A Troponin T Variant Linked with Pediatric Dilated Cardiomyopathy Reduces the Coupling of Thin Filament Activation to Myosin and Calcium Binding

Samantha Barrick, Lina Greenberg, and Michael Greenberg

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E21-02-0082
TITLE: A Troponin T Variant Linked with Pediatric Dilated Cardiomyopathy Decreases Cardiac Contractility by Reducing the Coupling of Thin Filament Activation to Myosin and Calcium Binding

Dear Dr. Greenberg:

As you can see, the reviewers are overall supportive of your manuscript. However, both request revisions that merit a round of re-review. In my own reading, I am optimistic that this can be accomplished in an expeditious fashion. Thanks for submitting for the special issue!

Sincerely,

Alexander Dunn
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Greenberg,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The manuscript by Barrick et al investigates the molecular and cellular effects of the R134G TnT mutation that is associated with pediatric-onset DCM. The work represents a logical continuation of investigating cardiomyopathy-causing thin filament mutations, using a suite of techniques and approaches outlined in previous research and review articles from the Greenberg Lab. The paper is well-written and data acquisition impressively thorough. Overall, it is nice manuscript that potentially extends our understanding of the R134G TnT variant beyond that of earlier studies. However, there are a few major and minor concerns with the study in its current form:

Major: IVM studies) The authors only report pCa50 values, the calcium concentration at which RTFs glide at half-max speed, i.e. the calcium sensitivity of RTF motility. The data presented in Fig 2 are normalized. Showing non-normalized data would be very useful for readers. Reporting the Vmax and the percent filaments moving (at least at max and min Ca) is recommended. Since in Fig 5F, KT at pCa 9 is significantly higher for the mutant, are more filaments possibly motile at low Ca? Would this contradict "hypocontractility" for the variant? Or might a 5-fold increase in KT at pCa 9 still be insufficient to activate more sliding RTFs? Presumably so, since it is stated on line 199 (and repeated in the discussion) that "At pCa 9, the level of thin filament activation is low despite the increased value of KT because most thin filaments adopt the blocked state."; however, these raw motility data should be shown. Also, qualitatively, the IVM data in Fig 2 would appear to show no/little difference in nH, yet the nH is reduced in Fig 5F by nearly half that of WT. This is a major finding of the study. Was a difference in nH unresolvable via IVM? If so, why? If the KT at saturating Ca for the mutant is so much lower than control, would that reduce mutant RTF Vmax or percent filaments moving at pCa 4 relative to WT? Gangadharan et al., 2017 did report significantly reduced max, R134G thin filament-activated ATPase activity. Also, in Fig 2 there appears to be no IVM data points collected above pCa 7.5?

Line 122) "A mutation-induced decrease in myosin crossbridge detachment kinetics would decrease RTF motility by decreasing the time myosin spends in the strongly bound state." It is recommended that the authors carefully check if this is accurate. After several reads, the current reviewer struggled with the statement. It seems that decreased detachment kinetics would INCREASE the time myosin spends strongly bound, and hence the opposite would be true. If it is correct, the authors might provide additional information to help clarify this sentence as well as add

appropriate citations. This would assist the general MBoC readership who may lack the expertise needed to fully comprehend the argument, and also help them better understand the subsequent sentence beginning on line 124.

pCa 4 KT values and "biological significance") In certain instances the biological significance of changes in particular indices (e.g. KW, line 216) is downplayed. However, a significantly lower KT at exceedingly high, non-physiological Ca concentrations, using non-physiological "homozygous" pools of mutant RTFs is reported as a potentially important disease-driving mechanism. Perhaps any notion of "biological significance" should be tempered. Relatedly, the authors claim (line 222) that "these molecular-level measurements demonstrate that the mutation induced decrease in calcium sensitivity observed in the in vitro motility assay is primarily caused by 1) reduced coupling of calcium binding to changes in tropomyosin positioning, resulting in reduced occupancy of the open state in the presence of calcium". However, this was not observed at more physiological, "biologically-significant" Ca (6.25) levels, where no statistically significant difference in KTs was found.

