nature portfolio

Peer Review File

A domain-swapped CaMKII conformation facilitates linker-mediated allosteric regulation

Corresponding Author: Professor Margaret Stratton

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)
The manuscript by Nguyen, B., et al., presents a structural analysis of the Ca2+/calmodulin-dependent protein kinase II (CaMKII). CaMKII's consist of an N-terminal kinase domain, followed by a variable linker region, and terminated by a C-terminal hub domain. The hub domain is an oligomerization domain and allows the CaMKII's to form dodecameric, tetradecameric and hexadecameric structures. The linker region is formed via alternative splicing producing CaMKII's with no linker (0) or up to 217 residues. Linker length can participate in modulating Ca2+/CaM sensitivity but is not compulsory.

This study reveals a crystal structure of human CaMKIIδ-0 with an observed linker region connecting the kinase and hub domains. Previously reported crystal structures of CaMKIIα-0 (no linker) failed to resolve the linker region. CaMKIIα with a 30 residue linker (CaMKIIα-30), consisting of exons 14 and 18, is the predominantly expressed variant. The human CaMKIIδ-0 structure is identical to CaMKIIα-0, however, the electron density for 13 residues between the kinase and hub domain were visible. The chain trace suggests a domain swapped arrangement, with the kinase domain interfacing with the hub domain of neighbouring subunit, an arrangement previously observed in PDB 3SOA). For the CaMKIIα-0 variant, activation of the enzyme requires relatively higher concentrations of Ca2+ relative to variants with exons 14 and 18.

The authors use mutational studies coupled with enzymatic activities, crystallography, SAXS, MD-simulations, in vivo FRET, and mass spectrometry. The suite of techniques is comprehensive, however, the analysis is not.

Given the availability of the CaMKIIα-0 crystal structure and knowing the domain swapped arrangement from CaMKIIδ-0, Modeller could have been used to make all atom models of CaMKIIα-(Exon 14) and CaMKIIα-(Exon 18) dodecamer. These models could be used to fit the SAXS data directly. The SEC-SAXS data is of high quality and sufficient resolution to provide new, molecular insights into the dynamics of CaMKII. Presumably, the hub and kinase domains can be treated as rigid bodies and MD simulations or conformational sampling restricted to the linker could be used to generate 1000's of alternative conformations to determine which conformations best fit the SAXS data. The GASBOR ab initio models are meaningless and should be excluded from the manuscript.

The in vivo FRET studies with ionomycin indicates a significant observed difference between exons 14 and 18, which asks how does this manifest itself structurally? The authors have the SAXS data and could readily demonstrate this through additional atomistic modelling. I believe this will elevate the paper substantially and set a new standard for structural studies of CaMKII. There are many previous studies of CaMKII's investigated by SAXS and they have fallen short of revealing molecular details through atomistic modelling of the solution state.

Furthermore, MD simulations are notoriously difficult to sample biological relevant, alternate conformations and often show force-field dependencies. Some kind of enhanced sampling is often required to assert that the simulations have sufficiently explored conformational space. The low RMSD over a 200 ns is not a good measure of domain stability. Most proteins, including incorrectly folded AI-models, will stay relatively close to their starting state in an MD simulation. A more direct validation of the simulation would be from fitting trajectories to the SAXS data. If no models sufficiently fit the SAXS data, the simulation is not realistic.

Exon 14 is negatively charged and shows activation at substantially lower Ca2+ concentrations than CaMKIIα-(Exon 18) variant which is positively charged. It is reasonable to assume charge neutralisation likely promotes a conformational

change leading to exon-14 variant to be more Ca2+ sensitive. There is a lost opportunity to understand the role of Ca2+ in modulating the structure of CaMKIIα-(Exon 14) and CaMKIIα-(Exon 18). SAXS studies in the presence and absence of 1000 nM Ca2+ could readily reveal conformational changes supporting compaction or increased flexibility (via Rg, Dmax, dimensionless Kratky plot, Porod exponent). Compactness is akin to being conformationally restricted and perhaps in the presence of Ca2+ with the exon 14 variant, we might promote a single, extended conformation of the enzyme. This may be outside the scope of the paper, but I would encourage the authors to consider more rigorous SAXS studies to delineate the structural changes likely to occur in presence of Ca2+.

My recommendation is to use GAJOE or multi-FOXS on the dodecamer structures of CaMKIIα-(Exon 14) and CaMKIIα-(Exon 18) to complete the SAXS analysis and to validate the MD simulations. I believe the paper provides novel insights on the missing linker region between the hub and kinase domains and can contribute significantly to future studies if the SAXS analysis/modeling is pursued. Overall, the research is rigorous and the manuscript is well written.

Minor point.
For Figures 1, 2 and 3, the font-sizes are very small, please consider a larger font.

Figure 3. SAXS reconstructions using GASBOR are uninformative. They show the expected symmetry as this is imposed on the modelling.

Figure 3E. Which software was used to make the Pair-distance distribution?

Figure 4C. Does the variance in the baseline FRET signal reflect flexibility between the kinase and HUB domain? Or is the variance related to measurement error? The variance is much more narrower for exon-14.

Figure 5, 2Fo-Fc map does not show density contoured at 0.8s (assuming s is sigma). Please clarify.

Figure 5 should be labelled “The CaMKIIδ-0 holoenzyme is comprised of domain-swapped dimers”.

(Remarks on code availability)
Code is fine, used to create the linker regions, reads well and is easy to follow. Will be useful to others wishing to build linker regions for other proteins

Reviewer #2

(Remarks to the Author)
Several structures of Ca+2/calmodulin-dependent protein kinase II (CaMKII) previously published are primarily focused on the kinase domain or the hub domain of the CaMKII complex. The role of the linker region that mediates the communication between the hub and kinase domain has not been addressed sufficiently. As a consequence, the implications of this kinase-hub linker region in the CaMKII specificity and activity is not well understood. Here, the authors present an X-ray crystallography structure of CaMKII holoenzyme consisting of domain-swapped dimers. This research suggests that not only are the lengths of the connector region important for the CaMKII sensitivity to calcium but also the sequence variations in the variable linker region impact the Ca+2 /CaM sensitivity. Previous low-resolution structures of CaMKII have displayed a poor resolution of the connector region. Although in the new structure the linker still retains high flexibility, the rather extensive linker manipulations have facilitated the formation of domain-swapped dimers of CaMKII and provide a possible another alternative way of CaMKII regulation.

There is sufficient evidence for the conclusions generated from the mutational analysis of selected residues in the lateral and equatorial interface of the hub domain. The methodology is interdisciplinary, combining analytical biochemistry, bioinformatics, protein engineering, and biophysics. Any conclusion made from the studies is correlated with previous research in the field for validation and justification of the outcomes.

The data collection and refinement statistics align well with the current highest resolution obtained. The methodology section, along with the supplement data and the structural information made available in the PDB database, outlines sufficient details for the reproduction of the studies. The data collection and refinement table illustrates acceptable R and Rfree values for the 2.3 Å resolution achieved. However, providing Rmeas values instead of Rmerge values in the table would help to more accurately access the quality of reflection data (Rmerge is not a reliable quality indicator). Furthermore, a slightly lower percentage of a.a in the preferred region in the Ramachandran plot would be good to explain (due to the flexible hub-kinase linker region?).The authors should also provide the PDB ID of the solved CaMKIIδ structure in the table or in the Figure 5 caption, as currently, the PDB code is not given anywhere in the paper. A validation report should also be provided to the reviewers. Similarly, it would be helpful for the readers to understand the experiment conditions better if the X-ray wavelength used to collect the data was given in Table 1 (Data collection and refinement statistics). In Fig. 5B, the electron density map is not shown (however, it's provided in Sup. Fig.2 but with different sigma level). The geometry is very tight (e.g. 0.004Å in bond lengths) and unrealistic, presumably to improve the statistics. The authors should provide a comment. Did the tight geometry improve the Rfree?

There are three conditions that gave crystals. The authors should clarify which condition was used for structure determination. Have all conditions given the same flexibility in the linker region?

Are there SAXS data with the same construct used for crystallisation? Are the mutated residues only involved in this reported crystal packing? It would be good if the authors provide some discussion how they can exclude the possibility of a crystallisation artefact.

The mass photometry data have been performed with proteins kept at -80 C. Fresh proteins should have been used as the freezing could and thaw could affect oligomerization of the proteins and equilibrium between various aggregation stages. Provide version of AcquireMP and DiscoverMP software.

In the introduction section, line 95, the author states that extending linker length increases flexibility, and based upon line 90, the longer 30-residue linker CaMKIIα activates more easily at lower concentration of Ca+2 /CaM than their CaMKIIα-0 counterparts. But this is not the case with CaMKIIβ-217, the CaMKIIβ-217 is as sensitive to Ca+2/CaM as CaMKIIβ-no linker (line 78). Does the current CaMKIIδ structure explain this phenomenon? A 3D structure comparison between the two would be good as an answer. It would be good to address this in the discussion section as well for the readers.

