

Paradoxical non-catalytic kinase functions are driven by inhibitor-induced displacement of autoinhibitory domains

Viviane Reber, Sabrina Keller, Stefanie Loosli, Yumi Arima, Tatjana Kleele, Paola Picotti, and Matthias Gstaiger

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Paola Picotti

Matthias Gstaiger

Dear Dr. Gstaiger,

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three reviewers who agreed to evaluate your manuscript. As you will see from the reports below, the reviewers find the study interesting and relevant. They raise, however, a series of concerns, which should be convincingly addressed in a major revision.

The reviewers' recommendations are relatively clear, so there is no need to reiterate the points listed below. All the issues raised by the reviewers need to be satisfactorily addressed. As you may already know, our editorial policy allows in principle a single round of major revision, and it is therefore essential to provide responses to the reviewers' comments that are as complete as possible. Please feel free to contact me in case you would like to discuss in further detail any of the issues raised by the reviewers.

On a more editorial level, we would ask you to address the following issues:

- Please provide a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

- Please provide individual production quality figure files as .eps, .tif, .jpg (one file per figure). When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen: <https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1>

- Please provide a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

- Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

- We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2' etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

For the figures and tables that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Each legend should be below the corresponding Figure/Table in the Appendix. Appendix figures and tables should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2, Appendix Table S1" etc. See detailed instructions regarding expanded view here: <https://link.springer.com/journal/44320/submission-guidelines#cms-Expanded-View-data>.

- Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database.

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also <https://link.springer.com/partners/embo-press/editorial-policies#Data%20availability%20statement>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843>)

- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

- At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

Additional information on source data and instruction on how to label the files are available <

<https://link.springer.com/journal/44320/submission-guidelines#cms-Source-data> >

- Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at < <https://link.springer.com/journal/44320/submission-guidelines#cms-Reference-guidelines> >.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy (<https://link.springer.com/partners/embo-press/editorial-policies#Competing%20interest%20disclosures>) and update your competing interests if necessary.

Please use the heading "Disclosure statement and competing interests".

- All Materials and Methods need to be described in the main text using our 'Structured Methods' format. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs.

Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines:

<https://link.springer.com/journal/44320/submission-guidelines#structuredmethods>.

When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but upload it as a separate file choosing the file type "Reagent Table".

-Regarding data quantification:

Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Please also include scale bars in all microscopy images.

- Please provide a "standfirst text" summarizing the study in one or two sentences (approximately 250 characters, including space), three to four "bullet points" highlighting the main findings and a "visual abstract" (exactly 550px width and 300 px height, PNG format) to highlight the paper on our homepage.

When you resubmit your manuscript, please download our CHECKLIST (<https://media.springernature.com/original/springer-cms/rest/v1/content/27825796/data/v1>) and include the completed form in your submission.

Please note that the Author Checklist will be published alongside the paper as part of the transparent process .

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

I look forward to receiving the revised manuscript soon.

Kind regards,
Jingyi

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (2nd Jul 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

<https://msb.msubmit.net/cgi-bin/main.plex>

IMPORTANT: Please read our guidelines for revised submissions: <https://link.springer.com/journal/44320/submission-guidelines#cms-Revised-submissions>

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Reviewer #1:

The manuscript "Paradoxical non-catalytic kinase functions are driven by 1 inhibitor-induced displacement of autoinhibitory domains" by Reber et al. described an integrated mass spectrometry approach combining LiP-MS, AP-MS, and proximity labeling-MS to map the inhibitor-induced kinase conformation and interaction changes. This work is very interesting and uncover the widespread mechanisms of kinase inhibitor-induced non-catalytic conformation alterations, which might greatly contribute to the rational design of more effective kinase inhibitors. I think this work can be published on MSB after minor revision.

1. All the Lip-MS experiments were performed after affinity purification. Although this AP approach is more native than in vitro analysis, the sequence and site coverage is a problem. So, can the authors cover all the interesting regions of kinase in ATP binding and autoinhibitory domains? The sequence region coverages for all the protein kinase analysis should be given in the manuscript.
2. How the authors evaluate the kinase conformation changes is directly related to the inhibitor binding or by other protein interactions? As the AP-MS experiments identified many interaction proteins. Maybe some invitro analysis by using purified proteins is still needed?
3. How the in vivo proximity labeling experiment and data analysis are conducted are not clear. They authors should describe the procedures in more detail. If CH₂O is used for in vivo labeling, how to avoid crosslinking, how to do database searching and realize confident results?
4. For AP-Lip-MS analysis, the conformation information of other proteins within the complexes could be also obtained. Can the authors also analysis and discuss these interface alterations.

Reviewer #2:

Summary

Reber et al. address an underexplored dimension of kinase pharmacology: the non-catalytic, "off-mechanism" effects of ATP-competitive inhibitors that is mediated through changes of kinase domain conformation and protein-protein interaction (PPI) changes. The authors develop a powerful proteomics framework using LiP-MS (limited proteolysis mass spectrometry; previously developed by the authors) applied to native affinity-purified protein complexes, and proximity labeling-MS to systematically map inhibitor-induced structural and interactome remodeling in a native cellular context. Using three Ser/Thr-kinases (CAMKK2, CHEK1, and PRKCA) with distinct autoinhibitory domain (AID) architectures, Reber and colleagues demonstrate that ATP-competitive kinase inhibitor binding induces displacement of the AID, promoting an open, active-like conformation despite catalytic inhibition. This structural transition rewires PPIs and drives diverse (paradoxical) non-catalytic phenotypes. This is nicely demonstrated in relevant cell biological assays for all three kinase-inhibitor pairs. The data is supported by mutational analyses, which allows to disentangle catalytic inhibition from conformational effects. The findings support a model in which the ATP-binding site acts as a central allosteric hub governing kinase conformation and interaction networks.

Overall, the study provides compelling evidence that kinase inhibitor-induced neomorphic PPIs may be a widespread and understudied feature with potentially important biological functions. The proposed proteomics pipeline represents a scalable and generalizable strategy to uncover such effects, with important implications for drug development, particularly in anticipating side effects and improving inhibitor selectivity.