While acknowledged as a limitation of the study, the lack of Ca handling/transient data for hiPSC-derived CMs makes data interpretation difficult. The hypocontractility and reduced shortening/lengthening velocities may simply result from tracking perturbed Ca behavior, which brings into question relevance of the cellular data. Hence, it may be premature to claim that (line 281) "At the cellular scale, these molecular defects lead to hypocontractility, which in turn causes changes in cellular morphology and disrupts sarcomeric organization."

Line 346) "In vitro measurements of tropomyosin-TnT binding affinity revealed that the R134G variant, along with several other DCM-causing mutations within the TnT N-terminal domain, decreases the affinity of TnT for tropomyosin (Gangadharan et al., 2017)." Please check the accuracy of this statement and, therefore, the subsequent arguments that follow. Gangadharan et al., 2017 indeed determined if the mutation changed TnT's affinity for Tm in vitro. The paper states that "Strikingly, the HCM mutants in regions 92-144, R92L, K124N, and R130C, significantly decreased the binding affinity for Tm (Fig. 1A and Table 2), while the DCM mutants R134G and R144W increased the affinity significantly (Fig. 1B)." Thus, one may argue that based on INCREASED affinity, the mutant TnT may stabilize Tm end-to-end bonds, which is seemingly inconsistent with the decreased cooperativity shown here by Barrick et al.

Minor: Line 57) The authors state "DCM is often caused by point mutations in proteins that regulate cardiac muscle contraction and mechanosensation, including troponin T (TnT)." "Often" may be overstated. Perhaps provide some numbers/reference to support this statement.

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Line 146) "Unexpectedly, we found that R134G has increased calcium binding to TnC [pCa50 (WT) = 6.29 {plus minus}
0.07, pCa50 (R134G) = 6.6 {plus minus}

0.1; p < .001] (Figure 4). Thus, the increased calcium binding sensitivity seen in R134G cannot explain the decrease in calcium sensitivity observed in the motility assay (Figure 2)." How might this discrepancy be explained? i.e. TnC-Ca affinity being high, yet RTF-Ca sensitivity low? Are there other examples of this in the literature? If not, should/could this potential novel observation be further investigated?

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Overall, the discussion could be abbreviated as it is quite repetitive.

Reviewer #2 (Remarks to the Author):

This is an outstanding manuscript that reports on the effects of a DCM associated variant in cardiac troponin T (TnT-R134G). The authors have assembled an impressive array of biophysical and physiological tools to study the mechanistic underpinnings of pathology associated with this mutation. I have a couple of suggestions for the authors to consider:
1) Lines 73-75: It is mentioned that there is a small number of patients carrying this variant - Are there refs that the author can add to scientifically support this statement? Another interesting point to consider is that different amino acid substitutions have been reported in this specific residue, e.g., R134H, R134S, R134C, R134P, R134Q and R134W (see clinvar).
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6) Line 302: I think two log units for physiological calcium transients is too large of a range. Calcium fluctuations in intact cardiac myocytes is in the range of 0.1 - 1 microM.
7) Lines 340-342: the following reference should be added to the sentence that describes TnT interaction with tropomyosin filaments on both side of the filament: Proc Natl Acad Sci U S A. 2021 Mar 30;118(13):e2024288118. doi: 10.1073/pnas.2024288118.
8) It is difficult to reconcile increased TF calcium binding affinity with impaired tropomyosin position to more active state. Is it possible that the R134G troponin complex is dissociating from actin-tropomyosin during the calcium titrations since troponin complex has a higher calcium binding affinity compared to TF?

We thank the reviewers for their supportive comments and constructive suggestions. We have provided our point-by-point responses to the reviewer's comments (reproduced in italics) below. The line numbers refer to the newly submitted manuscript. We are pleased to submit our revised manuscript for your consideration.

Reviewer #1 (Remarks to the Author):

The manuscript by Barrick et al investigates the molecular and cellular effects of the R134G TnT mutation that is associated with pediatric-onset DCM. The work represents a logical continuation of investigating cardiomyopathy-causing thin filament mutations, using a suite of techniques and approaches outlined in previous research and review articles from the Greenberg Lab. The paper is well-written and data acquisition impressively thorough. Overall, it is nice manuscript that potentially extends our understanding of the R134G TnT variant beyond that of earlier studies. However, there are a few major and minor concerns with the study in its current form:

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We thank the reviewer for the insightful suggestions. We now include the raw motility data and measurement of V_{max} (new Fig. 2B). Also, we now report the percent stuck filaments at pCa 4 and 9 (new Fig. 2A).

