nature portfolio

Peer Review File

Seven complete comparative maps of allosteric mutations in a protein family

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

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a large-scale experimental study in which the authors are able to distinguish the contributions to free energy from folding versus binding in a high throughput manner, allowing them to map the effect of all single and double mutations on binding free energies. The authors have previously published this map for a single PDZ domain, PSD95-PDZ3. The main new contribution in this work is to apply this to five members of the PDZ domain family with seven total binding interactions.

Although this work is incremental in the sense of not being qualitatively different, the generation of mutational sensitivity maps across multiple PDZ domains is first of its kind, allowing new insights.

The two main contributions of this work, in my opinion, are:

1. Mapping the locations of hotspots that are more conserved versus protein specific, and showing the rarity of gain-of-function allosteric mutations contributes to our understanding of the evolutionary programmability of protein signaling within a fixed fold family. Comparing across multiple PDZ allows for the building of a consensus pattern of modular allostery in which a mostly conserved core connects to protein-specific surface hotspots. To me, the most impactful consequence of this is enabling systematic comparison with the "sectors" predicted from coevolutionary analysis of sequence alignments. To be able to systematically show that the pattern of physical coupling matches that of coevolutionary constraints would constitute a highly meaningful contribution. The authors show the general consistency of the core amongst PDZ domains in figure 2 and the occurrences of allosteric residues shared by multiple PDZ domains are listed and shown in figures 4 and 5. They also list the statistically significant overlap of hotspot residues with the core and with the sector previously identified from sequence co-evolution. However, because the sector overlaps strongly with the core, it is not clear how closely the actual geometry of the hotspots mirror that of the sector. In particular, the sector consists of a contiguous region spanning part of the core and extending to select surface sites. A plot directly comparing the allosteric hotspots with the sector would therefore greatly improve the impact of this paper. In addition, although the authors argue that the heterogeneity of allosteric sites is highest at the surface, Figure 6 seems to show a fair amount of allosteric overlap amongst the different PDZ domains. It would be helpful to show the differential conservation of the core and surface quantitatively, for example by plotting the identities of the allosteric residues of all the PDZ domains as a function of distance to the surface.

2. The generated data is systematic, large-scale, and of high quality. The authors took care to quantiatively compare their predicted free energies against previous work, and their method isolates the effect of mutations on binding free energy, removing confounders such as effects on folding. The authors also quantified general trends in the data, such as the distance decay of allosteric signal, which corroborates other experimental findings on other proteins as well as molecular dynamics, NMR and evolutionary conservation studies. As the authors mention, this dataset will be valuable for future analysis. In particular, if this type of data can be more regularly produced in future for different proteins, that could potentially enable training of accurate machine-learning models for allosteric prediction and design of signaling proteins. The authors should provide a description of all the supplementary tables to facilitate future analysis of this data set.

If the authors can address the gaps in quantitative analysis of their data to support the claims, as mentioned in 1), this work will be of significance for the field of protein biophysics and molecular evolution.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This paper reports comparative DMS experiments of the effects of single amino acid mutations on the free energy of protein folding and peptide binding across multiple homologous PDZ proteins. This work applies the same experimental/computational pipeline developed in a number of recent papers by the Lehner group and here uses it in a comparative framework. The scope of the experiments is impressive and the data are of good quality, with more than adequate replicability and a good fit to the models used. However, I find that the way the experiments are presented and interpreted to be misleading. The meaning of the data and their contribution to the field need to be substantially reconceived for this to represent a constructive addition to the literature.

Understanding how mutations' effects change as protein families diverge is an important question in protein evolution and is also important for understanding the architecture by which protein functions – in this case, protein-protein interactions -- are physically and genetically determined. This is not the first paper to report changes in mutations' functional effects across homologous proteins using DMS – similar approaches have been applied to, for example, numerous viral proteins, TIM barrels, and bacterial and eukaryotic transcription factors (e.g., PMC9273037, PMC9429997, PMC4626756, PMC5343507, PMC9662819, PMC10515859, and others. The list includes comparative analysis of changing constraints over evolutionary divergence, the structural determinants of such changes, and the effects of mutations on allostery in allosterically regulated proteins (PMC9662819). Nevertheless, a strength of the current paper is its ability to deconvolute effects on binding from effects on stability using ddPCA, although it should be noted that a similar approach was also used in previous work on viral proteins (PMC7418704), although without the same comparative framework. This is also not the first paper to use DMS to show how mutations across a protein's sequence affect protein-protein interactions, including at sites far from the binding interface (see, for example, several of the papers described above, as well as the authors' own PMID 35388192, PMC5896888, etc.). The work in the current manuscript therefore does not break entirely new ground. However, the data are of a scope and quality that the paper has the potential to make a contribution to the field if the paper were focused more sharply on differences among homologous proteins in the sequence and structural determinants of protein-protein binding that allow this paper to go beyond prior work.