Major comments:

1. While I agree that kinase inhibitor-induced changes of (intra- and intermolecular) PPIs are understudied and some paradoxical effects of kinase inhibitor on signaling are mentioned in the Discussion section (paradoxical MAPK activation by BRAF inhibitors and JAK2 hyperphosphorylation by type I JAK1/2 inhibitors), one very prominent early example is missing and should be included: The Grzesiek lab demonstrated in 2013 that ATP competitive kinase inhibitors targeting BCR::ABL1 result in an open conformation, which enable its SH2 and SH3 domains to engage in PPIs (Skora et al. PNAS 2013; PMID: 24191057).
2. I was a bit puzzled that the authors focus entirely on type I kinase inhibitors. While type I inhibitors are indeed more common and induce an active open conformation of the kinase domain and are therefore prime candidates for the studied phenomenon, it has been shown that also type II inhibitors lead to AID displacement and disassembly of intramolecular PPIs. This was first demonstrated for type II BCR::ABL1 kinase inhibitors (see: Sonti et al. JACS 2018; PMID: 29319304); whereas in contrast type I TKIs (e.g. dasatinib, bosutinib, ...) kept BCR::ABL1 in an assembled conformation. I suggest to include these examples and thereby broaden the scope/impact of the manuscript. Hence, it can be expected that also binding of many type II inhibitors (and potentially also allosteric inhibitors) may also result in conformations that displace AID and induce neomorphic PPIs.
3. The manuscript focuses on kinase inhibitor-induced changes on native (unmutated) kinases. While this is important to study for possible side effects in "off-tissues" or uncover diminished therapeutic effect in the on-target cells/tissues, I lack a discussion and mention that the therapeutic targets of most kinase inhibitors are not native kinases, but active oncogenic kinases that are activated by point mutations, insertions/deletions, duplication, fusion partners etc. These oncogenic kinase variants have often already displaced AIDs, open/disassembled conformations and possibly a different subcellular localization. What is the authors expectation of the consequences of inhibitor-induced (further) changes in this common scenario?

Reviewer #3:

The authors set out to investigate small molecule inhibitor functions beyond catalytic inhibition using a multi-proteomic approach. Focussing on three examples with different structural architecture and three matching selective small molecule inhibitors, structural re-arrangements via LIP-MS and the change of the proximal proteome are investigated upon ligand treatment. The authors identify three separate consequences of ligand binding beyond inhibition: for CHEK1 dissociation from CLBP is identified. This causes mitochondrial fragmentation. PRKCA is re-localised to cell junctions and endosome-like organelles. CAMKK2 triggers AMPK sequestration. The study is overall of very high quality, elucidates novel mechanisms of action beyond inhibition of inhibitors and thus provides timely insights into additional/off-target consequences of small molecule binding. This provides important insights into small molecule actions and further substantiates the need of detailed dissection of inhibitor consequences beyond inhibition. At this point a few open points still, however, remain unclear.

Major points:

- The authors refer multiple times to neomorphic features. This would imply that a novel function of the protein is induced by small molecule binding. However, the authors do not specifically show any neomorphic features and instead it seems like endogenous interactions or functions are modulated by inhibitor binding?
- While the work is of high quality, all three inhibitors have IC50s in the nanomolar range, but are treated at 1 μ M. This hampers the strength of the drawn conclusions. Are the observed effects of direct or indirect nature? Could the authors please provide evidence that ligand binding is directly causing the observed non-catalytic effects.
- For PRKCA it would be interesting to see if mutated versions (inactive/constitutively active) occupy the same structural space to WT and how this correlates to the observed behavior upon ligand binding- if the hypothesis is correct Go6983 should lead to the identical structural changes.
- What percentage of loss of PRKCA in native lysis conditions upon Go6983 treatment can be explained by relocation to the cellular junctions vs the suggested insoluble organelles?

Minor points:

- Line 17: The abstract lacks a definition of AIDs.
- Line 24: The authors should consider rephrasing "highly prevalent" since here three examples are shown.
- Line 62: naming the inhibitors at this point may be beneficial, including reported EC50s or IC50s.
- Line 111: should include details as to binding affinity/inhibitory capacity and selectivity (see also comment on line 62).
- Line 361: it is unclear in what context "more pervasive feature" is meant.

Molecular Systems Biology, MSB-2026-13670

Paradoxical non-catalytic kinase functions are driven by inhibitor-induced displacement of autoinhibitory domains

Viviane Reber (1), Sabrina Keller (1), Stefanie A. Loosli (1), Yumi Arima (2), Tatjana Kleele (2), Paola Picotti (1), Matthias Gstaiger (1)

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2 Institute of Biochemistry, Department of Biology, ETH Zurich, Zurich, Switzerland

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Response to all reviewers:

We thank the reviewers for their supportive comments highlighting the impact and novelty of our study. We greatly appreciate that the reviewers took the time to review our work to provide insightful and constructive feedback that has truly strengthened the manuscript as we understand the time and effort it takes. To address the comments and questions, we have provided new experiments and analyses. In particular, we

- performed structural and interaction proteomics experiments on CAMKK2 using a negative control compound which no longer can bind CAMKK2 to confirm that measured effects depend on direct probe target interaction
- reanalyzed AP-LiP MS data of kinase interactors which revealed rabusertib induced structural changes in the CHEK1 interactor CLPB which helps to explain inhibitor induced mitochondrial fragmentation.
- included new proximity ligation dose response experiments with PRKCA inhibitor Gö 6983 which showed that the claimed changes in PRKCA proximity have a low median EC50 consistent with a direct inhibitor response
- performed a set of new AP-LiP-MS experiments on PRKCA mutants which confirmed structural changes within the PRKCA C2 domain as a main driver of membrane relocalization.
- all new MS raw data files have been uploaded to PRIDE. Reviewers may use the following login credentials to access the data pending publication: username: reviewer_pxd069830@ebi.ac.uk, password: Z6N5ue4ToxNo.