Since in Fig 5F, KT at pCa 9 is significantly higher for the mutant, are more filaments possibly motile at low Ca? Would this contradict "hypocontractility" for the variant? Or might a 5-fold increase in KT at pCa 9 still be insufficient to activate more sliding RTFs? Presumably so, since it is stated on line 199 (and repeated in the discussion) that "At pCa 9, the level of thin filament activation is low despite the increased value of KT because most thin filaments adopt the blocked state."; however, these raw motility data should be shown.

Based on the reviewer's suggestion, we quantified the percent stuck filaments at pCa 4 and 9, and we do not see a difference at pCa 9 ($p = 0.91$, new figure 2A). This is consistent with our proposal that the increase in KT at pCa 9 has little effect on thin filament activation. We now highlight this point on lines 331-337:

"At low calcium (pCa 9), the larger value of K_T for R134G signifies increased thin filament activation relative to WT; however, the overall level of activation for both WT and R134G is low since both K_B and K_T are small (≤ 0.3), indicating that most of the regulatory units are in the blocked state. Consistent with this observation, the fraction of stuck filaments in the in vitro motility assay at pCa 9 is the same for both the WT and mutant troponin. Therefore, the increase in K_T at pCa 9 is not sufficient to explain the increase the motility of RTFs containing R134G troponin (Figure 2)."

Also, qualitatively, the IVM data in Fig 2 would appear to show no/little difference in nH, yet the nH is reduced in Fig 5F by nearly half that of WT. This is a major finding of the study. Was a difference in nH unresolvable via IVM? If so, why?

We did not detect a significant difference in n_H from the motility data; however, the Hill coefficient is not well-resolved from the fit, as indicated by wide confidence intervals. In contrast, the pCa_{50} values are well-defined by the fit. Therefore, we are not confident in our ability to resolve differences in n_H from our data. We now report the n_H values in the text on lines 115-116. We also note the challenges comparing the cooperativity measured in different assays on lines 366-375:

"Our measurements also revealed a decrease in the cooperativity of myosin binding to thin filaments regulated by R134G TnT, relative to WT. This is important because full thin filament activation depends on both calcium and myosin binding (Houmeida et al., 2010). This finding is qualitatively consistent with the decreased Hill coefficient of actomyosin ATPase activity observed for R134G TnT relative to WT (Gangadharan et al., 2017). While we did not observe a statistically significant change in the Hill coefficient in the motility assay, this could be due to the large uncertainty in determining this value. Moreover, it should be noted that the Hill coefficients measured for motility, ATPase activity, and myosin binding to RTFs in equilibrium titrations are not necessarily equivalent."

If the KT at saturating Ca for the mutant is so much lower than control, would that reduce mutant RTF Vmax or percent filaments moving at pCa 4 relative to WT? Gangadharan et al., 2017 did report significantly reduced max, R134G thin filament-activated ATPase activity.

We see a decrease in V_{max} for the mutant relative to WT (new Fig. 2B) and an increase in the percent stuck filaments at pCa 4 (new Fig. 2A). Moreover, we found that the ADP release rates (Fig. 3) were the same for WT and R134G. Therefore, the change in motility speed is likely due to changes in attachment kinetics (limited by the rate of phosphate release and strong binding) rather than detachment kinetics (limited by the rate of ADP release). This makes sense because regulatory proteins gate the association of myosin and the thin filament. Therefore, our results are consistent with the studies of Gangadharan et al., who saw decreased ATPase activity, indicative of a slower rate of phosphate release and strong binding for R134G regulated thin filaments. We now highlight this point on lines 344-353:

"A mutation-induced reduction in thin filament activation at saturating calcium concentrations may also help explain the shift in calcium sensitivity of RTF motility (Figure 2). The motility rate in the absence of regulatory proteins is frequently assumed to be limited by the rate of actomyosin dissociation (Huxley, 1990); however, we do not observe a change in the rate of ADP release with the mutant protein (Figure 3). Therefore, it is likely that the mutant is affecting attachment kinetics, which is reasonable since the transition to strong binding is regulated by troponin and tropomyosin. Therefore, the reduced maximal speed in the motility assay is consistent with the reduced

actin-activated ATPase rate measured with R134G regulated thin filaments at fully activating calcium concentrations (Gangadharan et al., 2017)."