1. My major concern about this paper is that the purpose of the experiments and the interpretation of the data are described in a way that obscures rather than illuminates their meaning and their relationship to existing knowledge. The authors center their manuscript around the topic of allostery – the genetic basis of allostery, the ways in which that basis is conserved or variable during evolution, and how allostery itself evolves.. This focus is the organizing principle of the manuscript, the justification for the work, and the interpretation of the data. Allostery means regulation of a protein's activity (catalysis, binding a biological ligand) by binding an effector ligand at a site on the protein distinct from the active site. This is the classic definition that goes back to Monod, whom the authors cite, and which was subsequently formalized biophysically in the classic papers of Monod-Wyman-Changeux and Koshland-Nemethy-Filmer. This definition has been reiterated many times, including in the more recent review by Motlagh-Wraby-Hilser, which is the first citation in the current manuscript. The latter papers begins, "Allostery is the process by which biological macromolecules (mostly proteins) transmit the effect of binding at one site to another, often distal, functional site, allowing for regulation of activity." This definition is in every biochemistry textbook and is central to modern understanding of how proteins work and are regulated. The PDZ proteins that the authors study are not allosteric or allosterically regulated. Their functional activity at their "active site" is to bind peptides, but this activity is not regulated by binding to a second effector ligand or by any other form of regulation (such as post-translational modification) discussed in the manuscript. The data in the current manuscript do not address how allostery evolves, how it is encoded in protein sequence, how that encoding changes over time, etc., because the proteins studied are not allosteric. (I note that some papers have used an expanded definition of allostery that refers to changes in protein conformation caused by ligand-binding, irrespective of whether activity is regulated by the binding event. This "structural" definition of allostery has been applied to PDZ domains. Whatever the merits of this definition, it is not the one the authors of the current manuscript use, and it would not accurately describe the subject addressed by the authors' data.)

Instead of following this well-established definition (or even the looser structural definition) , the current manuscript redefines an allosteric protein as one in which mutations that are far from the active site alter the protein's function – in this case, that means that mutations away from the peptide binding surface affect binding affinity. This does not make these proteins allosteric, because allostery refers to regulation of a protein (or, by the structural definition, to conformational change). A mutation changes the protein's sequence, creating a new protein; this is not a form of regulation of the activity of an existing protein (or a change in its conformation). It is meaningless and confusing to call mutations that affect binding in a nonallosteric protein "allosteric" mutations, because the protein remains nonallosteric both before and after the mutation. Genetic perturbation of a nonallosteric protein – such as in the DMS experiments reported here – reveal the genetic basis of peptide binding, not the basis of allostery. I recognize that the authors have used their re-definition in some of their previous papers, and that there are some other papers in the literature that have also used it. But this redefinition is problematic: to conflate allosteric regulation and the effects of mutation on protein-peptide binding muddies our understanding of both topics.

The paper's claims to experimentally dissect the genetic basis of allostery and changes in that basis among homologs

should therefore be removed from the paper. This will require a fundamental reframing to focus on the questions that the experiments do address – the genetic basis of peptide binding and how that basis changes among homologs.

2. This reframing of the paper will also require the authors to clarify what is novel in their work relative to other work on the genetic derterminants of peptide binding. Many of the results in the manuscript retread issues already shown in prior work (including that of the authors). For example, the fact that certain residues within binding interfaces – particularly those that are fully buried near the “center” of an interface rather than near its edge -- are more likely when mutated to affect ligand binding or specificity has already been established. So has the fact that mutations at sites distant from the binding interface can affect binding, as well as the fact that these couplings follow predictable trends in terms of distance from the binding surface, structural relationship to the protein core, etc. I found myself wondering frequently where the novel impact of this paper lies. Eventually in reading the manuscript we do arrive at the most novel and impactful data, which is the comparative analysis among homologs of the distribution of sites that affect binding at a distal surface. I would suggest that in their revision the authors should focus more on these topics and bring out the significance for evolution, the physical basis of protein function, and therapeutics more clearly.

