex22 (…)”
Should cite PMID: 29754825 since the PDB structure 6C9M was used.
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7. Extended Data Fig. 2A “After administration of cyclohexamide to inhibit protein translation, the NAA10 protein was measured by a specific antibody and normalized to vinculin.”
This sentence is repeated in the legend, please correct.
>> Thank you very much for pointing this out. We corrected it.

8. Extended Data Fig. 8 showing qPCR data: Figure is mislabelled as Extended Data Fig. 6, please correct to Fig. 8.
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9. Line 303: “Consistent predictions and previous reports, the majority of Nt-acetylated proteins were substrates for NatA (Fig. 8a, b).”
Correct typo; “Consistent with”
>> Thank you for pointing it out. We corrected the typo.

10. Line 339: “While predominantly described in males, NAA10-related syndrome cases have been described in heterozygous females12,14.”
Please re-write. This statement is incorrect, there are more female than male cases described to date. For example, one recent study (PMID: 37130971) described 48 female cases and 8 male cases.
>> Thank you for the comment. We revised it as follows: “While predominantly described in males, NAA10-related syndrome cases have been described in heterozygous females” → “While originally described in males, the increasing number of NAA10-related syndrome cases have been described in heterozygous females”

11. Several places in the manuscript: please change ‘mass spectroscopy’ to ‘mass spectrometry’ which is the correct term.
>> Thank you for pointing those out. We corrected them. (Lines 303, 309, 315, 326, and 420)

12. For all mass spectrometry data supporting Figure 8, please adjust supplemental data for better readability and understanding. Add tables to clearly show which proteins are detected as Nt-acetylated, N-terminal sequence etc. As it stands, it is difficult for the general reader to get an overview of the data and to verify the statements made in the text. Also remember to refer to each supplemental Figure and Table in the main text.
>> We added Extended Data Tables 2 and 3 for the description of Nt-acetylated peptides with HYTANE and LATE, respectively. We added the notification. (Lines 305, 308, and 310)

13. Line 304: “The remaining subset of identified proteins were predicted to be substrates for NatB based on the alpha-peptide sequence of either ME, or MD (Fig. 8c).”

Please double-check and verify that no NatC/E/F-type proteins were detected.
>> We apologize for the confusion. Our statement was not accurate. We detected a few NatC/E/F-type proteins, as shown in Extended Data Tables 2 and 3. We revised this part as follows: “The remaining subset of

identified proteins were predicted to be substrates for NatB, or NatC/E/F based on the alpha-peptide sequence." (Lines 307-308)

14. Line 310: "To determine if the NAA10-R4S variant impacts NatA enzymatic activity in cardiomyocytes, we normalized Nt-acetylated peptides by mass-spectroscopy to internal peptides for each identified protein and ranked them by the degree of Nt-acetylation (Fig. 8f). While not all the original set of Nt-acetylated proteins could be normalized, we demonstrated that 68% of NatA targets showed a reduction in Nt-acetylation (Fig. 8g). Proteins associated with nuclear envelope structure or transport including RAN and MATR3 and structural proteins including FHL1, SPTAN1, and CFL2 demonstrated decreased Nt-acetylation in NAA10R4S/Y-iPSC-CMs (Fig. 8f). These data support the hypothesis that the NAA10-R4S variant significantly impairs the function of NatA and reduces global Nt-acetylation in affected iPSC-derived CMs."

Please re-write to make it clear that SPTAN1 is not a NatA substrate (but a NatB substrate).
>> Thank you for pointing those out. We mistakenly used the data which included all the substrates. We excluded NatB substrates and corrected Fig. 8f and 8g accordingly. And we revised the part as follows:

1. *68% → 69%*
2. *SPTAN1 → deleted*

15. Line 318: "In addition to the identification of differentially Nt-acetylated proteins in our NAA10R4S/Y-iPSC-CMs, we sought to investigate the impact of NatA dysfunction on total protein expression. Quantitative mass-spectroscopy of total proteins isolated from WT and eNAA10R4S/Y-iPSC-CMs demonstrated differential expression of 84 proteins (Fig. 8h)."