Some spelling and syntax errors need attention, for example, change “disfunction” to “dysfunction” (line 57). change “his” to “their”(line 385), and correct “small angle” to “small-angle” (lines 37 and 89). Change “xon 18” to “Exon 18” (line 465), in line 211 add `P´to 4222, and a few more typos are present, suggesting proof-reading the whole material is required one more time. Correct 'ul' in l. 627.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In the manuscript, Nguyen et al. describe the structural and biophysical characterisation of the linker-mediated allosteric regulation of CaMKII. As part of this, the authors present X-ray crystal structure evidence CaMKII(delta)-0 forms a dodecameric holoenzyme alongside assignment of junction between the regulatory and hub domains. As part of this work, the authors employ a FRET-based biosensor “Camui” to characterise the conformational change of CaMKII with exon 14 or exon 18 in the presence of Ca/CaM.
Could the authors comment on the large size of the additional fluorescent proteins added to the N and C-terminus of CaMKII, do these influence protein complex formation and function? Both mseCFP and mCitrine YFP have a molecular weight of 27kDa each with CaMKII having a molecular weight of ~50 kDa. Could the authors comment on if they have biophysical evidence the protein with the two fluorescent proteins forms a dodecameric holoenzyme as depicted in figure 4A/B with both FP’s on the N- & C-termini. With CFP and YFP, the complex will be 1.25 MDa?
Could the authors comment on panels H, G & I of figure 4 as to the traces which appear to yield no conformational response for the FRET experiment.
Could the authors clarify the simultaneous measurement of Camui FRET and Ca2+ level using rhodamine-2. Based on the emission of mCitrine and excitation of Rhod-2(Ca2+) overlap?

(Remarks on code availability)
n/a

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #5

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(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for addressing my comments. The paper is much more impactful. Whilst the suggested MD simulations were not performed, I suggest the authors seek collaborations for future studies as this will provide new insights when coupled with the SAXS experiments. Supplemental figure R1 is superb, findings in response to Ca2+/CaM show how SAXS could be readily used to investigate structural properties without ab initio modeling. I have a minor comment:

Please verify the following. R2 shows Exon-14 variant forms a compact globular state at 20 uM CaM whereas in the absence of CaM, the variant is less compact/globular (shifted Guinier Kratky peak). The dimensionless Kratky plot in R2 B suggest compaction at 20 uM CaM, not consistent with the text "We observed both exon 14 and exon18 holoenzymes underwent
decompaction in the presence of Ca2+/CaM ", can you validate the data is plotted/labelled correctly? Exon-18 figures are consistent with author's statement. I suspect Figure R2 is correctly labeled whereas Figure R1 is not and that the results are reversed, that in the absence of Ca2+/CaM, exon-18 variant is compact/globular whereas exon-14 variant is not. Exon-14 variant becomes compact/globular at elevated Ca2+/CaM concentrations. This is consistent with the linkers being differentially charged.

Overall, this is a solid manuscript and I support publication

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I'm satisfied with the revision. Only a little comment that the authors could have tried refmac for refinement (e.g., at the final stages) as phenix seems to give very bad statistics for rsrz.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
In the manuscript, Nguyen et al. describe the structural and biophysical characterization of the linker-mediated allosteric regulation of CaMKII. I am happy with the responses from the authors tom my previous comments and have no additional comments or concerns in relation to the manuscript. As such I am happy to recommend the manuscript be accepted.

(Remarks on code availability)
The code is easy to read and understand and operates as presented

Reviewer #6

(Remarks to the Author)
The connector between the hub and the kinase domains of CaMKII contains the region that is sensitive to Ca2+/CaM. The accessibility of Ca2+/CaM to this Ca2+/CaM footprint and consequently, the sensitivity of CaMKII to Ca2+/CaM can be modulated by varying the length of the rest of the linker between the hub and the kinase domains. In this manuscript, the authors use multiple linker constructs and techniques (1) to show that the sequence of the linker, specifically the location of charges, can also strongly modulate the Ca2+/CaM sensitivity of CaMKII and (2) to crystallize a zero-length linker version of CaMKIIδ (CaMKIIδ-0) and show that it is composed of 3D-domain-swapped dimers. The authors then discuss a model of allosteric communication using (1) and (2).
Comments on the MD simulations:

(1) Currently the code availability statement reads: “CaMKII models and all scripts supporting the MD simulations can be found on Github (https://github.com/kairongdong/camk2_linker_modeling).”
I was not able to find this directory and the models and scripts seem to be at:
https://github.com/kairongdong/p1_camk2_linker_effect_AA

Can the authors please check and correct.

(2) MD simulations though short, have been performed with a standard protocol and the partially assembled CaMKII constructs seem appropriate. Were the partial CaMKII constructs held in place by a harmonic constraint or did the edge units not drift away (especially relevant to the higher temperature of the simulations) because the simulation time was short and the simulation box imposed constraints? This should be clarified in the methods section.

(3) The main observation from the MD simulations seems to be in Fig. 7A right panel. This shows the molecular interactions between the pattern of +ve charges of the regulatory segment with the pattern of –ve charges in the exon 18 linker which leads to a more compact exon 18 CaMKII conformation. The experimental evidence shows that the position and polarity of charges in the linker region matter. However, the MD simulations are necessary to give a possible molecular picture of why they matter.

(4) An alignment of the swapped and unswapped structures of CaMKII (cam2d_exon18.pdb and cam2a_exon18.pdb for instance, from the authors' constructs) indicates that a change in conformation of only 3-5 residues in the linker leads to domain-swapping. So, almost all the linker composition effects that the authors see (both experimentally and in simulations) should hold for both the swapped and the unswapped conformations. I could only find Supplementary Fig. S9 as evidence for domain-swapping in the longer (non-zero) linker constructs. However, the unswapped structures in Fig. S9 are created using a different sequence background than the swapped structures. There is also a difference in the total number of residues in the swapped and the unswapped structures. These give rise to local differences in conformation away from the site of the linker (in the starting structures) which the small simulation times are unlikely to change. The authors also don't provide a clear molecular explanation for how the change in conformation of the linker (due to un-swapping) affects the interactions between exon 18 (and reverse exon 18) and the regulatory segment as in Fig. 7A. So, it is possible that effects other than domain-swapping cause the effects in Fig. S9. The authors should re-analyze their unswapped simulation data and answer these questions but also add a caveat that the lack of difference between the exon 18 variant and the reverse exon 18 variant in Fig. S9 could arise from the different starting structures (sequences) in the unswapped variants. Also, see general comment below.

3D-domain-swapping propensity can also vary based on linker (hinge) length and composition. Given this, it is unclear why every linker variant of CaMKII should show 3D-domain-swapping. Additionally, CaMKII linkers are not static and extend and collapse during functon(as shown by the authors in this manuscript) and this could lead to a switch between a swapped and an "un"-swapped conformation. Such switching could lead to an even richer allosteric landscape for CaMKII.

If the authors are convinced from their data that all their CaMKII variants show domain-swapping, then this should be very clearly written in a section emphasizing the specific data that shows this. If however they think that swapping and un-swapping (either dynamically within a single structure or different domain-swapping propensities in different linkers) is a possibility, then this should also be discussed.

(Remarks on code availability)
The github README file needs to be edited and expanded for both clarity, grammar and correctness.

Version 2:

Reviewer comments:

Reviewer #6

(Remarks to the Author)
I am not entirely convinced that domain-swapping is central to this story of linker pattern mediated functional modulation and perhaps neither are the authors because they are reluctant to analyse their MD simulations further to understand the molecular basis for what domain-swapping is doing. That being said, I agree with the other reviewers that this manuscript has sufficient new and interesting data that it should be published.

(Remarks on code availability)

Reviewer #1 (Remarks to the Author):

The manuscript by Nguyen, B., et al., presents a structural analysis of the Ca2+/calmodulin-dependent protein kinase II (CaMKII). CaMKII's consist of an N-terminal kinase domain, followed by a variable linker region, and terminated by a C-terminal hub domain. The hub domain is an oligomerization domain and allows the CaMKII's to form dodecameric, tetradecameric and hexadecameric structures. The linker region is formed via alternative splicing producing CaMKII's with no linker (0) or up to 217 residues. Linker length can participate in modulating Ca2+/CaM sensitivity but is not compulsory.

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Thanks to the reviewer for their comments and helpful suggestions.

We agree about the GASBOR models and have removed them. We also agree that one could potentially build ensembles of atomistic models that are consistent with the SAXS data, even though explicit solvent simulations would be computationally too expensive (and likely not necessary) for this. Nonetheless, reconstruction atomistic ensembles based on SAXS data is beyond the scope of this manuscript in our opinion. We hope to expand this for future studies to the full complex and use our SAXS data more extensively to study various conformations adopted in solution.

We also want to note that, in this work, atomistic MD simulations were used to mainly investigate the conformational properties of the dynamic linker and how its interactions with the folded domains may modulate the conformational equilibrium of dodecamer. The simulation construct of four hub domains and two kinase domains is a minimal one that captures all interdomain interactions and local environment required for studying the linker and kinase-hub interactions.

The in vivo FRET studies with ionomycin indicates a significant observed difference between exons 14 and 18, which asks how does this manifest itself structurally? The authors have the SAXS data and could readily demonstrate this through additional atomistic modelling. I believe this will elevate the paper substantially and set a new standard for structural studies of CaMKII. There are many previous studies of CaMKII's investigated by SAXS and they have fallen short of revealing molecular details through atomistic modelling of the solution state.