Also, in Fig 2 there appears to be no IVM data points collected above pCa 7.5?

We collected IVM data over the range of pCa 9-4. The measurements at pCa > 6.5 are perhaps not clear from the figure because we did not observe gliding filaments at these low calcium concentrations. As described in the methods, we assigned a speed of 0 to calcium concentrations that did not support continuous, directional filament gliding; thus, there are no error bars associated with these measurements. We have updated the figures to enlarge these points; however, they are partially obscured by the fitted line. We now mention that points collected at pCa 6.75, 7, 8, and 9 are likely obscured by the line. We have also edited the text to explicitly address the measurements at low calcium values on lines 568-570.

Line 122) "A mutation-induced decrease in myosin crossbridge detachment kinetics would decrease RTF motility by decreasing the time myosin spends in the strongly bound state." It is recommended that the authors carefully check if this is accurate. After several reads, the current reviewer struggled with the statement. It seems that decreased detachment kinetics would INCREASE the time myosin spends strongly bound, and hence the opposite would be true. If it is correct, the authors might provide additional information to help clarify this sentence as well as add appropriate citations. This would assist the general MBoC readership who may lack the expertise needed to fully comprehend the argument, and also help them better understand the subsequent sentence beginning on line 124.

We thank the reviewer for pointing this out. We have edited the manuscript to correct this typographical error and to further explain our logic. The sentence (lines 138-140) now reads, "A mutation-induced increase in the rate of myosin crossbridge detachment would decrease thin filament activation by decreasing the time myosin spends in the activating, strongly-bound state."

pCa 4 KT values and "biological significance") In certain instances the biological significance of changes in particular indices (e.g. KW, line 216) is downplayed. However, a significantly lower KT at exceedingly high, non-physiological Ca concentrations, using non-physiological "homozygous" pools of mutant RTFs is reported as a potentially important disease-driving mechanism. Perhaps any notion of "biological significance" should be tempered. Relatedly, the authors claim (line 222) that "these molecular-level measurements demonstrate that the mutation induced decrease in calcium sensitivity observed in the in vitro motility assay is primarily caused by 1) reduced coupling of calcium binding to changes in tropomyosin positioning, resulting in reduced occupancy of the open state in the presence of calcium". However, this was not observed at more physiological, "biologically-significant" Ca (6.25) levels, where no statistically significant difference in KTs was found.

We appreciate the reviewer's point and we have removed the reference to biological significance in this section. We also now state (lines 354-357): "Although pCa 9 and 4 are extreme values relative to the typical range of calcium concentrations in a

physiological calcium transient, pCa 6-7 (Bers, 2002), we believe that measurements at pCa 9 and 4 provide meaningful insight into relaxed and fully activated conditions, respectively."

While acknowledged as a limitation of the study, the lack of Ca handling/transient data for hiPSC-derived CMs makes data interpretation difficult. The hypocontractility and reduced shortening/lengthening velocities may simply result from tracking perturbed Ca behavior, which brings into question relevance of the cellular data. Hence, it may be premature to claim that (line 281) "At the cellular scale, these molecular defects lead to hypocontractility, which in turn causes changes in cellular morphology and disrupts sarcomeric organization."

We agree that the cellular data are consistent with molecular hypocontractility but do not unambiguously demonstrate that the molecular defects are the direct cause of the cellular hypocontractility. We have edited the manuscript to carefully make this distinction. These changes can be found on lines 413-416:
"Our molecular studies suggest that the initial molecular insult driving disease pathogenesis is altered thin filament regulation that decreases thin filament activation, which is consistent with the decreased sarcomeric contractility observed in our stem cell-derived cardiomyocyte model of R134G".