We restructured the supplementary figures and tables in accordance with editorial guidelines as expanded view (EV) figures that are shown in the main text and additional figures and tables provided in an appendix. In addition, we have included detected mutant peptides in AP-LiP-MS data visualizations of kinase mutants (Figure 3A). All changes in the main text are highlighted in red.

All details are provided below.

Point-by-point response:

Reviewer #1:

The manuscript "Paradoxical non-catalytic kinase functions are driven by 1 inhibitor-induced displacement of autoinhibitory domains" by Reber et al. described an integrated mass spectrometry approach combining LiP-MS, AP-MS, and proximity labeling-MS to map the inhibitor-induced kinase conformation and interaction changes. This work is very interesting and uncover the widespread mechanisms of kinase inhibitor-induced non-catalytic conformation alterations, which might greatly contribute to the rational design of more effective kinase inhibitors. I think this work can be published on MSB after minor revision.

1. All the Lip-MS experiments were performed after affinity purification. Although this AP approach is more native than in vitro analysis, the sequence and site coverage is a problem. So, can the authors cover all the interesting regions of kinase in ATP binding and autoinhibitory domains? The sequence region coverages for all the protein kinase analysis should be given in the manuscript.

We more clearly indicated in our revised Fig. 1B, C and D, Fig. 3A, Fig. 5B, Fig. EV1C, Fig. EV2B and Fig. EV4E how the peptides identified by AP-LiP-MS cover the various annotated protein domains of the analyzed kinases and also added information on the % of sequence coverage. We also provide information on the domain level coverage in Appendix Tables S1, S3 and S5.

2. How the authors evaluate the kinase conformation changes is directly related to the inhibitor binding or by other protein interactions? As the AP-MS experiments identified many interaction proteins. Maybe some invitro analysis by using purified proteins is still needed?

We thank the reviewer for suggesting a more precise evaluation of the measured structural changes. In our view, the goal and advantage of AP-LiP-MS over other techniques is to measure structural changes induced by inhibitors in the context of native human kinases reflecting physiological PTMs and in association with kinase binding proteins. Purified recombinant proteins used for classical *in vitro* target engagement studies lack binding partners but also PTMs. This compromises their use to investigate relevant probe- induced structural changes. In fact, a recent study showed that ligandability can be modulated by PTMs¹. We agree with the reviewer that the measured structural changes in response to inhibitors may be affected also by the presence/absence of these interactors (in addition to PTMs). We therefore included a sentence at page 4 line 124 in the first result section to clarify this point: "Since we analyze natively purified kinases, it is possible that some of these inhibitor-induced conformational changes may depend on the presence or absence of kinase binding partners or PTMs."

3. How the in vivo proximity labeling experiment and data analysis are conducted are not clear. They authors should describe the procedures in more detail. If CH2O is used for in vivo labeling, how to avoid crosslinking, how to do database searching and realize confident results?

We have better organized the corresponding subsections in the *Methods* section to make it clear how we conducted and analyzed our biotin proximity labeling experiments. CH2O was not used for *in vivo* labeling.

We used CH2O exclusively for chemical modification of the SrepTactin sepharose beads prior to the pulldown step to reduce the amount of streptavidin peptides following on bead digestion of *in vivo* proximity labeling samples based on a previously published protocol².

4. For AP-Lip-MS analysis, the conformation information of other proteins within the complexes could be also obtained. Can the authors also analysis and discuss these interface alterations.

We greatly appreciate this suggestion to identify structurally informative peptides of kinase interactors in our AP-Lip-MS analysis. Unfortunately, peptide coverage for kinase interactors was much lower compared to bait peptides (Fig. R1A), which limits the detection of structural changes by our method. For both CAMKK2 and PRKCA, none of the peptides from kinase interactors passed our statistical criteria for significance. However, AP-Lip MS identified significant changes in the CHEK1 interactor CLPB (Fig. R1B), which dissociated from CHEK1 upon treatment with rabusertib. We observed changes in a region with no functional annotation, in an ANK repeat which mediates protein-protein interactions, and in a segment that regulates disaggregase activity. This suggests that CLPB might interact with CHEK1 via these regions, and that CHEK inhibition leads to structural changes that could alter mitochondrial disaggregase activity of CLPB. This, in turn, could help to explain the observed changes in mitochondrial morphology upon CHEK1 inhibition. We have added the AP-Lip-MS data on CLPB in Fig. 5B and described this finding in the revised manuscript on page 9 line 275 as it supports our hypothesis that the change in the CHEK1-CLPB complex underlies the change in mitochondrial morphology.