3. A reframing like this would still allow the authors to speculate in the discussion about the relevance of their findings for allostery. It is well known by now that mutations far from the binding surface can affect peptide binding in nonallosteric proteins. As the authors of this manuscript note, such findings in nonallosteric proteins can inform our understanding of how allostery works biophysically and how it evolves. For example, the fact that mutations can affect peptide binding at a distant surface implies that the mutated regions and the binding surface are conformationally coupled. It is possible that mutations in the former region, if they were to confer binding to an effector, might plausibly confer allostery on the protein – an important finding for evolution and for engineering novel allosteric proteins. Similarly, if an effector can be engineered to bind to such a coupled surface, even without mutations, this would represent an allosteric drug that can modulate the activity of a protein that is not endogenously allosterically regulated. These points have already been made repeatedly in the literature, so if the authors of the current manuscript raise these issues, they should have something particular to say about the insights provided by their data that adds to the existing body of knowledge in this area. Since the novelty of this paper lies in the scope of its comparative analysis and the isolation of effects on binding from those on folding , I would hope that the authors would address the specifics of the apparent lability across homologs of this kind of conformational coupling and its implications for engineering, evolution, and therapeutics.

4. In the discussion, the authors identify several “important insights into the evolution of allostery,” but many of these are not new insights. The authors do cite other work as if it is confirmatory of their findings, but I think it is more accurate to say that the authors’ findings on some of these topics are confirmatory of the prior work. For example, the distance-dependence decay from the active site of mutation effects on bindings is already established. Similarly, the paper claims to “reveal a modular allosteric architecture via a conserved allosteric core connecting to protein-specific extensions to surface sites.” As the authors note, this directly echoes prior work on PDZ domains and other proteins in which sets of covarying residues structurally extend from the protein core to protein surfaces. This is therefore not a new insight. The authors should identify what was uncertain in prior analyses and clearly state the contribution made by their experiments that was not already settled by prior work.

5. The authors claim to identify “gain of function” mutations, but the definition of this term is problematic. The authors appear to use this phrase to refer to mutations that increase a protein’s affinity for a ligand that it already binds. The term normally refers not to refinement or amplification of an existing functon(as the authors use it) but to the acquisition of an entirely new function – e.g., binding to a ligand to which the protein previously had no detectable binding or affinity. I would suggest finding a different term to avoid confusion.

6. The final paragraph of the discussion seems out of place. It seems potentially better suited to the introduction, because it states why the type of analyses the authors are conducting is relevant to developing therapeutics, but it does not reflect on the implications of the authors’ particular findings for this area. Either the latter should be developed with specific reference to the data, moved to the introduction, or omitted.

7. The paper says that “our data suggest that allostery primarily evolves via the gain and loss of periprheral extensions to a conserved allosteric core.” Because none of their proteins or mutants are allosteric, their data do not support this statement.

8. Given the scope of the experiments and the nature of the technology, I think that the replicability of the experiments is more than reasonable. However, some of the correlation coefficients -- especially to the biophysical measurements -- are very modest, and when squared some of them would indicate a nontrivial amount of unexplained variance attributable either to measurement noise or model misfit. Acknowledgement of the cases in which the correlations are only moderate would enhance the transparency of the paper.

9. The authors present correlations across replicates for their experimental measurements, but there may be much greater uncertainty in the estimates of binding and folding energies, which are fit to a complex nonlinear model. As these energies are the object of interest in the paper that drive all the subsequent interpretations, it would be worth characterizing the replicability of these inferences or at least the extent to which the inferred energies predict the observations. Extended data Fig. 2c presents these fits, and some of them appear by eye to be relatively modest, although the correlation coefficients do not seem to be presented. In addition to noise, another source of mis-fitting could be epistasis: for an additive model to fit the data extremely well would imply virtually no epistasis other than that imposed by the global nonlinearity, and this would be somewhat surprising. I would therefore like to see more detailed quantification of the fits and if necessary a more cautious description in the text.