Line 346) "In vitro measurements of tropomyosin-TnT binding affinity revealed that the R134G variant, along with several other DCM-causing mutations within the TnT N-terminal domain, decreases the affinity of TnT for tropomyosin (Gangadharan et al., 2017)." Please check the accuracy of this statement and, therefore, the subsequent arguments that follow. Gangadharan et al., 2017 indeed determined if the mutation changed TnT's affinity for Tm in vitro. The paper states that "Strikingly, the HCM mutants in regions 92-144, R92L, K124N, and R130C, significantly decreased the binding affinity for Tm (Fig. 1A and Table 2), while the DCM mutants R134G and R144W increased the affinity significantly (Fig. 1B)." Thus, one may argue that based on INCREASED affinity, the mutant TnT may stabilize Tm end-to-end bonds, which is seemingly inconsistent with the decreased cooperativity shown here by Barrick et al.

We thank the reviewer for correcting this error. The DCM mutants were associated with <u>increased</u> affinity for tropomyosin, as the reviewer correctly points out. However, it is not clear that this effect is inconsistent with decreased cooperativity of myosin binding to RTFs. We feel that the most important point is that TnT mutants can affect the Tm end-to-end bonds, and we do not attempt to predict or explain the direction of this effect.

Minor: Line 57) The authors state "DCM is often caused by point mutations in proteins that regulate cardiac muscle contraction and mechanosensation, including troponin T (TnT)." "Often" may be overstated. Perhaps provide some numbers/reference to support this statement.

We have updated this statement (lines 57-59) to clarify that <u>familial DCM</u> is often caused by mutations in genes <u>involved in</u> contraction and mechanosensation. We have also added a reference regarding identified genetic causes of DCM.

Line 92) "Porcine cardiac actin is identical to human actin, and porcine cardiac myosin is 97%

identical to human..." Please change to "Porcine cardiac actin is identical to human CARDIAC actin, and porcine cardiac myosin is 97% identical to human CARDIAC MYOSIN..."

Fixed.

Line 109) "This result suggests that the R134G variant might demonstrate decreased force per sarcomere during a calcium transient..." The variant will not demonstrate decreased force, the myocytes expressing the variant might demonstrate decreased force.

Fixed.

Line 156) "The blocked state, in which tropomyosin blocks the strong myosin-binding site on the thin filament, is significantly occupied only at low calcium concentrations." This currently reads as though there is a separate strong vs weak S1 binding site on actin.

We have made the requested changes to the manuscript.

Line 146) "Unexpectedly, we found that R134G has increased calcium binding to TnC [pCa50 (WT) = 6.29 {plus/minus} 0.07, pCa50 (R134G) = 6.6 {plus minus} 0.1; p < .001] (Figure 4). Thus, the increased calcium binding sensitivity seen in R134G cannot explain the decrease in calcium sensitivity observed in the motility assay (Figure 2)." How might this discrepancy be explained? i.e. TnC-Ca affinity being high, yet RTF-Ca sensitivity low? Are there other examples of this in the literature? If not, should/could this potential novel observation be further investigated?

We agree that this is an interesting result, directly demonstrating reduced coupling between calcium binding to troponin C and activation of the thin filament. To the best of our knowledge, our observation of increased calcium binding affinity with decreased thin filament activation is a novel mechanism. That being said, the concept that cardiomyopathy mutations in the troponin T tail region can allosterically affect calcium binding and/or the coupling between the troponin complex and tropomyosin was proposed by the Tardiff group (e.g., PMID: 26957598 and PMID: 31387947). We have included discussion of this important point on lines 309-319:

"The R134G variant decreased the calcium sensitivity of RTF motility (Figure 2) despite the increased affinity of TnC for calcium within RTFs containing R134G TnT, relative to WT (Figure 4). The effect of a mutation in TnT on calcium binding by TnC is consistent with previous work showing that two mutations at the R92 position of troponin T differentially altered calcium binding through a complex network of allosteric interactions (Williams et al., 2016). Our data suggest that the R134G mutation alters allosteric communication among thin filament proteins such that the coupling between tropomyosin positioning and calcium binding to troponin C is reduced. This is consistent with a study showing that the nearby hypertrophic cardiomyopathy mutation ΔE160 can affect the coupling between the troponin core complex and the tropomyosin overlap region (Abdullah et al., 2019)."