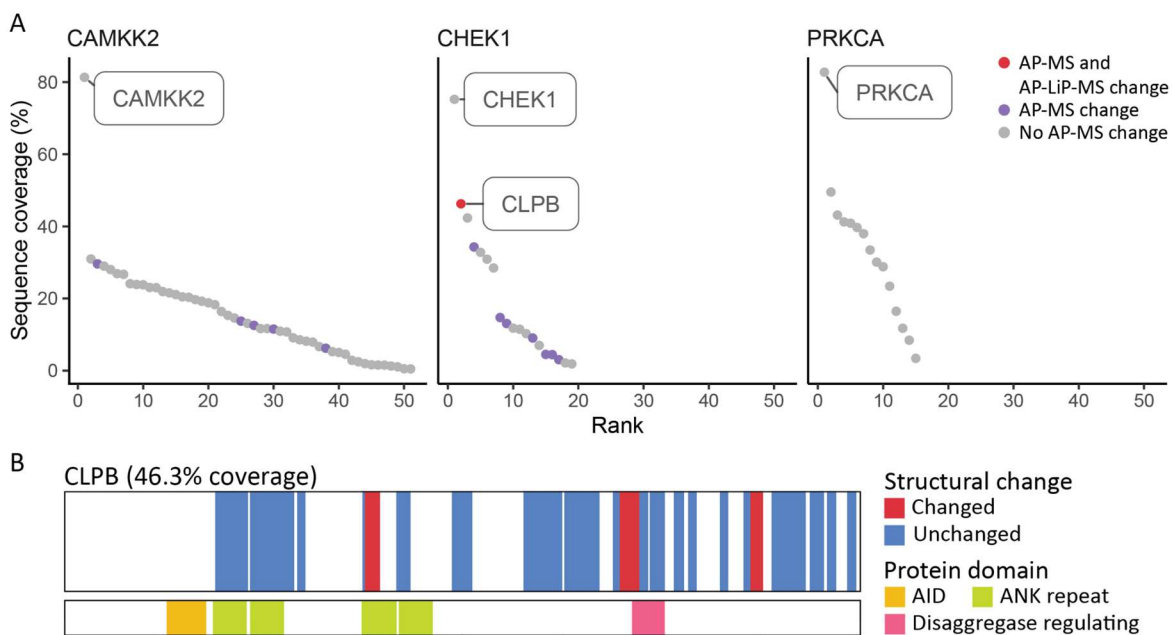


Figure R1. AP-Lip-MS changes in interactors. A) Comparison of sequence coverage for interactors of CAMKK2, CHEK1 and PRKCA. Proteins that show significant changes in AP-MS and AP-Lip-MS experiments are shown in red, those changing in AP-MS experiments only are shown in purple and all other interactors are shown in grey. B) Structural changes detected for CLPB upon CHEK1 inhibition with rabusertib. All AP-Lip-MS experiments were performed in triplicates and p-values were calculated using a moderated t-test followed by multiple testing corrections using the procedure of Benjamini-Hochberg.

Reviewer #2:

Summary

Reber et al. address an underexplored dimension of kinase pharmacology: the non-catalytic, "off-mechanism" effects of ATP-competitive inhibitors that is mediated through changes of kinase domain conformation and protein-protein interaction (PPI) changes. The authors develop a powerful proteomics framework using LiP-MS (limited proteolysis mass spectrometry; previously developed by the authors) applied to native affinity-purified protein complexes, and proximity labeling-MS to systematically map inhibitor-induced structural and interactome remodeling in a native cellular context. Using three Ser/Thr-kinases (CAMKK2, CHEK1, and PRKCA) with distinct autoinhibitory domain (AID) architectures, Reber and colleagues demonstrate that ATP-competitive kinase inhibitor binding induces displacement of the AID, promoting an open, active-like conformation despite catalytic inhibition. This structural transition rewires PPIs and drives diverse (paradoxical) non-catalytic phenotypes. This is nicely demonstrated in relevant cell biological assays for all three kinase-inhibitor pairs. The data is supported by mutational analyses, which allows to disentangle catalytic inhibition from conformational effects. The findings support a model in which the ATP-binding site acts as a central allosteric hub governing kinase conformation and interaction networks.

Overall, the study provides compelling evidence that kinase inhibitor-induced neomorphic PPIs may be a widespread and understudied feature with potentially important biological functions. The proposed proteomics pipeline represents a scalable and generalizable strategy to uncover such effects, with important implications for drug development, particularly in anticipating side effects and improving inhibitor selectivity.

Major comments:

1. While I agree that kinase inhibitor-induced changes of (intra- and intermolecular) PPIs are understudied and some paradoxical effects of kinase inhibitor on signaling are mentioned in the Discussion section (paradoxical MAPK activation by BRAF inhibitors and JAK2 hyperphosphorylation by type I JAK1/2 inhibitors), one very prominent early example is missing and should be included: The Grzesiek lab demonstrated in 2013 that ATP competitive kinase inhibitors targeting BCR::ABL1 result in an open conformation, which enable its SH2 and SH3 domains to engage in PPIs (Skora et al. PNAS 2013; PMID: 24191057).

We thank the reviewer for pointing out the early example on the BCR:ABL1 inhibitor which we overlooked. We now included the work of Skora et al. in line 444 of the discussion of our revised manuscript on page 14 (see also response to question 2 below).

2. I was a bit puzzled that the authors focus entirely on type I kinase inhibitors. While type I inhibitors are indeed more common and induce an active open conformation of the kinase domain and are therefore prime candidates for the studied phenomenon, it has been shown that also type II inhibitors lead to AID displacement and disassembly of intramolecular PPIs. This was first demonstrated for type II BCR::ABL1

kinase inhibitors (see: Sonti et al. JACS 2018; PMID: 29319304); whereas in contrast type I TKIs (e.g. dasatinib, bosutinib, ...) kept BCR::ABL1 in an assembled conformation. I suggest to include these examples and thereby broaden the scope/impact of the manuscript. Hence, it can be expected that also binding of many type II inhibitors (and potentially also allosteric inhibitors) may also result in conformations that displace AID and induce neomorphic PPIs.

We thank the reviewer for noting this important point and agree that the described inhibitor responses may not be restricted to type I but also occur for type II inhibitors and potentially also allosteric inhibitors. We included the suggested examples and expanded on this point to broaden the scope of non catalytic effects in our discussion page 14, line 444: “While we and others have observed AID displacement and PPI rearrangements most commonly for type I inhibitors, there is evidence that also type II tyrosine kinase inhibitors (TKIs) can induce an open conformation. This was first demonstrated for the type II inhibitor imatinib against ABL kinase, which induced a disassembled state of the SH3-SH2-kinase core of ABL³. On the other hand, type I TKIs against ABL like dasatinib or bosutinib bound ABL in the assembled state⁴. This shows that structural modulation by kinase inhibitors is a phenomenon that also occurs for other types of inhibitors.” We also highlight that also type II inhibitors can show similar effects in line 456.

3. The manuscript focuses on kinase inhibitor-induced changes on native (unmutated) kinases. While this is important to study for possible side effects in "off-tissues" or uncover diminished therapeutic effect in the on-target cells/tissues, I lack a discussion and mention that the therapeutic targets of most kinase inhibitors are not native kinases, but active oncogenic kinases that are activated by point mutations, insertions/deletions, duplication, fusion partners etc. These oncogenic kinase variants have often already displaced AIDs, open/disassembled conformations and possibly a different subcellular localization. What is the authors expectation of the consequences of inhibitor-induced (further) changes in this common scenario?