Please make sure that these data are properly and clearly presented in supplemental and referred to.
>> We added Extended Data Table 5. And for the accuracy, we revised the statement as follows: "Quantitative mass-spectrometry of total proteins isolated from WT and eNAA10$^{R4S/Y}$-iPSC-CMs demonstrated differential expression of 84 peptides (56 proteins) (Fig. 8h and Extended Data Tables 4 and 5). (Lines 325 - 327)

16. Please assess all affected NatA substrates in NAA10 R4S cells (both specific proteins mentioned in the text, and the whole dataset with reduced Nt-acetylation) and their abundance (up, down, or no regulation) and discuss these data in the context of the N-degron pathways: whether or not proteins with less Nt-acetylation seems to be more or less stable (see for example: PMID: 35145090, PMID: 33523899, PMID: 37891180, PMID: 38519774, PMID: 39264755)
>> Thank you very much for the comment. We investigated the relationship between Nt-acetylated peptides and internal peptides of the same proteins (Extended Data Figure 13). Interestingly, we found internal peptides, which represent total proteins, were increased in NAA10 R4S cells while Nt-acetylated peptides were reduced. This result can be explained by a previous report (PMID: 35145090), which describes that while depletion of NatA accelerates the degradation of the substrates, it triggers a concomitant increase in translation as a feedback reaction. Since we did not inhibit the protein synthesis in this study, we did not observe the direct effect of defective NatA on protein degradation. However, increased internal peptides in our study may result from the increased translation which could overwhelm the degradation effect. This would be a very interesting area for future research.

We added following statements:
(Lines 318-320) Interestingly, parallel coordinate plots for Nt-acetylated peptides and internal peptides of the same proteins demonstrated that 67% of the internal peptides were upregulated overall while Nt-peptides were

downregulated (Extended Data Fig. 13).
(Lines 418-422) In a fraction of isolated proteins from NAA10-mutant iPSC-CMs, reduced Nt-acetylation levels were associated with total protein downregulation likely through N-degron pathway-mediated protein degradation[68]. However in the remaining fraction of proteins with decreased Nt-acetylation, corresponding internal peptides were increased suggesting a translationally mediated feedback system to maintain overall protein levels[69].

Reviewer #4 (Remarks to the Author):

The authors have submitted a revised manuscript with sufficient revisions. The authors adequately addressed all of my concerns and present an exciting study that will make a meaningful impact on the field.

>> We thank the reviewer for their thoughtful comments.

Reviewer #5 (Remarks to the Author):

After evaluating Reviewer 3'S concerns, and as requested, my overall comment and evaluation of the rebuttal is that the authors have a made significant effort in addressing the concerns of Reviewer #3. My comments on the authors' rebuttal on the two main concerns of the reviewer are listed below in blue font. Importantly, as noted in their rebuttal of Reviewer #3 concern1, the authors should reconstruct (and be less categorical) on the sentence on lack of QT prolongation and should refer to the relevant article provided therein (PMID 26256814). Otherwise, this rebuttal overall, is fine by me.

Important concerns of Reviewer #3 in the authors' revision:

Concerns of reviewer #3's (black font)
Authors' rebuttal (red font)
My comment on adequacy of author' rebuttal (blue font)

Author's revised response (*initialized green font*)

(Concern 1)
That the mechanistic link between calcium dysregulation and the APD and arrhythmic phenotype remains unexplored. This is critical to do so that a complete mechanistic picture can be formed and two phenotypes, one of APD alteration, and one of calcium overload, can be reconciled. For example, how does the elevated intracellular calcium lead to APD alterations (or does it?). Is this the cause of the arrhythmias?

(Authors' response)
Based on our data and the literature, elevated diastolic calcium levels do not contribute to AD prolongation. This is supported by the observation that there is no QT prolongation in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), despite clear evidence of increased calcium leak through the RyR2 channel and elevated diastolic calcium levels (PMC6750809 and PMC10311407).
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The authors (Yoshinaga et al) should be less categorical on the comment on elevated diastolic calcium and APD prolongation given changes in, and elevation of intracellular calcium can in some instances lead to changes in APD and arrhythmogenesis.
Importantly, I disagree with a statement above regarding a lack of QT prolongation in patients with CPVT

because it is not strictly correct! Although for the most part, QT interval in CPVT patients is typically normal, it has been reported however, that some CPVT patients with pathogenic RyR2 mutations have significant QT prolongation and, in a few such cases, may have been misdiagnosed as concealed long QT syndrome (LQTS), given that both diseases (LQTS as CPVT) are associated with syncope or sudden death following stress or swings in emotional states (PMID: 26256814) Interestingly, for the treatment of such CPVT patients showing QT prolongation, the normalization with flecainide perhaps may be relevant and supportive of authors' contention on the role of ion channel dysfunction in APD prolongation underlying the arrhythmogenesis they investigated.

>>We thank the reviewer for highlighting the possibility of phenotypic crossover between CPVT and long QT in certain cases. We agree that patients can present with more of a mixed picture such as with pathogenic calmodulin variants and other Ca2+ handling proteins. We have included a comment in the discussion (lines 411-415 in the revised manuscript) and the appropriate citation.