Our in cellulo FRET experiments with exon 14 and exon 18 recapitulated our biochemical finding that exon 18 is less sensitive to calcium. We have two pieces of evidence indicating that this is due to structural changes within the CaMKII complex. 1) Our SAXS data show that exon 14 has a larger R_g compared to exon 18, consistent with previous CaMKII work showing that more compact structures (like exon 18 and CaMKIIalpha-0) are more difficult to activate. 2) The baseline FRET measurements for our Camui constructs are quite different – where Camui-exon 18 is significantly higher (Fig 4). This also indicates that exon 18 is a more compact structure, bringing the N- and C-termini closer together in the autoinhibited state (higher baseline FRET).

We agree with the reviewer that building models of the full holoenzyme and using these to do atomistic modelling with our SAXS data is a good idea, however we think this is beyond the scope of the current work. Additionally, using a generated model (using Modeller for example) and fitting this into SAXS generated densities can be fraught with error, and we would want to do this in a more comprehensive way – perhaps most thoroughly through collaboration with a lab who does this rigorously.

Furthermore, MD simulations are notoriously difficult to sample biological relevant, alternate conformations and often show force-field dependencies. Some kind of enhanced sampling is often required to assert that the simulations have sufficiently explored conformational space. The low RMSD over a 200 ns is not a good measure of domain stability. Most proteins, including incorrectly folded AI-models, will stay relatively close to their starting state in an MD simulation. A more direct validation of the simulation would be from fitting trajectories to the SAXS data. If no models sufficiently fit the SAXS data, the simulation is not realistic.

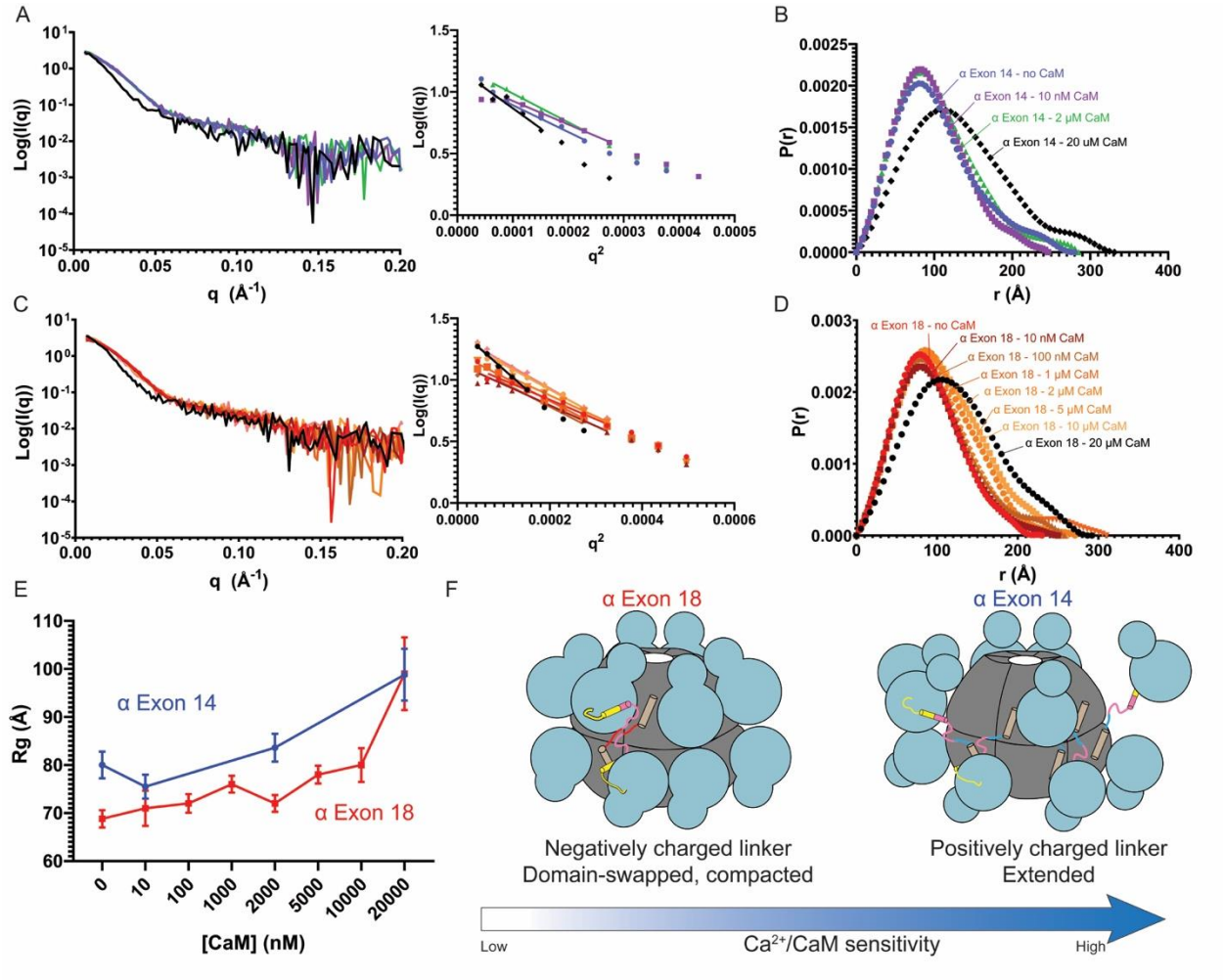
We agree that atomistic simulations are unlikely to be able to sample large conformational dynamics of large complexes like CaMKII dodecamers, even with enhanced sampling. As mentioned above, the objective of atomistic MD simulations in this work is mainly to investigate the conformational properties of the dynamic linker and its interactions with the folded domains, which is captured in a minimal construct with four hub domains and two kinase domains. The 200-ns simulations are reference

simulations to show that the construct and the charmm36m force field do not have obvious problems capturing the experimentally determined conformational state. Such a control is important for comparison to subsequent simulations of various with different linkers. These simulations are not meant as a rigorous validation of the entire dodecamer structure itself. We hope to pursue atomistic modeling based on SAXS data in future studies.

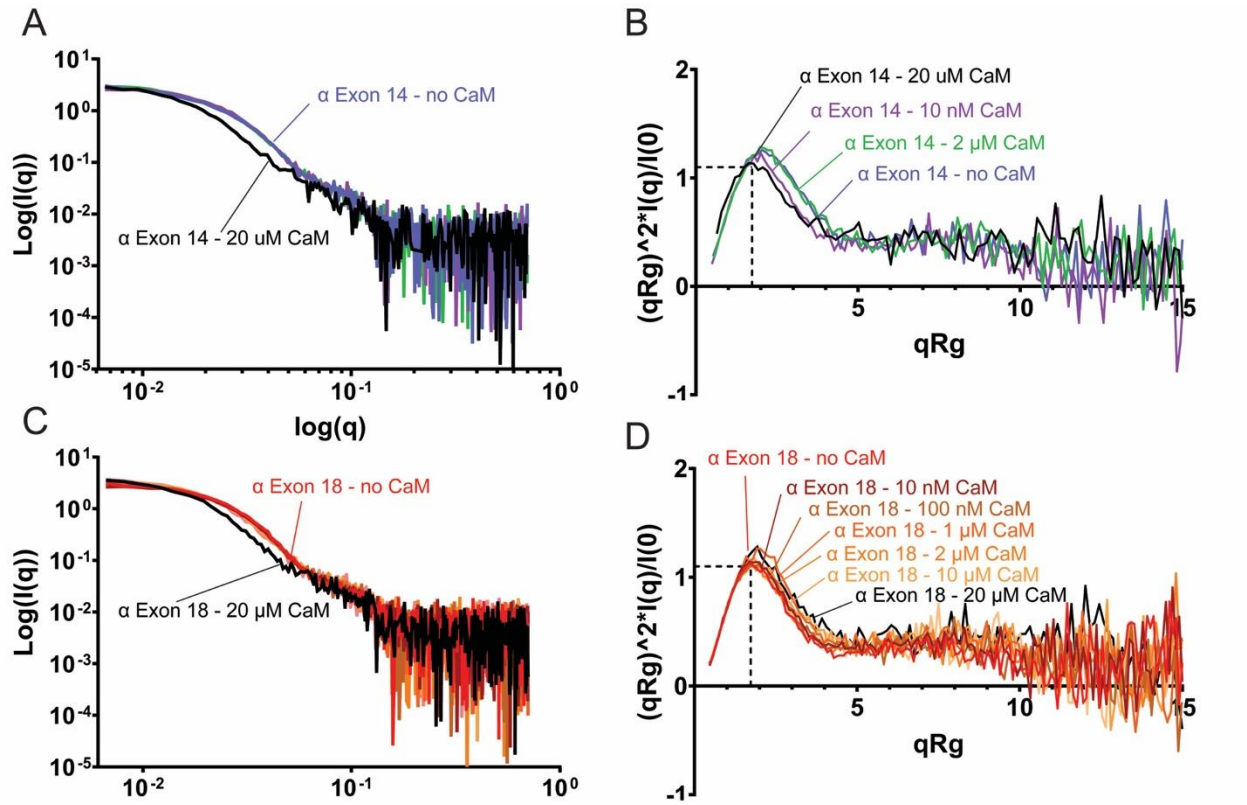
Exon 14 is negatively charged and shows activation at substantially lower Ca2+ concentrations than CaMKIIα-exon 18 variant which is positively charged. It is reasonable to assume charge neutralisation likely promotes a conformational change leading to exon-14 variant to be more Ca2+ sensitive. There is a lost opportunity to understand the role of Ca2+ in modulating the structure of CaMKIIα-exon 14 and CaMKIIα-exon 18. SAXS studies in the presence and absence of 1000 nM Ca2+ could readily reveal conformational changes supporting compaction or increased flexibility (via Rg, Dmax, dimensionless Kratky plot, Porod exponent). Compactness is akin to being conformationally restricted and perhaps in the presence of Ca2+ with the exon 14 variant, we might promote a single, extended conformation of the enzyme. This may be outside the scope of the paper, but I would encourage the authors to consider more rigorous SAXS studies to delineate the structural changes likely to occur in presence of Ca2+.