We thank the reviewer for suggesting a discussion on the importance of disease-associated target variants in profiling inhibitor responses. In our study we presented one example of a disease-associated target variant (CAMKK2 R311C) which indeed demonstrated that inhibitor induced PPI changes can depend on the disease mutant context. This is of course even more important for the analysis on how oncogenic target kinase variants are affected by inhibitors. We added text on page 14 line 462 in the discussion section to clarify this and to discuss the need to include most common disease associated target variants to better understand the inhibitors mechanism of action in the disease context: “Most of our analysis focused on inhibitor-induced changes of the corresponding WT kinase, which is particularly important to study both possible on-target side effects in healthy tissues or lack of efficacy in diseased tissues. The disease-associated CAMKK2 R311C mutant showed that responses to inhibitors may depend on the specific disease variant, which may affect their conformation, PPIs or subcellular localization⁵. Since most therapeutic kinase inhibitors aim to target kinases that are mutated in oncogenic contexts, it will be important to expand multimodal proteomic profiling also towards disease-associated target variants. In this regard...”

Reviewer #3:

The authors set out to investigate small molecule inhibitor functions beyond catalytic inhibition using a multi-proteomic approach. Focussing on three examples with different structural architecture and three matching selective small molecule inhibitors, structural re-arrangements via LIP-MS and the change of the proximal proteome are investigated upon ligand treatment. The authors identify three separate consequences of ligand binding beyond inhibition: for CHEK1 dissociation from CLBP is identified. This causes mitochondrial fragmentation. PRKCA is re-localised to cell junctions and endosome-like organelles. CAMKK2 triggers AMPK sequestration. The study is overall of very high quality, elucidates novel mechanisms of action beyond inhibition of inhibitors and thus provides timely insights into additional/off-target consequences of small molecule binding. This provides important insights into small molecule actions and further substantiates the need of detailed dissection of inhibitor consequences beyond inhibition. At this point a few open points still, however, remain unclear.

Major points:

1. The authors refer multiple times to neomorphic features. This would imply that a novel function of the protein is induced by small molecule binding. However, the authors do not specifically show any neomorphic features and instead it seems like endogenous interactions or functions are modulated by inhibitor binding?

We thank the reviewer for the suggestion to be more careful when using the term “neomorphic” since many of our reported inhibitor responses can indeed be explained by modulation of an already existing kinase function. To avoid confusion, we removed the term “neomorphic” in lines 76, 391 and 473 in the revised manuscript.

2. While the work is of high quality, all three inhibitors have IC50s in the nanomolar range, but are treated at 1 μ M. This hampers the strength of the drawn conclusions. Are the observed effects of direct or indirect nature? Could the authors please provide evidence that ligand binding is directly causing the observed non-catalytic effects.

We thank the reviewer for acknowledging the quality of our work. The reviewer raises an important question that is relevant for all studies analyzing the effects of small molecule inhibitors. In order to detect inhibitor effects *in vivo*, we used concentrations higher than the known biochemical IC50 values to overcome known factors that reduce their *in vivo* capacity (millimolar intracellular ATP concentration, cellular uptake, pharmacokinetics). The 1 μ M inhibitor concentration used in our study is well within the range of concentrations used in the literature for measuring *in vivo* responses⁶⁻⁸. We have also provided additional experimental evidence to support our claim that observed non-catalytic effects are direct consequences of ligand-target interactions.

To ensure specificity of the reported effects for CAMKK2, we used the corresponding negative control compound SGC-CAMKK2-1N, a compound that is structurally related to the active inhibitor SGC-CAMKK2-1 but does not bind CAMKK2⁶. In contrast to the active SGC-CAMKK2-1 inhibitor this molecule did not

cause changes in CAMKK2 structure or interaction with AMPK suggesting that the measured SGC-CAMKK2-1 effects depend on direct inhibitor binding (Fig R2). This new information is now included in the revised version in Fig. EV2 and described in the manuscript on page 5 line 153: “To ensure that the observed changes are specific, we used the negative control compound SGC-CAMKK2-1N⁶ (Fig. EV2A). This compound is structurally similar to the inhibitor SGC-CAMKK2-1 but does not bind CAMKK2. We found that this compound does not induce any structural changes in CAMKK2 (Fig. EV2B, Appendix Fig. S4A, B) or alter its interactome (Fig. EV2C, Appendix Fig. S4C). Thus, the changes observed upon treatment with SGC-CAMKK2-1 are likely specific.”