Our data strongly supports that APD prolongation is caused by the combination of increased late INa and decreased IKs which are both canonical mechanisms of APD prolongation and increased risk of arrhythmogenesis, as in long QT syndrome.
My Comment:
For the most part, I agree with the rebuttal above)
Elevated intracellular calcium levels are frequently associated with heart failure and HCM but not directly to APD prolongation We demonstrated that diastolic calcium levels were elevated secondary to impaired Ca reuptake and extrusion as observed in heart failure. We did not see the evidence of Ca2+ leak from SR, which is known to be the major cause of arrhythmogenesis in RyR2-based disorders such as CPVT. We propose that arrhythmia is largely caused by ion channel dysfuncton(Nav1.5 and Kv7.1). Our data supports the hypothesis that elevated diastolic Ca2+ is caused by dysregulation of Ca2+ reuptake and Ca2+ extrusion, as observed in heart failure. Therefore, while there is dysregulation of Ca2+ signaling, our data supports that APD prolongation secondary to ion channel dysregulation is the primary mechanism for arrhythmogenesis in our models. Indeed, we only observed early after depolarizations (EADs) which are associated with APD prolongation and long QT syndrome as opposed to delayed after depolarizations (DADs) which are associated with increased calcium leak during our extension electrophysiologic analyses. We have included this important observation in the discussion and its implications for the mechanism of arrhythmia in NAA10 dysfunction.
My Comment:
I do agree with authors' rebuttal in the statement above and the addition as edits in the manuscript discussion is important and should include additional references to buttress this contention.

>>We have included these comments into the discussion and added an appropriate reference. (Line 402)

(Concern 2) The authors do put forward some new experiments showing differential protein expression levels of Nav1.5 and KCNQ1 (with a marked increase in Nav1.5) at the membrane. While the Nav1.5 data is most interesting; it is unclear why expression of Kv7.1 at the membrane was not 'assed' in iPSC-CMs (as it was with Nav1.5) and why an over expressed HEK293 cell model was used? Taking a step back, the link between NAA10 function and ion channel trafficking alterations remains unexplored and would seem to be a critical mechanistic link.
Unfortunately, the antibodies for KCNQ1 were insufficiently sensitive to detect native channel in surface biotinylation studies of iPSC-CMs. As an alternative, we developed an assay with a Myc-tag cloned into one of the extracellular loops of KCNQ1 to unambiguously detect its surface expression. To prevent competition with untagged KCNQ1, we performed the assay heterologous expression paradigm with NAA10 knockdown (Figures 4l and 4m). These data strongly support a direct trafficking defect of KCNQ1 with NAA10 dysfunction as a mechanism for reduced KCNQ1 function and impaired IKs. Indeed, reduced trafficking of KCNQ1

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My Comment:
I agree with authors' rebuttal in the statement above regarding difficulty and the frustration in detecting and adequately quantifying KCNQ in iPSC-CMs. Data presented in Fig 4 albeit with some limitations, are suggestive of defective trafficking as proposed by the authors.

>>We thank the reviewer for their insightful comment and have included a statement regarding the limitation of low-expression KCNQ1 levels in iPSC-CMs as the reviewer states, necessitating alternative methods to detect surface ion channel expression. (Lines 218-219).

Reviewer #6 (Remarks to the Author):

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Third set of reviews and Point-by-Point responses (in red).

Reviewer #1 (Remarks to the Author):

Previous comments have been properly addressed.
Figure 8 panels f and g need explanatory text to be understandable.
Figure 8 legend does not match the figure panels (legend for panel g belongs to figure panel
h etc).

We added text in the figure 8 legend and confirmed the proper corresponding statements to the appropriate panel in the figure.

Edited text in the figure 8 legend is now:
" **f.** Scatter plot of the Nt-acetylated proteins with the relative ratio of Nt-acetylated peptides normalized by total protein. Each protein was ranked according to the order of the relative ratio. Proteins known as causative to heart diseases are highlighted in red. **g.** The normalized relative ratio of Nt-acetylated peptides from **f.**, showing a significant reduction in Nt-acetylation in eNAA10$^{R4S/Y}$-iPSC-CMs (-0.25 ± 0.08; mean ± SEM; p = < 0.0001; one sample Wilcoxon test). **h.** Volcano plot...."

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We thank the reviewer for their previous comments and suggestions.

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That the mechanistic link between calcium dysregulation and the APD and arrhythmic phenotype remains unexplored. This is critical to do so that a complete mechanistic picture can be formed and two phenotypes, one of APD alteration, and one of calcium overload, can be reconciled. For example, how does the elevated intracellular calcium lead to APD alterations (or does it?). Is this the cause of the arrhythmias?