Thanks to the reviewer for this comment - we have now collected SAXS data for CaMKII-exon14 and –exon18 in the presence of increasing concentrations of Ca^{2+}/CaM (see below). We observed both exon 14 and exon18 holoenzymes underwent decompaction in the presence of Ca^{2+}/CaM ***(Supplemental figure R1)****. The R_g values of exon14 are consistently higher than exon 18 until they both reach ~99 Å under saturating Ca^{2+}/CaM, suggesting a similar and uniform extended conformation between exon14 and exon18, as you have mentioned.*

Porod exponent analysis showed that addition of Ca^{2+}/CaM decreases the Porod exponents for both exon14 and exon18, indicating increased flexibility. Dimensionless Kratky plots showed that addition of Ca^{2+}/CaM increases the globular characteristic for CaMKII-exon14, perhaps due to a decrease in charge repulsion and less disruption from the positively charged Exon 14 linker. On the other hand, CaMKII-exon18 exhibits an increase in disorder as concentration of Ca^{2+}/CaM increases, perhaps due to the decrease in linker-regulatory segment interactions ***(Supplemental figure R2)****.*



Supplemental Figure R1. *CaMKIIα-exon 18 holoenzymes undergo a larger decompaction than CaMKIIα-exon 14 holoenzymes upon activation. Scattering traces of CaMKIIα-exon 14 (A) and CaMKIIα-exon 18 (C) are shown, and inlets are the Guinier regions used to fit the R_g values. Pair distribution functions of CaMKIIα-exon 14 and CaMKIIα-exon 18 are shown in (B) and (D), respectively. (E) R_g values of CaMKIIα-exon 14 and CaMKIIα-exon 18 at different concentrations of Ca^{2+}/CaM. Data are shown as the fitted R_g standard deviation of the fit function (RAW v2.1.4). (F) Models showing a domain-swapped, compacted CaMKIIα-exon 18 holoenzyme that is less sensitive to Ca^{2+}/CaM than an extended CaMKIIα-exon 14 holoenzyme.*



***Supplemental Figure R2**. Porod and Kratky analyses of CaMKII-exon14 (A, B) and CaMKII-exon18 (C, D) in the presence of Ca^{2+}/CaM. Plots were generated in RAW v2.1.4.*

My recommendation is to use GAJOE or multi-FOXS on the dodecamer structures of CaMKIIα-exon 14 and CaMKIIα-exon 18 to complete the SAXS analysis and to validate the MD simulations. I believe the paper provides novel insights on the missing linker region between the hub and kinase domains and can contribute significantly to future studies if the SAXS analysis/modeling is pursued. Overall, the research is rigorous and the manuscript is well written.

Minor point.
For Figures 1, 2 and 3, the font-sizes are very small, please consider a larger font.
We have increased font sizes.

Figure 3. SAXS reconstructions using GASBOR are uninformative. They show the expected symmetry as this is imposed on the modelling.
We agree and have removed the reconstructions from the main figure.

Figure 3E. Which software was used to make the Pair-distance distribution?
We used GNOM program BioXTAS RAW v2.1.4 to calculate the Pair-distance distribution – this is now detailed in the methods section.

Figure 4C. Does the variance in the baseline FRET signal reflect flexibility between the

kinase and HUB domain? Or is the variance related to measurement error? The variance is much more narrower for exon-14.
The reviewer brings up a good point – we think the lower variance for exon-14 is due to structural fluctuations that are all at relatively low FRET values (i.e. less dynamic range here). Whereas the structural fluctuations within the exon-18 construct are at higher FRET values allowing us to observe these changes. We have added this to the discussion section as well.

Figure 5, 2Fo-Fc map does not show density contoured at 0.8s (assuming s is sigma). Please clarify.
We have removed the density from this figure and included it in Supplemental Fig. 2 where sigma is 0.7 Å.

Figure 5 should be labelled “The CaMKII□-0 holoenzyme is comprised of domain-swapped dimers”.
This has been edited.

Reviewer #1 (Remarks on code availability):

Code is fine, used to create the linker regions, reads well and is easy to follow. Will be useful to others wishing to build linker regions for other proteins

Reviewer #2 (Remarks to the Author)

Several structures of Ca+2/calmodulin-dependent protein kinase II (CaMKII) previously published are primarily focused on the kinase domain or the hub domain of the CaMKII complex. The role of the linker region that mediates the communication between the hub and kinase domain has not been addressed sufficiently. As a consequence, the implications of this kinase-hub linker region in the CaMKII specificity and activity is not well understood. Here, the authors present an X-ray crystallography structure of CaMKII holoenzyme consisting of domain-swapped dimers. This research suggests that not only are the lengths of the connector region important for the CaMKII sensitivity to calcium but also the sequence variations in the variable linker region impact the Ca+2 /CaM sensitivity. Previous low-resolution structures of CaMKII have displayed a poor resolution of the connector region. Although in the new structure the linker still retains high flexibility, the rather extensive linker manipulations have facilitated the formation of domain-swapped dimers of CaMKII and provide a possible another alternative way of CaMKII regulation.

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Thanks to the reviewer for the nice summary and constructive comments.

The data collection and refinement statistics align well with the current highest resolution obtained. The methodology section, along with the supplement data and the structural information made available in the PDB database, outlines sufficient details for the reproduction of the studies. The data collection and refinement table illustrates acceptable R and Rfree values for the 2.3 Å resolution achieved. However, providing Rmeas values instead of Rmerge values in the table would help to more accurately access the quality of reflection data (Rmerge is not a reliable quality indicator).

We have added the Rmeas values: 0.162 (1.691) as well as the R_{pim} (the precision-independent measurement of R) to Table 1.

Furthermore, a slightly lower percentage of a.a in the preferred region in the Ramachandran plot would be good to explain (due to the flexible hub-kinase linker region?).

The reviewer is right, we attribute the 94.9% of amino acids in the preferred Ramachandran range is due to the less ordered parts of the structure – including the connector region and other disordered loops within the kinase domain.

The authors should also provide the PDB ID of the solved CaMKIIδ structure in the table or in the Figure 5 caption, as currently, the PDB code is not given anywhere in the paper. A validation report should also be provided to the reviewers. Similarly, it would be helpful for the readers to understand the experiment conditions better if the X-ray wavelength used to collect the data was given in Table 1 (Data collection and refinement statistics).

The PDB code (8USO) and wavelength (0.979) have been added to Table 1. We also added the PDB code to the Figure 5 legend. The validation report from the PDB is also uploaded.

In Fig. 5B, the electron density map is not shown (however, it's provided in Sup. Fig.2 but with different sigma level).

Thanks to the reviewer for pointing this out – we actually removed the density here and instead include it only in Supp Fig 2.

The geometry is very tight (e.g. 0.004Å in bond lengths) and unrealistic, presumably to improve the statistics. The authors should provide a comment. Did the tight geometry improve the Rfree?

Approximately 35 rounds of standard reciprocal space refinement (interspersed with manual model inspection and correction) were performed in the program package Phenix. For low to medium resolution datasets, we have observed the best results when using an automatic target function and automatic optimization of the X-ray terms to the stereochemistry and atomic displacement (ADP) weights. As a result, both the R_{free} value and the stereochemistry of the model are optimized. One could obtain a lower R_{free} value but with worse model stereochemistry (i.e., Ramachandran plot statistics,

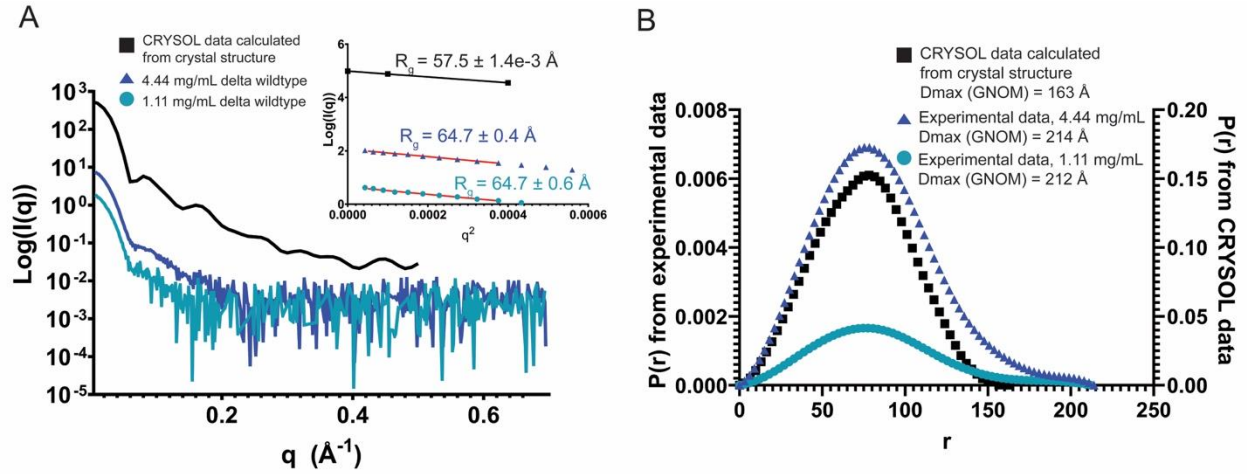
rotamer outliers, clashscore, etc.), but this refinement protocol resulted in the best balance of minimizing the overall discrepancy index to the model stereochemistry.