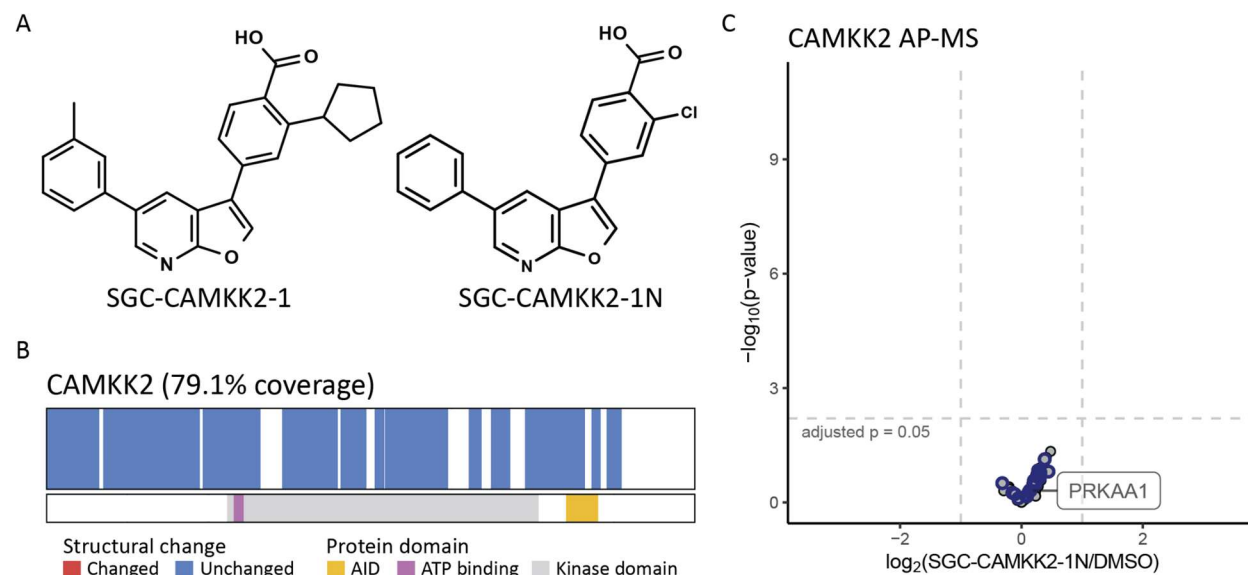


Figure R2. The negative control compound SGC-CAMKK2-1N does not induce structural or interaction changes in CAMKK2. A) Structures of the inhibitor SGC-CAMKK2-1 and the negative control compound SGC-CAMKK2-1N. B) Structural changes observed with AP-LiP-MS for CAMKK2 treated with SGC-CAMKK2-1N. C) Volcano plot illustrating changes in CAMKK2 interactors upon inhibition with SGC-CAMKK2-1N measured by AP-MS. Known CAMKK2 interactors are highlighted with a blue border. AP-LiP-MS experiments were performed in technical triplicates and AP-MS experiments were performed in biological triplicates. P-values were calculated using a moderated t-test followed by multiple testing corrections using the procedure of Benjamini-Hochberg.

Such a negative control compound is not available for the probes used to investigate CHEK1 and PRKCA, prompting us to apply other strategies.

The CHEK1 inhibitor rabusertib was ranked among the highest in selectivity among 243 kinase inhibitors tested in a chemoproteomics study with no off targets being detectable for rabusertib across 253 kinases even at high concentrations⁹. This does not fully exclude other indirect drug effects but demonstrates exceptional target selectivity. We reported that the CHEK1-CLPB complex dissociates after 4 h *in vivo* treatment with 1 μM rabusertib. By AP-LiP-MS, we observed structural changes in CLPB in CHEK1 pulldowns after only 5 minutes of rabusertib treatment of purified complexes *in vitro* (see Fig. R1B), suggesting that the observed CLPB dissociation from CHEK1 is direct. We also observed that the constitutively active CHEK1 L449R mutant no longer dissociates from CLPB, showing that the effect does

indeed depend on CHEK1 directly. Furthermore, in our manuscript we provided evidence that rabusertib-induced mitochondrial fragmentation cannot be explained by indirect effects such as DNA damage or CDK1 inactivation induced by rabusertib (Fig. EV3, Fig. 5).

For the PRKCA inhibitor Gö 6983, we addressed this question by measuring Ca^{2+} release upon Gö 6983 treatment and performing dose-response proximity labeling experiments. Since membrane association of PRKCA is regulated via Ca^{2+} -signaling¹⁰, we already asked whether Gö 6983 may affect membrane relocalization indirectly via increasing intracellular Ca^{2+} levels. However no Ca^{2+} increase could be detected upon inhibitor treatment which excludes such an indirect mechanism (Fig. EV4B). However, we acknowledge that this does not rule out non-specific effects via other mechanisms. We therefore performed an additional series of dose-response proximity labeling MS experiments following the well-established rationale that specific on target effects occur at lower concentrations than non-specific effects^{11, 12}. We treated miniTurbo-PRKCA expressing cells with 6 different concentrations between 100 pM to 10 μM and plotted the response curves of all the proteins which changed their proximity to PRKCA across the concentration range (Fig. R3). The proteins we reported to change in Fig. 6A had a median half maximum effective concentration (EC50) of 382 nM, while all other proteins changed proximity only at much higher concentrations with a median EC50 of 2.1 μM . The difference between these groups is highly significant ($p = 1.8 \times 10^{-11}$, two-tailed Mann Whitney U test). This supports the view that the measured effects are highly specific.