(Authors' response)

Based on our data and the literature, elevated diastolic calcium levels do not contribute to AD prolongation. This is supported by the observation that there is no QT prolongation in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), despite clear evidence of increased calcium leak through the RyR2 channel and elevated diastolic calcium levels (PMC6750809 and PMC10311407).

My Comment:

The authors (Yoshinaga et al) should be less categorical on the comment on elevated diastolic calcium and APD prolongation given changes in, and elevation of intracellular calcium can in some instances lead to changes in APD and arrhythmogenesis.

Importantly, I disagree with a statement above regarding a lack of QT prolongation in patients with CPVT because it is not strictly correct! Although for the most part, QT interval in CPVT patients is typically normal, it has been reported however, that some CPVT patients with pathogenic RyR2 mutations have significant QT prolongation and, in a few such cases, may have been misdiagnosed as concealed long QT syndrome (LQTS), given that both diseases (LQTS as CPVT) are associated with syncope or sudden death following stress or swings in emotional states (PMID: 26256814) Interestingly, for the treatment of such CPVT patients showing QT prolongation, the normalization with flecainide perhaps may be relevant and supportive of authors' contention on the role of

ion channel dysfunction in APD prolongation underlying the arrhythmogenesis they investigated.

Our data strongly supports that APD prolongation is caused by the combination of increased late I_{Na} and decreased I_{Ks} which are both canonical mechanisms of APD prolongation and increased risk of arrhythmogenesis, as in long QT syndrome.

My Comment:

For the most part, I agree with the rebuttal above)

Elevated intracellular calcium levels are frequently associated with heart failure and HCM but not directly to APD prolongation We demonstrated that diastolic calcium levels were elevated secondary to impaired Ca reuptake and extrusion as observed in heart failure. We did not see the evidence of Ca2+ leak from SR, which is known to be the major cause of arrhythmogenesis in RyR2-based disorders such as CPVT. We propose that arrhythmia is largely caused by ion channel dysfunction (Nav1.5 and Kv7.1). Our data supports the hypothesis that elevated diastolic Ca2+ is caused by dysregulation of Ca2+ reuptake and Ca2+ extrusion, as observed in heart failure. Therefore, while there is dysregulation of Ca2+ signaling, our data supports that APD prolongation secondary to ion channel dysregulation is the primary mechanism for arrhythmogenesis in our models. Indeed, we only observed early after depolarizations (EADs) which are associated with APD prolongation and long QT syndrome as opposed to delayed after depolarizations (DADs) which are associated with increased calcium leak during our extension electrophysiologic analyses. We have included this important observation in the discussion and its implications for the mechanism of arrhythmia in NAA10 dysfunction.

My Comment:
I do agree with authors' rebuttal in the statement above and the addition as edits in the manuscript discussion is important and should include additional references to buttress this contention.

(Concern 2) The authors do put forward some new experiments showing differential protein expression levels of Nav1.5 and KCNQ1 (with a marked increase in Nav1.5) at the membrane. While the Nav1.5 data is most interesting; it is unclear why expression of Kv7.1 at the membrane was not 'assed' in iPSC-CMs (as it was with Nav1.5) and why an over expressed HEK293 cell model was used? Taking a step back, the link between NAA10 function and ion channel trafficking alterations remains unexplored and would seem to be a critical mechanistic link.

Unfortunately, the antibodies for KCNQ1 were insufficiently sensitive to detect native channel in surface biotinylation studies of iPSC-CMs. As an alternative, we developed an assay with a Myc-tag cloned into one of the extracellular loops of KCNQ1 to unambiguously detect its surface expression. To prevent competition with untagged KCNQ1, we performed the assay heterologous expression paradigm with NAA10 knockdown (Figures 4l and 4m). These data strongly support a direct trafficking defect of

KCNQ1 with NAA10 dysfunction as a mechanism for reduced KCNQ1 function and impaired I_{Ks}. Indeed, reduced trafficking of KCNQ1 channels to the plasma membrane is a known mechanism for several KCNQ1 variants associated with long QT syndrome (PMC5842040). Respectively, we feel that our data supports dysregulated ion channel trafficking as a mechanism for the observed electrophysiologic changes, which agrees with other regulatory mechanisms for ion channel function. We would of course be happy to consider any additional specific experiments or changes to our manuscript, but none were provided by the reviewer.

My Comment:

I agree with authors' rebuttal in the statement above regarding difficulty and the frustration in detecting and adequately quantifying KCNQ in iPSC-CMs. Data presented in Fig 4 albeit with some limitations, are suggestive of defective trafficking as proposed by the authors.