There are three conditions that gave crystals. The authors should clarify which condition was used for structure determination. Have all conditions given the same flexibility in the linker region?

Apologies for this confusion. In the methods we have now indicated that condition #1 (0.1 M pH 6.5 Bis-Tris, 16% PEG 2K, 0.2 M sodium malonate dibasic) was used for final structure determination and refinement. All conditions show the same flexibility in the linker region.

Are there SAXS data with the same construct used for crystallisation?

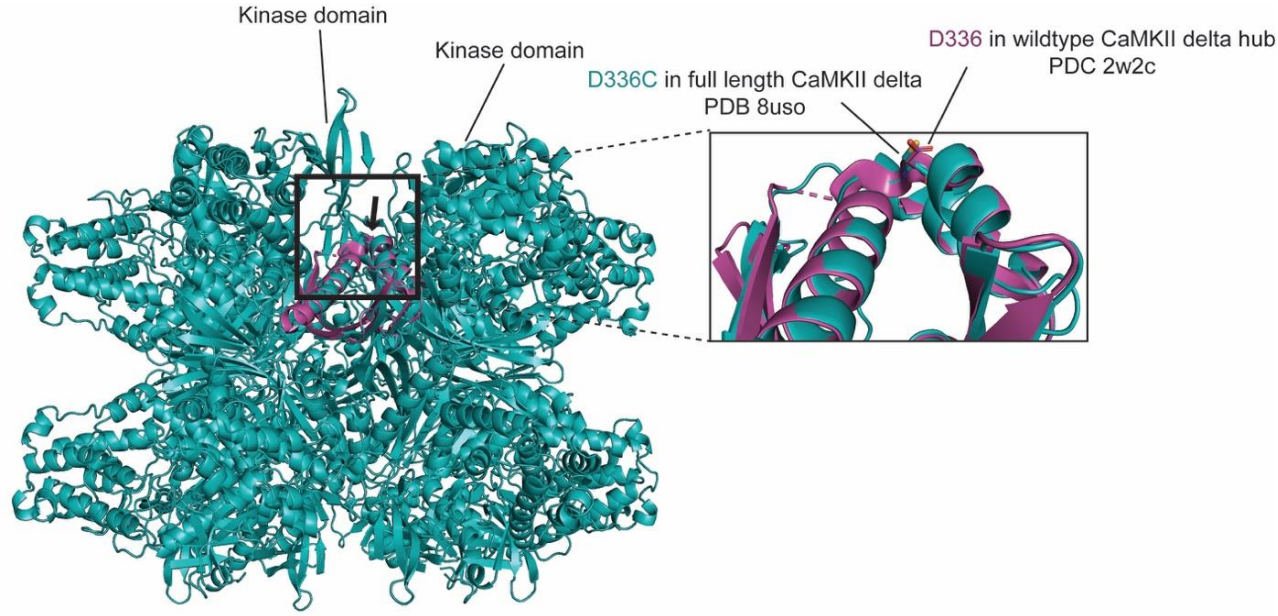
We have now collected SAXS data using the crystallization construct. We compared the experimental data with the calculated scattering pattern from the crystal model using CRYSOL (v2.8.3). The experimental R_g and Dmax values are higher than the theoretical R_g value, possibly due to the interconversion between closed and extended conformations of the holoenzyme in solution ***(Supplemental Figure R3).***



Supplemental Figure R3. *SAXS traces of CaMKIIδ-0 construct used in crystallography in comparison with the calculated SAXS trace from the crystal structure. The Guinier (A) and pair distribution function (B) analyses were performed in RAW v2.1.4.*

Are the mutated residues only involved in this reported crystal packing? It would be good if the authors provide some discussion how they can exclude the possibility of a crystallisation artefact.

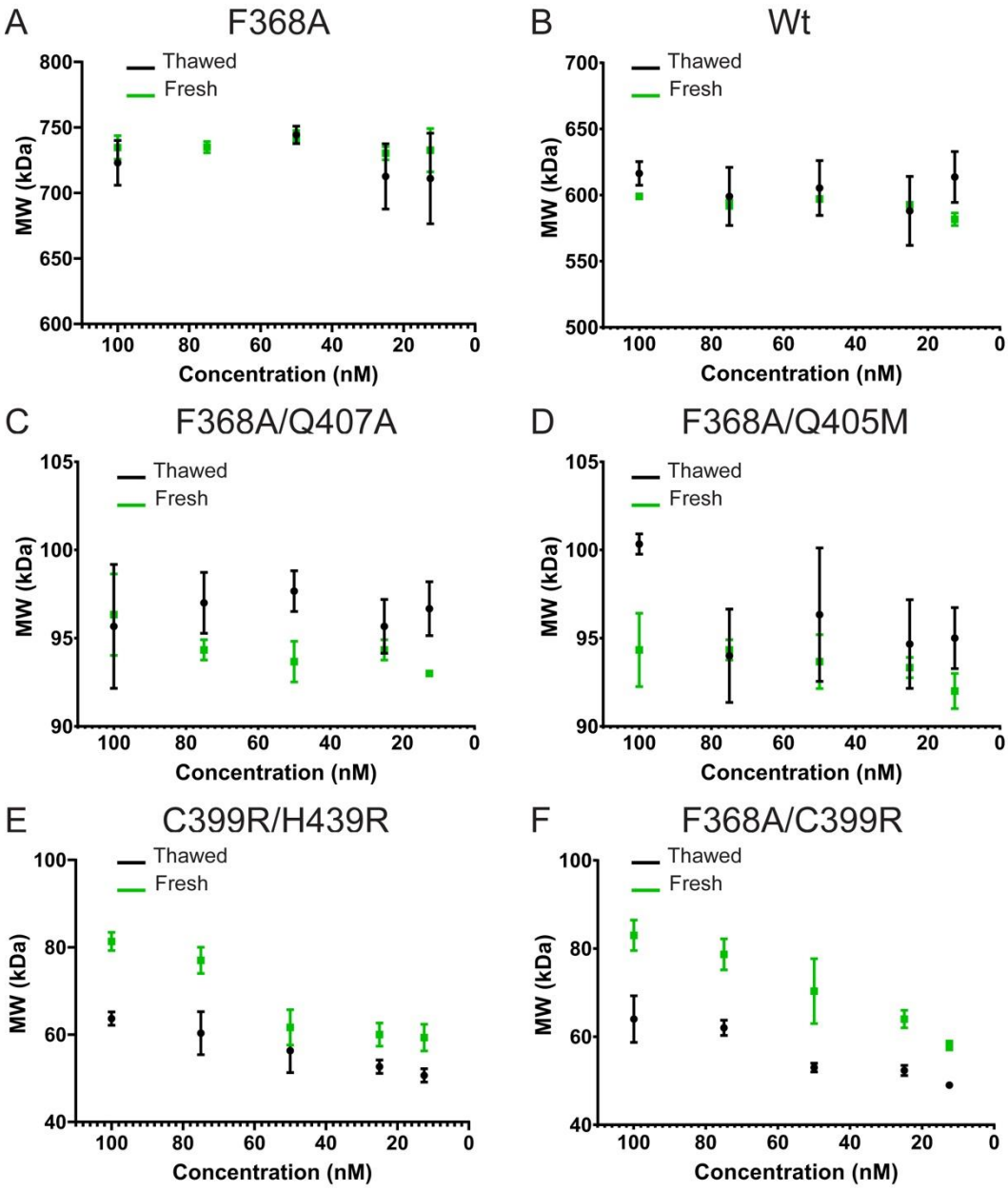
There is a single point mutation (D336C) in the construct used to calculate the crystal structure. This residue is not involved in crystal packing in the reported lattice. An overlaid model between a hub subunit from the crystal structure of WT CaMKII delta hub (PDB 2W2C) and our new structure (PDB 8USO) showed minimal differences (all atom RMSD = 1.025, ***Supplemental Figure R4***).