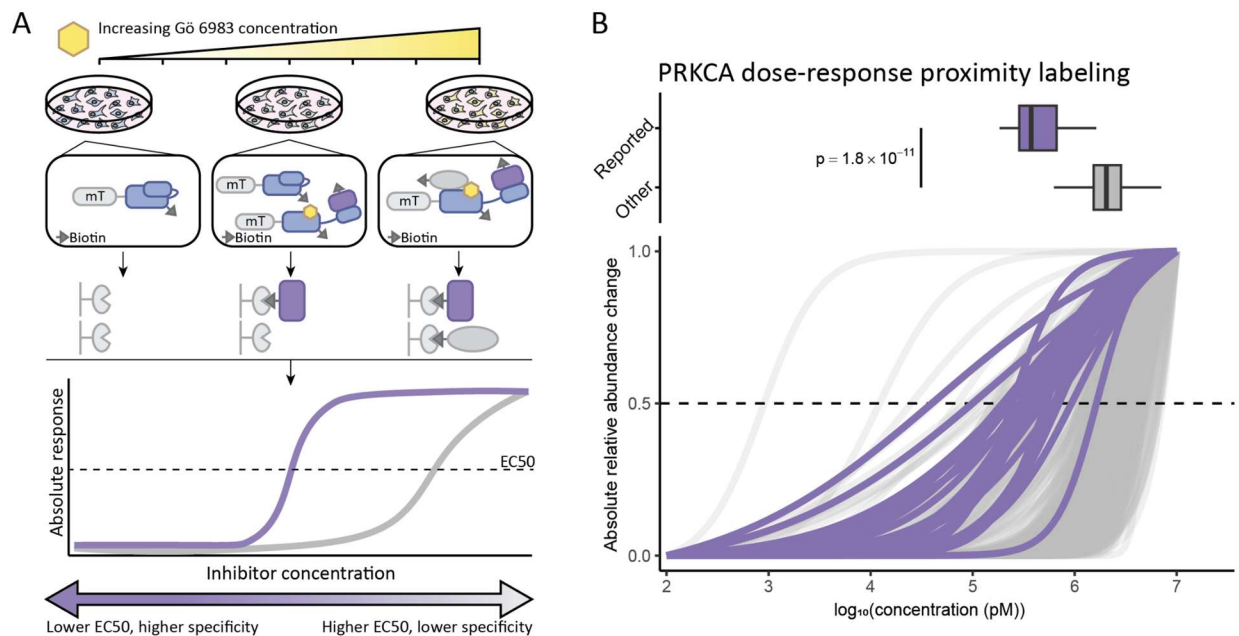


Figure R3. Dose-response *in vivo* proximity labeling shows that relocalization of PRKCA in response to Gö 6983 occurs at lower EC50s than other effects. A) Schematic representation of the dose-response *in vivo* proximity labeling workflow. B) Dose-response curves for proteins changing with increasing Gö 6983 concentrations. Proteins reported to change in Fig. 6A are shown in purple, all other proteins are shown in grey. For each dose, biological quadruplicates were used. The p-value comparing EC50s was calculated using a two-sided Mann Whitney U test.

These new results have been added to the manuscript in Fig. EV4C, D and are described starting at page 11, line 339: “To investigate whether the observed relocalization is specific, we followed previous approaches that have established that specific, on-target effects occur at lower concentrations compared to indirect off-target effects^{11, 12}. We therefore performed a dose-response in vivo proximity labeling experiment (Fig. EV4C) with 6 treatment concentrations ranging from 100 pM to 10 μ M and computed the half-maximal effective concentration (EC50) of all proteins for which we obtained a high-correlation and significantly changing fitted sigmoidal curve (Fig. EV4D, Appendix Fig. S9). We found that the changes reported in Fig. 6A occurred at significantly lower concentrations (median EC50 = 382 nM) compared to all other proteins (median EC50 = 2.1 μ M; $p = 1.8 \times 10^{-11}$, two-tailed Mann Whitney U test).”

3. For PRKCA it would be interesting to see if mutated versions (inactive/constitutively active) occupy the same structural space to WT and how this correlates to the observed behavior upon ligand binding- if the hypothesis is correct Go6983 should lead to the identical structural changes.

We initially shared the view of the reviewer that the mutants should be structurally similar to the WT and thus undergo similar structural changes when treated with Gö 6983. In order to test this hypothesis, we performed a new set of AP-LiP MS experiments including the PRKCA variant D436A and R22A/A25E (Fig. R4A). While we had less overall protein coverage in the mutants, we noticed that the only peptides affected across all three experiments mapped to the C2 domain, which mediates PRKCA membrane binding¹³. The shared structural alterations in the C2 domain in WT and mutant PRKCA correlate well with the similar inhibitor induced proximity changes to membrane proteins. Together with the proposed role of the C2 domain in membrane targeting via direct interactions with phosphatidylinositol 4,5-bisphosphate¹⁴, these results strengthen our model in which the inhibitor causes structural alterations in the C2 domain that drive membrane relocalization.

In contrast to PRKCA WT, Gö 6983 did not change the AID in the mutants. While this could be expected for the constitutively active R22A/A25E mutant¹⁵, we did not expect this for D463A as we assumed it would have a closed conformation like PRKCA WT. To explore why we did not observe structural changes in the AID of the D463A mutant, we generated AlphaFold 3 models of the PRKCA KD-AID interaction following the same protocol as for CAMKK2-PRKAA1 (see *Methods*; Fig. R4B, C). These models suggest that D463 is located in the KD interface interacting with the AID. We believe that the D463A mutation might thus disrupt this domain-domain interaction, similar to the R22A/A25E mutant. This could explain why we do not see structural changes in the AID upon Gö 6983 treatment. Our AP-LiP-MS data together with the AlphaFold 3 prediction suggest that the D463A and R22A/A25E mutants do not occupy the same structural space as the WT but rather have unique conformations and thus undergo different structural changes upon Gö 6983 treatment. These new insights do not change our main conclusions about how Gö 6983 induces relocalization of PRKCA to the membrane via structural changes in the C2 domain. The new AP-LiP-MS results are now included in Fig. EV4E, Appendix Fig. S10, Appendix Table S5 and referenced on page 11 line 352 of the revised manuscript: “AP-LiP MS of PRKCA WT and mutants identified shared structural changes within the C2 domain when inhibited (Fig. EV4E, Appendix Fig. S10, Appendix Table S5) suggesting that membrane relocalization may be driven by structural alterations in the C2 domain.”