Line 202) "We estimated the occupancy of each state from the equilibrium constants determined for WT and R134G and found that R134G decreased the occupancy of myosin-bound states by

31% at pCa 4." Presenting all these data (percent B-, C-, M-state occupancies) would be useful.

> We initially provided this estimate to give a more intuitive description of the magnitude of the mutation's effect, compared to the observed change in K_T. However, our estimates of the occupancy of each state from the determined equilibrium constants did not consider the cooperativity of thin filament activation. Upon further reflection, this simplification is unjustified given the effect of R134G on thin filament cooperativity, such that attempting to quantify state occupancies may be misleading. We have therefore removed the numerical estimate for occupancy of the myosin-bound states.

Line 451) "In addition, we used homozygous isogenic lines to facilitate the comparison between the molecular and cellular levels, whereas patients are usually homozygous." Patients are not usually homozygous.

> We have corrected this typographical error.

Overall, the discussion could be abbreviated as it is quite repetitive.

> We have reorganized and abbreviated the discussion.

Reviewer #2 (Remarks to the Author):

This is an outstanding manuscript that reports on the effects of a DCM associated variant in cardiac troponin T (TnT-R134G). The authors have assembled an impressive array of biophysical and physiological tools to study the mechanistic underpinnings of pathology associated with this mutation. I have a couple of suggestions for the authors to consider:

We thank the reviewer for their enthusiasm and support.

1) Lines 73-75: It is mentioned that there is a small number of patients carrying this variant - Are there refs that the author can add to scientifically support this statement? Another interesting point to consider is that different amino acid substitutions have been reported in this specific residue, e.g., R134H, R134S, R134C, R134P, R134Q and R134W (see clinvar).

We thank the reviewer for bringing this interesting point to our attention. The small number of patients referred to the study conducted by Hershberger et al. We have edited this statement to clarify that there is a small number of patients known to carry this specific mutation. We thank the reviewer for raising the interesting point about different mutations at this same position. We now discuss this point on lines 70-80: "…we examined the molecular and cellular consequences of a variant in troponin T, R134G, which has been implicated in pediatric-onset DCM (Hershberger et al., 2008) (Figure 1). Several variants at the R134 position have been implicated in cardiomyopathy, with clinical significance of individual variants ranging from uncertain to likely pathogenic (Landrum et al., 2018). We chose to study the likely pathogenic R134G variant, which was shown to segregate with DCM in a patient cohort (Hershberger et al., 2009). The small number of patients known to carry this specific variant makes it difficult to definitively determine pathogenicity from the currently available clinical data (Hershberger et al., 2009; Landrum et al., 2018)."

2) Line 130, 133 and 135: actomyosin should read acto-S1 or actomyosin S1.

We thank the reviewer for improving the precision of the language in the manuscript. We have edited the manuscript to specify actomyosin S1 when referencing experimental results. In the sentence extrapolating the results of our biochemical experiments (utilizing S1) to the in vitro motility assay (which utilizes full-length myosin), we have retained the original phrasing for simplicity.

3) Lines 202-204: it is stated that R134G decreased the occupancy of myosin-bound states by 31%. This finding is somehow similar to the one reported in J Biol Chem. 2010 Jun 4;285(23):17371-9. doi: 10.1074/jbc.M109.064105. How does the decreased occupancy of myosin bound states compare to the maximum speed in the in vitro motility assay? The JBC 2010 paper showed that the reduction of the number of myosin binding site in the TF caused by a troponin DCM mutation is comparable to the reduction of force of contraction and ATPase activation.

We thank the reviewer for pointing out this reference, which we have included in support of the concept of reduced contractility due to fewer accessible binding sites. In our revised manuscript, we now show the absolute change in motility speed (new Fig. 2B) in addition to the normalized speed. Consistent with the reviewer's suggestion, we see a reduction in the maximal speed in the motility assay, and we now discuss this point in the revised manuscript in both the results (lines 113-115) and the discussion (lines 204-218).