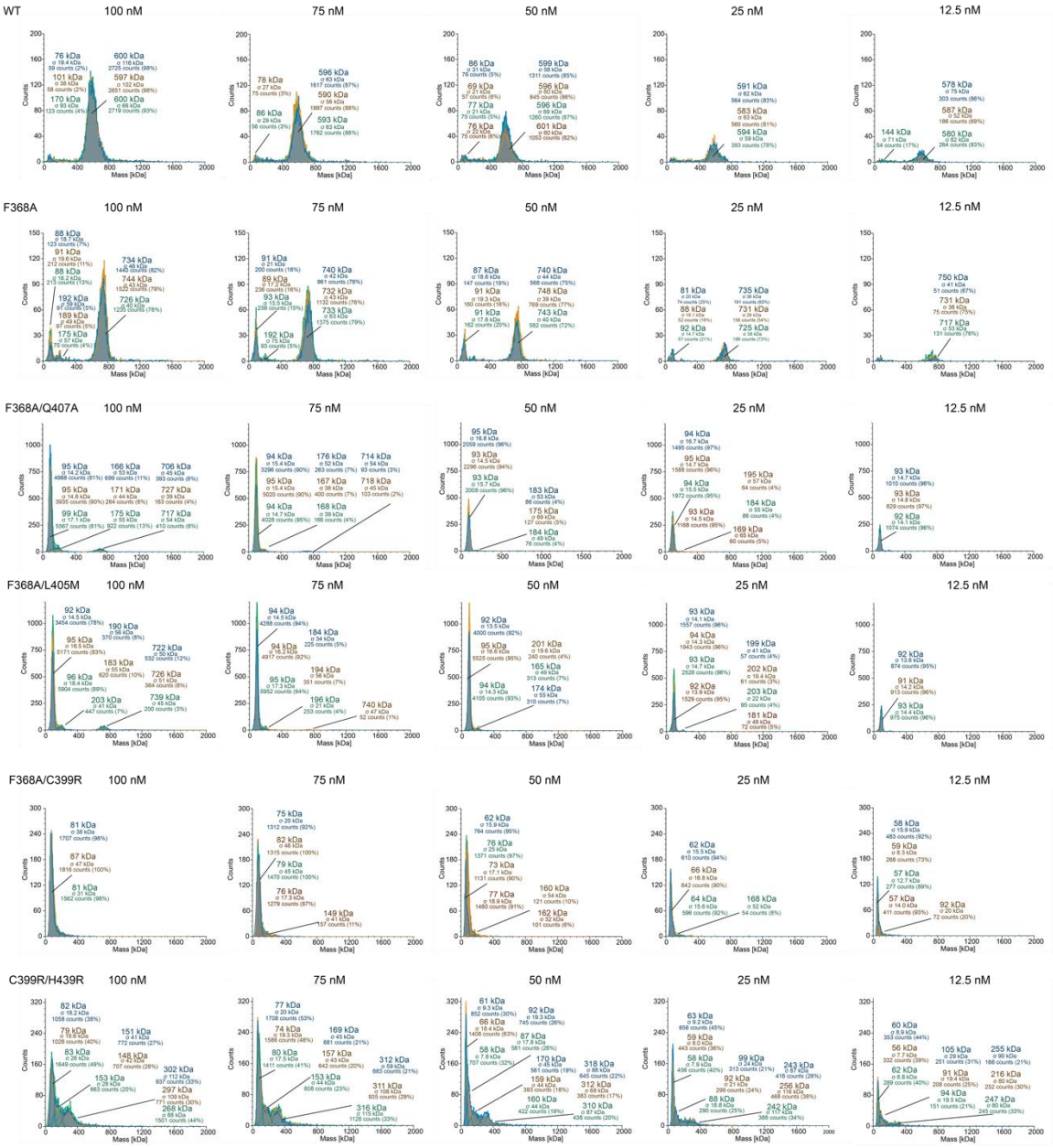
Supplemental Figure R4. *Alignment between the full-length CaMKIIδ-0 holoenzyme (PDB 8USO) and a subunit of CaMKIIδ hub structure (PDB 2W2C). The figure was generated in Pymol v2.3.3.*

The mass photometry data have been performed with proteins kept at -80 C. Fresh proteins should have been used as the freezing could and thaw could affect oligomerization of the proteins and equilibrium between various aggregation stages. Provide version of AcquireMP and DiscoverMP software.

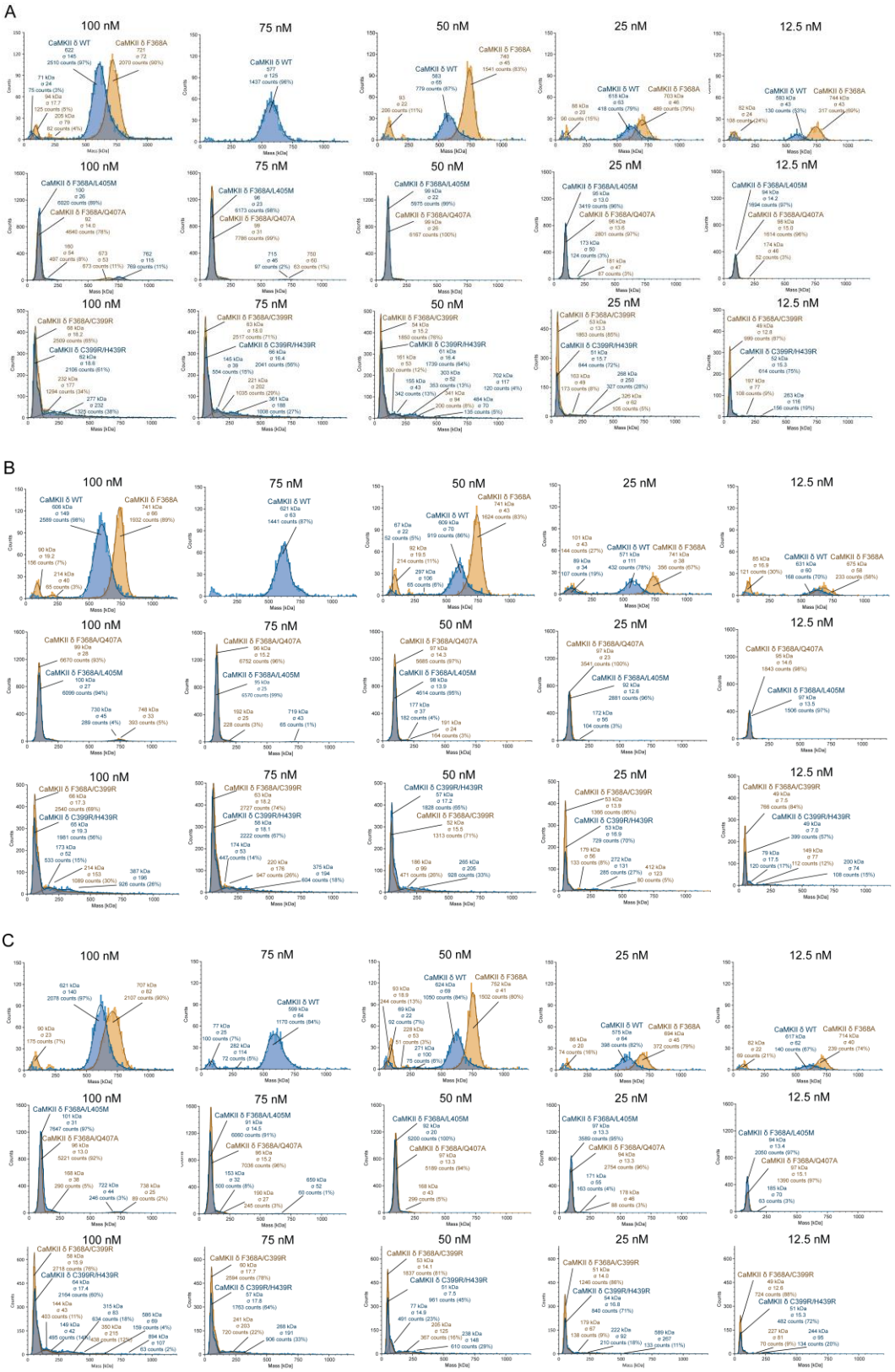
*We have now repeated all MP experiments with freshly purified protein. We observed similar results for wildtype, F368A, F368A/L405M, and F368A/Q407A. There was a 10-20 kDa difference in the molecular weight of the predominant populations between fresh and thawed proteins in F368A/C399R and C399R/H439R mutants (**Supplemental Figure R5**). The differences in molecular weight decreased at lower concentrations. The freeze/thaw may affect these mutants' oligomerization properties. We observed ~50-60% of C399R/H439R mutant forming oligomers in the freshly purified sample compared to 30-40% in the thawed sample (**Supplemental Figure R6**). However, we draw similar conclusions to those obtained from thawed protein samples, where we showed that the kinase-hub interactions dissociate as the hubs dissociate, and kinase-hub interactions may contribute to holoenzyme formation. We have updated our result section and the main figure to include the freshly purified proteins. Of note, the concentration of protein used in our kinase assay is ~13 nM, at which concentration the oligomeric states of the fresh protein and thawed proteins were similar. Software versions of MP are now included in the Methods section.*



Supplemental Figure R5*. Freshly purified delta-0 wildtype and mutants were examined for their oligomeric states by mass photometry, in comparison to proteins thawed from -80°C.*



Supplemental Figure R6*. Raw data of freshly purified protein used to generate Supplemental Figure R5. Three or four replicates of data are shown in each panel.*



Supplemental Figure R7*. Raw data of thawed protein used to generate Supplemental Figure R5. Each panel (A, B, C) represents one replicate.*

In the introduction section, line 95, the author states that extending linker length increases flexibility, and based upon line 90, the longer 30-residue linker CaMKIIα activates more easily at lower concentration of Ca+2 /CaM than their CaMKIIα-0 counterparts. But this is not the case with CaMKIIβ-217, the CaMKIIβ-217 is as sensitive to Ca2+/CaM as CaMKIIβ-no linker (line 78). Does the current CaMKIIδ structure explain this phenomenon? A 3D structure comparison between the two would be good as an answer. It would be good to address this in the discussion section as well for the readers.