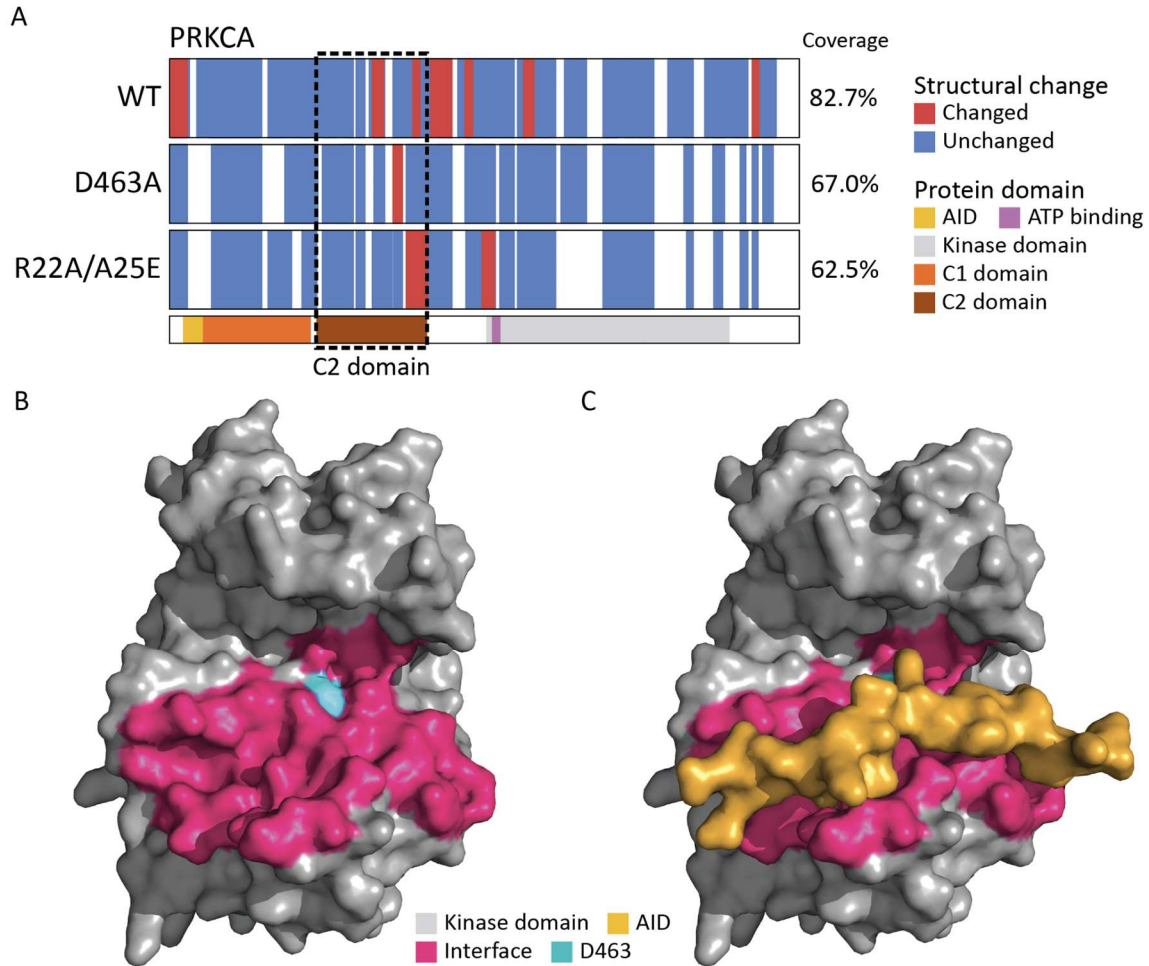


Figure R4. PRKCA mutants undergo structural changes in the C2 domain. A) Gö 6983 induced structural changes obtained by AP-LiP-MS in wild type and mutant PRKCA. The panel for PRKCA WT was previously shown in Fig. 1D. AP-LiP-MS experiments were performed in technical triplicates. P-values were calculated using a moderated t-test followed by multiple testing corrections using the procedure of Benjamini-Hochberg. B, C) AlphaFold 3 predictions of the PRKCA KD-AID interaction shown without (B) and with the AID (C). The catalytic residue D463 lies in the predicted interaction interface with the AID.

4. What percentage of loss of PRKCA in native lysis conditions upon Gö6983 treatment can be explained by relocalisation to the cellular junctions vs the suggested insoluble organelles?

We were also curious about the distribution of PRKCA after Gö 6983 treatment. Using MS PRKCA abundance measurement in native lysates we estimate that 75% of PRKCA relocates into insoluble subcellular structures upon inhibitor treatment (Fig. EV4A). Super-resolution microscopy data of Gö 6983-treated HEK293 cells expressing EGFP-PRKCA was acquired as z-stacks as described in *Methods*. Deconvolved z-stacks were segmented in Ilastik ¹⁶ and signal intensity was integrated per cell over the volumes of the membranes and foci. Experiments were done in technical triplicates using 38 images per

cell. We estimate that 83.7% (SD = 8.0%) of PRKCA was present at junctional membranes and 16.2% (SD = 8.0%) at dot-like organelles in response to Gö 6983.

Minor points:

1. Line 17: The abstract lacks a definition of AIDs.

Thank you for noting the lacking abbreviation. We have added it to the abstract.

2. Line 24: The authors should consider rephrasing "highly prevalent" since here three examples are shown.

We have replaced "highly prevalent" with "likely to occur in other kinases as well" to tone down our statement.

3. Line 62: naming the inhibitors at this point may be beneficial, including reported EC50s or IC50s.

We added the name of the inhibitors and their known IC50s in line 60 of the revised manuscript.

4. Line 111: should include details as to binding affinity/inhibitory capacity and selectivity (see also comment on line 62).

We added more details on available half maximum inhibitory capacity in line 60 and references to available selectivity data in line 63.

5. Line 361: it is unclear in what context "more pervasive feature" is meant.

We agree the term is not clear in this context and we replaced it with "could be a common feature" in line 383 of the revised manuscript.

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15th Jun 2026

Manuscript Number: MSB-2026-13670R

Title: Paradoxical non-catalytic kinase functions are driven by inhibitor-induced displacement of autoinhibitory domains

Author: Viviane Reber

Sabrina Keller

Stefanie Loosli

Yumi Arima

Tatjana Kleele

Paola Picotti

Matthias Gstaiger

Dear Dr. Gstaiger,

Thank you for sending us your revised manuscript. We have now heard back from the reviewer who was asked to re-evaluate your study. As you will see below, the reviewer is now satisfied with the modifications made and supports publication. Before we can formally accept your manuscript, we would ask you to address the following editorial-level issues:

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3. "Disclosure statement and competing interests" should be renamed to "Disclosure and Competing Interests Statement".
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9. Sections need to be named and the order should be corrected to: Title page - Abstract - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure Legends - Table(s) - Expanded View Figure Legends.

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Kind regards,
Jingyi

Jingyi Hou, PhD

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Reviewer #3:

The authors have addressed all raised points with very detailed experiments, including orthogonal approaches when chemical probes were not available. The manuscript points to features of kinase inhibitors beyond inhibition, which are important to highlight for the research community. I would hence recommend its acceptance for publication.

All editorial and formatting issues were resolved by the authors.

18th Jun 2026

Manuscript number: MSB-2026-13670RR

Title: Paradoxical non-catalytic kinase functions are driven by inhibitor-induced displacement of autoinhibitory domains

Dear Dr. Gstaiger,

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

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Kind regards,
Jingyi

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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Yes	Materials and Methods, Data availability