In terms of the quantitative change seen in myosin-bound states, we have removed our attempt to quantify the decrease in occupancy of myosin-bound states in the manuscript due to our concern that our calculation did not appropriately account for changes in thin filament cooperativity. Please see our response to reviewer 1 for more details.

4) Lines 206-214: to which figure is this paragraph referring?

This paragraph is also referring to Figure 5. We apologize for the confusion and have added the appropriate figure reference.

5) In figure 6, I am wondering if the R134G cells display more sarcomeres compared to WT and what their diastolic sarcomere length are.

We thank the reviewer for this interesting question. Based on the reviewer's question, we went back to our data and we examined the sarcomere length of fixed cells on both glass and physiological stiffness hydrogels (10 kPa). This is not necessarily the same as the diastolic sarcomere length, since accurately determining sarcomere length would require live cell imaging of cells containing a fluorescent sarcomeric reporter (which would need to be engineered into the cell line). Based on our analysis, we see that the sarcomere length of R134G is longer on glass ($p = 0.004$), but not significantly different on hydrogels ($p = 0.342$). We now include this point (lines 261-263) and methods describing our analysis procedure (lines 712-713) in the paper and have added the data to Table 1 and Figure 6.

In terms of determining the number of sarcomeres, this was more challenging. We tried several approaches, including using the approach developed by Kit Parker's lab (PMID: 25733020). Unfortunately, the unique sarcomeric morphology of these cells (see Fig. 6), caused these algorithms to be less accurate. Qualitatively, the R134G cells are larger, but there also appear to be larger regions that lack sarcomeres compared to the WT cells. Based on these challenges, we do not feel comfortable drawing any firm conclusions about whether the R134G cells contain more sarcomeres.

6) Line 302: I think two log units for physiological calcium transients is too large of a range. Calcium fluctuations in intact cardiac myocytes is in the range of 0.1 - 1 microM.

We agree that pCa 6-7 is a better representation of the range of calcium concentrations during a transient and have updated the manuscript accordingly.

7) Lines 340-342: the following reference should be added to the sentence that describes TnT

interaction with tropomyosin filaments on both side of the filament: Proc Natl Acad Sci U S A. 2021 Mar 30;118(13):e2024288118. doi: 10.1073/pnas.2024288118.

We agree and have added the suggested reference, which was not available at the time of initial submission.

8) It is difficult to reconcile increased TF calcium binding affinity with impaired tropomyosin position to more active state. Is it possible that the R134G troponin complex is dissociating from actin-tropomyosin during the calcium titrations since troponin complex has a higher calcium binding affinity compared to TF?

R134G TnT has an increased affinity for actin-tropomyosin relative to WT (Gangadharan et al., 2017), suggesting that it would be unlikely for the R134G troponin to dissociate from actin-tropomyosin to a greater extent than WT troponin. Moreover, we do not believe that the R134G troponin complex is dissociating from actin-tropomyosin in our experiments because we observe calcium-based regulation in our other molecular-level measurements.

We believe that the most likely mechanism involved is partial uncoupling of calcium binding to troponin C from tropomyosin movement along the thin filament. The concept that cardiomyopathy mutations in the troponin T tail region can allosterically affect calcium binding and/or the coupling between the troponin complex and tropomyosin has been proposed by the Tardiff group (e.g., PMID: 26957598 and PMID: 31387947). Based on this mechanism, a reduction in coupling would mean that more calcium binding to troponin C would be required to reach the same level of thin filament activation, consistent with our results. We have expanded our discussion of this point on lines 309-319, as detailed in the response to Reviewer 1.

RE: Manuscript #E21-02-0082R
TITLE: "A Troponin T Variant Linked with Pediatric Dilated Cardiomyopathy Reduces the Coupling of Thin Filament Activation to Myosin and Calcium Binding"

Dear Dr. Greenberg,

Thank you for the comprehensive revision of your manuscript. I am delighted to say it can be accepted for publication at MBoC. Thanks very much for submitting this study to our journal. I'm very much looking forward to seeing it in print.

Best wishes,

Alex

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Greenberg:

Congratulations on the acceptance of your manuscript.

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