In summary, the data in this paper show in detail how the effects of mutations on peptide binding can change among evolutionarily related proteins. The authors should reframe their paper to explore the significance of these observations without casting them as a direct investigation of allostery, which is not valid. I think that in general authors should have broad leeway in determining how to present their results; moreover, definitions of terms are not written in stone but rather represent meanings, agreed upon by the community, that allow us to think and communicate clearly with each other. After consideration, however, I feel that the authors' use of the term allostery does not accurately convey the meaning and significance of their work, and that the redefinition proposed would lead to less clarity in our understanding of the genetics and structural foundations of protein function, regulation, and evolution.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Marti-Aranda and Lehner present a comparative study of the mutations tuning binding affinity and stability across a collection of seven protein-peptide interactions. This data-rich paper enables — for the first time to our knowledge — a comprehensive examination of how allosteric networks are conserved or diverge across homologs. The methods used to measure allosteric maps are state of the art and have been sufficiently validated (Faure et al., 2022). Specifically, the ability of ddPCA + Mochi to approximate in vitro binding free energies is strong. The authors study the PDZ domain, which is a well-explored model system for ligand binding and allostery, allowing them to place their results in dialog with other previous work. The main shortcoming of the PDZ domain is that the potential for long range communication is somewhat limited by the relative small size of the domain. Key results are that: (1) allostery in the protein core is largely conserved, while surface hotspots vary and (2) allosteric maps of the same protein binding different ligands are more similar to each other than maps of different PDZ domains binding the same ligand. These results are well-supported by the presented data. From these observations, the authors propose a model in which allostery evolves via gain and loss of peripheral extensions to a conserved allosteric core. This aligns with some earlier proposals in the field, but the dataset from Marti-Aranda provides some of the most extensive and direct evidence that this model might be correct. Taken together, we anticipate the results and data will be of interest to a broad community of scientists working in molecular evolution, allosteric regulation, protein engineering and signaling. We propose a few changes to the paper that we feel would increase the impact, interpretability and utility of the work.

Major comments:

1.Clarity of figures. In this work the authors face a considerable challenge of presenting a large and multi-dimensional data set. While the paper includes ample figure panels and supplementary materials, at times it is hard to appreciate the main conclusions from the graphic presentation. In particular, it is challenging to visually compare the results (ΔΔGb and ΔΔGf) across domains. As a few sub-points/suggestions on how to achieve this:

a.Variability in the spatial distribution of allosteric hotspots is the key result that needs to be communicated visually, but this was not conveyed satisfactorily in Figures 2, 5, and 6. Figure 2C only shows the surface of ΔΔGb in an orientation focused on the binding site. In Figures 5 and 6, the annotations somewhat obscure the differences. We propose either representing ΔΔGb as colored VDW spheres on top of ribbon structures in different orientations with limited annotation or plotting two maps on the same structure. Positions in common could be shown in one color, while differing positions are shown in contrasting colors. Possibly one or two representative pairs could illustrate the concept simply, while the full data is presented separately. Alternatively, differences between two allosteric maps could be plotted directly using a diverging color map.

b.In figure 2A the authors present all of the ΔΔGb and ΔΔGf data as heat maps. While this is very thorough, heat maps can be challenging to visually compare, and do not allow error in the measurements to be easily represented. We propose making this a supplemental figure, and instead showing a more compact comparison of the ΔΔGb and ΔΔGf between domains. One idea would be to show a heat map of the correlation coefficients between ΔΔGb (or ΔΔGf) in one protein interaction versus another to allow rapid comparison of which domains exhibited more or less similar mutational profiles. Another possibility would be to show correlation plots (with error bars) or difference maps for select pairs of domains, to emphasize which mutations are similar or divergent across homologs.

2.Limited discussion. The brief discussion summarizes results and highlights the implications for drug discovery, but would benefit from a deeper treatment of the broader significance of the findings. The authors might speculate on mechanisms of allostery that underpin the observed differences in allosteric maps. Could the variation across homologs be explained by a single physical model, or are multiple models required? What physical differences might distinguish allosteric mechanisms in the core versus the periphery? And what is the relationship between the conserved/non-conserved allosteric hotspots in this dataset and sequence conservation in the protein family — are conserved allosteric hotspots more conserved in sequence space as well? In general it feels like there is a little bit of a missed opportunity to synthesize the data with sequence evolution patterns in the PDZ family.