This is a question that we hope to answer soon! From our previous work (Sci Signaling, 2020, PMID 32694170), we found that the identity of the hub domain also plays a role in regulating Ca^{2+} sensitivity. We found that CaMKIIδ behaved similarly to CaMKIIα and indeed could be due to similarities in structure as determined by hub/linker/kinase interactions. We are currently carrying out a series of cryoEM experiments coupled with biochemistry to fully understand the structure(s) of these autoinhibited holoenzymes.

Some spelling and syntax errors need attention, for example, change “disfunction” to “dysfunction” (line 57). change “his” to “their”(line 385), and correct “small angle” to “small-angle” (lines 37 and 89). Change “xon 18” to “Exon 18” (line 465), in line 211 add `P´to 4222, and a few more typos are present, suggesting proof-reading the whole material is required one more time. Correct 'ul' in l. 627.

These have all been fixed.

Reviewer #3 (Remarks to the Author)

In the manuscript, Nguyen et al. describe the structural and biophysical characterisation of the linker-mediated allosteric regulation of CaMKII. As part of this, the authors present X-ray crystal structure evidence CaMKII(delta)-0 forms a dodecameric holoenzyme alongside assignment of junction between the regulatory and hub domains. As part of this work, the authors employ a FRET-based biosensor “Camui” to characterise the conformational change of CaMKII with exon 14 or exon 18 in the presence of Ca/CaM.

Could the authors comment on the large size of the additional fluorescent proteins added to the N and C-terminus of CaMKII, do these influence protein complex formation and function? Both mseCFP and mCitrine YFP have a molecular weight of 27kDa each with CaMKII having a molecular weight of ~50 kDa. Could the authors comment on if they have biophysical evidence the protein with the two fluorescent proteins forms a dodecameric holoenzyme as depicted in figure 4A/B with both FP’s on the N- & C-termini. With CFP and YFP, the complex will be 1.25 MDa?

Thanks to the reviewer for bringing up this important point. Since its development in 2005 (Takao 2005, PMID: 15788767), Camui has been used in many different systems to monitor CaMKII activity – including neurons and cardiomyocytes (Erickson et al., 2011; Otmakhov et al., 2015b, 2015a).The original paper characterized holoenzyme

with the addition of the GFP and YFP both in vitro, in cell lysates, and in mammalian cells (Takao 2005, PMID: 15788767), and indeed the protein forms a >1000 kDa comlex and is responsive to CaMKII-specific activation and inhibition treatments.

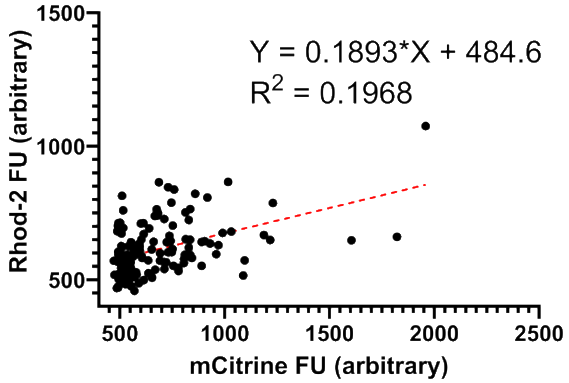
However, to directly answer the question, we do not have in vitro evidence that Camui forms a dodecamer as shown in Figure 4. Given that the Camui construct will be overexpressed in oocytes, it is statistically likely that it will homo-oligomerize as opposed to oligomerizing with endogenous protein. To the reviewer's point, Camui introduces large fluorescent proteins that may affect oligomerization and/or dynamics. We would prefer to use a substrate-based sensor like the one we developed (FRESCA, Ardestani et al. 2019; PMID: 31201271), however Camui provides a nice way to observe a single CaMKII variant at a time in a system which naturally expresses CaMKII, which is the experiment we needed to do here. Because the 2 Camui constructs are identical other than the linker region, we think this is a viable comparison, even with the caveats introduced by the Camui design.

Could the authors comment on panels H, G & I of figure 4 as to the traces which appear to yield no conformational response for the FRET experiment.

Each trace corresponds to data collected from a single oocyte. Indeed, there are cell to cell variations, which is why some responses (both Ca^{2+} and FRET) are worse than others. We include all data in our baseline analysis (Fig. 4C).

Could the authors clarify the simultaneous measurement of Camui FRET and Ca2+ level using rhodamine-2. Based on the emission of mCitrine and excitation of Rhod-2(Ca2+) overlap?

*Each channel (Blue FP, Yellow FP, and Rhod-2) is imaged sequentially. In our experiment, we excite CFP directly at 436 nm and monitor emission of CFP (~480 nm) and FRET induced citrine emission (~530 nm). Rhod-2 is maximally excited with 550 nm light. The reviewer is correct that there is some overlap here, however, this would have to be a result of FRET between citrine and Rhod-2, which is unlikely. In the experiment we do not directly excite citrine. To be sure, we checked the Rhod-2 and mCitrine emissions, which have a low correlation value (**Supplemental Figure R8**).*



***Supplemental Figure R8**. Correlation analysis between basal mCitrine and Rhod-2 emission during the 1st minute of the experiment. Each data point represents an individual oocyte (142 total oocytes). Linear regression analysis was performed in Prism8.*

Reviewer #1 (Remarks to the Author):

I thank the authors for addressing my comments. The paper is much more impactful. Whilst the suggested MD simulations were not performed, I suggest the authors seek collaborations for future studies as this will provide new insights when coupled with the SAXS experiments. Supplemental figure R1 is superb, findings in response to Ca2+/CaM show how SAXS could be readily used to investigate structural properties without ab initio modeling. I have a minor comment:

Please verify the following. R2 shows Exon-14 variant forms a compact globular state at 20 uM CaM whereas in the absence of CaM, the variant is less compact/globular (shifted Guinier Kratky peak). The dimensionless Kratky plot in R2 B suggest compaction at 20 uM CaM, not consistent with the text "We observed both exon 14 and exon18 holoenzymes underwent decompaction in the presence of Ca2+/CaM ", can you validate the data is plotted/labelled correctly? Exon-18 figures are consistent with author's statement. I suspect Figure R2 is correctly labeled whereas Figure R1 is not and that the results are reversed, that in the absence of Ca2+/CaM, exon-18 variant is compact/globular whereas exon-14 variant is not. Exon-14 variant becomes compact/globular at elevated Ca2+/CaM concentrations. This is consistent with the linkers being differentially charged.

Overall, this is a solid manuscript and I support publication

We thank the reviewer for the constructive comments.

Our overall understanding of the exon 18 versus exon 14 structures is that exon 18 will behave as a compact protein whereas exon 14 will behave as a more extended protein. The R_g data supports this as exon 14 displays a larger R_g in the absence of Ca^{2+}/CaM. As expected, both constructs reach a full open state (highest R_g) in the presence of saturating Ca^{2+}/CaM (20 uM). The Kratky plots indicate that exon 18 shape does not change significantly with and without Ca^{2+}/CaM. This also fits our hypothesis because the compact protein is more globular, and Ca^{2+}/CaM binding does not disrupt its globular state. The Kratky plots indicate that exon 14 shifts from a less globular state to a more globular state in the presence of saturating Ca^{2+}/CaM. We interpret this to mean that the extension of the kinase domains away from the hub domain in the exon 14 protein may behave more as a multidomain protein. But the reviewer is correct that further SAXS analysis and MD will benefit our understanding of this system moving forward. We have changed the language of the sentence "We observed both exon 14 and exon 18 holoenzymes underwent decompaction in the presence of Ca^{2+}/CaM" to avoid any confusion.

Reviewer #2 (Remarks to the Author):

I'm satisfied with the revision. Only a little comment that the authors could have tried refmac for refinement (e.g., at the final stages) as phenix seems to give very bad statistics for rsrz.

We appreciate the reviewer's suggestion regarding the use of Refmac for refinement. Phenix is widely regarded as a standard tool in the field, and the refinement statistics we obtained were within acceptable parameters and approved by the PDB. That said, we recognize that different refinement programs may yield improvements in specific metrics such as RSRZ. We will certainly consider incorporating comparative refinements using Refmac in future studies to further optimize model quality.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5 (Remarks to the Author):

In the manuscript, Nguyen et al. describe the structural and biophysical characterization of the linker-mediated allosteric regulation of CaMKII. I am happy with the responses from the authors tom my previous comments and have no additional comments or concerns in relation to the manuscript. As such I am happy to recommend the manuscript be accepted.

Reviewer #5 (Remarks on code availability):

The code is easy to read and understand and operates as presented

Reviewer #6 (Remarks to the Author):

The connector between the hub and the kinase domains of CaMKII contains the region that is sensitive to Ca2+/CaM. The accessibility of Ca2+/CaM to this Ca2+/CaM footprint and consequently, the sensitivity of CaMKII to Ca2+/CaM can be modulated by varying the length of the rest of the linker between the hub and the kinase domains. In this manuscript, the authors use multiple linker constructs and techniques (1) to show that the sequence of the linker, specifically the location of charges, can also strongly modulate the Ca2+/CaM sensitivity of CaMKII and (2) to crystallize a zero-length linker version of CaMKIIδ (CaMKIIδ-0) and show that it is composed of 3D-domain-swapped dimers. The authors then discuss a model of allosteric communication using (1) and (2).

Comments on the MD simulations:

(1) Currently the code availability statement reads: "CaMKII models and all scripts supporting the MD simulations can be found on Github (https://github.com/kairongdong/camk2_linker_modeling)."

I was not able to find this directory and the models and scripts seem to be at:
https://github.com/kairongdong/p1_camk2_linker_effect_AA
Can the authors please check and correct.

We apologize for this mistake. The depository has now been updated and moved to a new, permanent location (https://github.com/mdlab-um/camk2_linkers). The README files have also been updated to provide a clearer description of key files (e.g., initial and equilibrated structure models). All MD and analysis scripts are still included, but in sub-directories for clarity.

(2) MD simulations though short, have been performed with a standard protocol and the partially assembled CaMKII constructs seem appropriate. Were the partial CaMKII constructs held in place by a harmonic constraint or did the edge units not drift away (especially relevant to the higher temperature of the simulations) because the simulation time was short and the simulation box imposed constraints? This should be clarified in the methods section.

We have revised the Method section to clarify that no restraint was imposed during the production simulations at both 300K and 400K. Due to the limited simulation timescale, we did not observe dissociation of edge units.

(3) The main observation from the MD simulations seems to be in Fig. 7A right panel. This shows the molecular interactions between the pattern of +ve charges of the regulatory segment with the pattern of –ve charges in the exon 18 linker which leads to a more compact exon 18 CaMKII conformation. The experimental evidence shows that the position and polarity of charges in the linker region matter. However, the MD simulations are necessary to give a possible molecular picture of why they matter.

Thanks for this comment, we agree that the modeling and simulations in the current work give a possible molecular picture of why charge polarity and position matter in CaMKII activation.

(4) An alignment of the swapped and unswapped structures of CaMKII (cam2d_exon18.pdb and cam2a_exon18.pdb for instance, from the authors' constructs) indicates that a change in conformation of only 3-5 residues in the linker leads to domain-swapping. So, almost all the linker composition effects that the authors see (both experimentally and in simulations) should hold for both the swapped and the unswapped conformations.

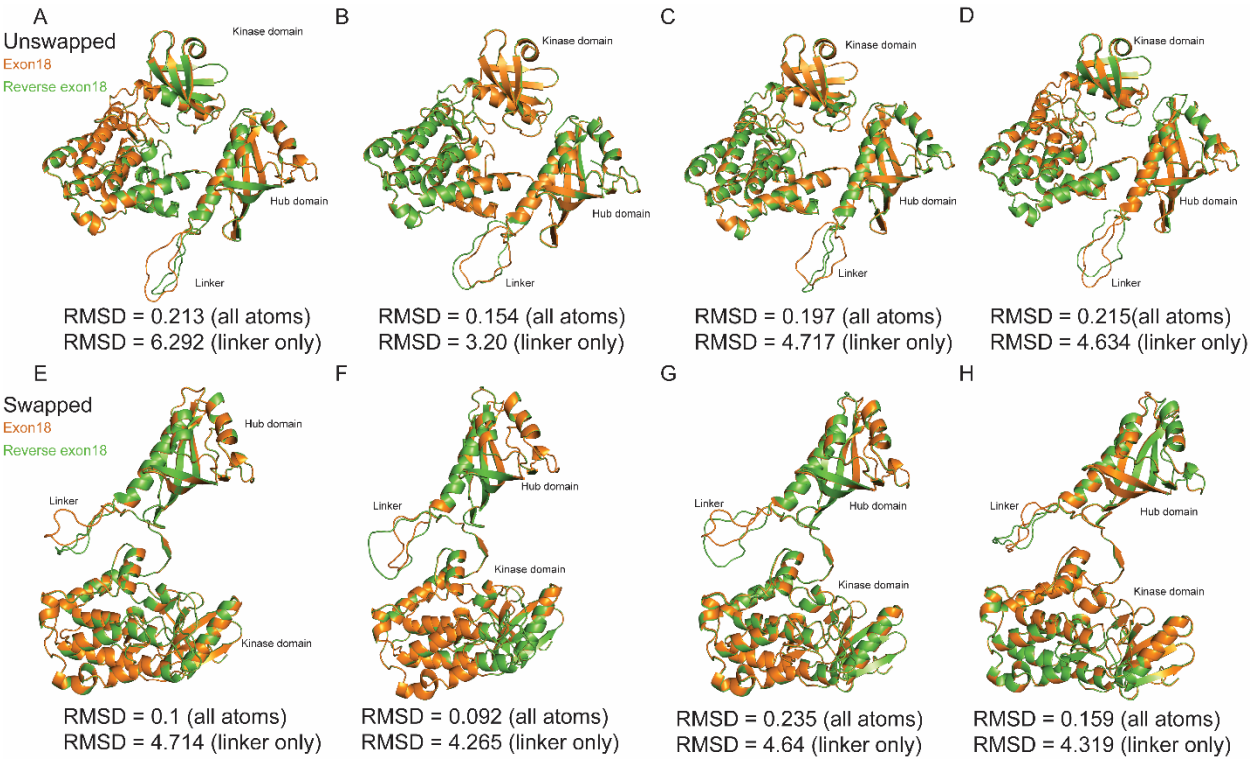
I could only find Supplementary Fig. S9 as evidence for domain-swapping in the longer (non-zero) linker constructs. However, the unswapped structures in Fig. S9 are created using a different sequence background than the swapped structures. There is also a difference in the total number of residues in the swapped and the unswapped structures. These give rise to local differences in conformation away from the site of the linker (in the starting structures) which the small simulation times are unlikely to change.

Indeed, the reviewer is correct that swapped versus unswapped structures are quite subtle in an overlay of static structures. A very difficult question to answer here is "what is domain swapping

facilitating?". While we cannot directly answer this question, the simulations comparing different linkers in the swapped versus unswapped conformations show that only in the swapped conformation do we see any differences in the fluctuation of the regulatory segment. Even on a relatively short timescale, these simulations indicate that domain swapping facilitates this interaction between linker and regulatory segment.

The authors also don't provide a clear molecular explanation for how the change in conformation of the linker (due to un-swapping) affects the interactions between exon 18 (and reverse exon 18) and the regulatory segment as in Fig. 7A. So, it is possible that effects other than domain-swapping cause the effects in Fig. S9. The authors should re-analyze their unswapped simulation data and answer these questions but also add a caveat that the lack of difference between the exon 18 variant and the reverse exon 18 variant in Fig. S9 could arise from the different starting structures (sequences) in the unswapped variants. Also, see general comment below.

We thank you for raising this point. We have calculated the RMSD values for the starting structures of the swapped and the unswapped conformations of exon 18 and reverse exon 18 (Supp. Fig. R1). As the reviewer points out, there are differences in the starting structures of the linker regions in both swapped and unswapped. However, despite having different starting structures, in the unswapped conformation - exon 18 and reverse exon 18 linkers exhibit similar levels of fluctuation throughout the simulation as shown in Fig. S9. Whereas in the swapped conformation, there are changes in these fluctuations. Therefore, we think that the lack of a difference observed in Fig. S9 is very likely due to domain swapping. We think that pursuing the molecular details of the effects of unswapping would require further studies and something we are pursuing.



Supplemental Figure R1. *Structural alignments between exon 18 (orange) and reverse exon 18 (green) in unswapped (A-D) and swapped (E-H) conformations at the beginning of the simulation. Each panel shows an alignment (Pymol) of each subunit of exon 18 and reverse exon 18 extracted from the tetramers of unswapped or swapped conformations.*

3D-domain-swapping propensity can also vary based on linker (hinge) length and composition. Given this, it is unclear why every linker variant of CaMKII should show 3D-domain-swapping. Additionally, CaMKII linkers are not static and extend and collapse during function (as shown by the authors in this manuscript) and this could lead to a switch between a swapped and an “un”-swapped conformation. Such switching could lead to an even richer allosteric landscape for CaMKII.

We appreciate this insightful comment on CaMKII. We agree that switching between unswapped and swapped conformations would provide a rich allosteric landscape for CaMKII, and this could be the reason for domain swapping in all CaMKII variants. So far, such a transition between swapped and unswapped has not been shown, and we are pursuing further experiments to better understand this.

If the authors are convinced from their data that all their CaMKII variants show domain-swapping, then this should be very clearly written in a section emphasizing the specific data that shows this. If however they think that swapping and un-swapping (either dynamically within a single structure or different domain-swapping propensities in different linkers) is a possibility, then this should also be discussed.

We appreciate this comment, and this is a crucial question! We do know from cryoEM, for example, that there are significant dynamics within the holoenzyme structure. Our new structure unambiguously supports a domain-swapped conformation, specifically for CaMKII delta. The previous structure of CaMKII alpha had sufficiently poor density at the connector segment that we are unable to unambiguously say whether it is swapped or not. Our MD simulations of the domain swapped conformation were performed using CaMKII delta swapped structure. And the results of the simulations corroborate the biochemical data done in CaMKII alpha, providing evidence for a domain swapped conformation in CaMKII alpha. To properly determine this, we must solve additional structures to high resolution and ideally use cryoEM to better understand the dynamic landscape within the holoenzyme structure. We added a few sentences in the discussion (end of first paragraph) to clarify our thinking on this.

Reviewer #6 (Remarks on code availability):

The github README file needs to be edited and expanded for both clarity, grammar and correctness.

This has been done.