They should also discuss related work that has addressed how the distribution of hotspots and pathways changes upon mutation (Leander et al., PNAS 2020), ligand binding (Glasgow et al., Nat. Commun. 2023), across homologs (Salinas and

Ranganathan, eLife 2018; Leander et al., eLife 2022), and local sequence contexts (Laursen et al., PNAS 2022). All of this relates to the same overarching problem: variability in the spatial distribution of allostery. Finally, the discussion should expand on the evolutionary implications of a modular allosteric architecture. Does such modularity accelerate evolutionary adaptation?

3.Justification of allosteric hotspot definition. The authors define allosteric hotspots by comparing the inferred |ΔΔGb| to the expected effect based on distance from the active site. It is not clear why this choice was made over the simpler method of identifying allosteric sites as positions outside the binding site with median |ΔΔGb| comparable to the median effect of binding site mutations (as used in some of the authors' own prior work).

Along the same lines, in the section "Allostery beyond distant dependent decay" the authors compare the ΔΔGb-residuals between domains, why compare the residuals rather than the values themselves? This implies there is some kind of difference in the scaling or dynamic range of the different datasets?

4.Data accessibility. It would be helpful to have a table of the allosteric hotspot positions for each domain peptide interaction, either in excel or PDF format. This will be easier to parse than the raw data if someone wants to quickly map the relevant positions to a structure. Likewise, we appreciated that the measurements are provided in machine-readable (CSV) format in supplemental tables, but these tables really need some accompanying legend or description that describes what the different headers mean. While the meaning of some columns is clear, others are ambiguous (is ddg the ΔΔGb or ΔΔGf?) or unclear (what is scHAmin_ligand?)

Minor comments:
1. A more informative title would be "Variation of allostery across a protein family." The use of the word "evolution" may lead some readers, including us, to initially expect a study on how allostery emerges through mutation and selection.

2. Top of page 3, "More than 270 PDZ domains in 155 different proteins…" How are the 155 proteins defined? Are these in the human genome or across some broader definition of the protein family?

3. Results section, "Generating seven complete allosteric and energetic maps" – the experimental "blocks" are referenced throughout the figures and explained in the methods, but need some succinct description / explanation in the results so that the reader can better interpret the figures as they go (without having to skip to the methods section to understand the blocks definition).

4. When the authors reference Fig. 3A, they state that "The data for the seven PDZ domain interactions is consistent with the hotspot concept, with a bimodal distribution of ΔΔGb values." However, the presence of bimodality is less obvious for the PSD95-PDZ2 CRIPT and NHERF-PDZ1 DAP1 interactions. Is this an artifact of how the data are visualized (and if so can the figure be improved), or do these domains/interactions have different mutational distributions? Was there some statistical fitting to establish bimodality for these distributions?

5. Results section, "A conserved allosteric core". It is interesting that the authors compare to the previously calculated PDZ sector, however this concept (what is the sector/how is it calculated?) likely needs a little more introduction for a broad audience.

6. The presentation in figure 3b is somewhat confusing, this figure might benefit from a more comprehensive legend. We might have missed this in the text, but why do some positions depict only a few bars, but others include all the interactions? Does this reflect missing measurements? Or only significant measurements are shown?

7. Unusual usage of "energy landscape." An energy landscape is typically defined as a function describing the energy (or free energy) along collective variables or other coordinates. Since no alternative definition is provided here, the meaning is unclear.

8. Typo: "Plotting absolute ΔΔGb against. the Euclidean distance" → remove the period after against.

9. Typo: page 6, "Interface hotspot evolution" results section, line 8 → hostposts instead of hotspots.

10. Typo: page 6, "Seven comparative allosteric maps" → "focussed" instead of "focused"

11. Reference check: Allosteric drugs and mutations: chances, challenges, and necessity. Current Opinion in Structural Biology 62, 149–157 (2020). Please confirm whether this reference includes authors.

(Remarks on code availability)
We took a cursory look at the code, it appears reasonably well documented but we did not try running though all of the analyses ourselves.

Reviewer #4

(Remarks to the Author)

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my comments and concerns in their revised draft. I recommend publication of the article.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
As I noted previously, this paper reports data on an interesting topic: the effects of all possible single amino acid replacements across a PDZ domain on binding affinity for peptide ligands and how those effects change among evolutionarily related PDZ domains. The data are comprehensive and quantitative, and they isolate effects on binding energy from those on protein stability. Although the results of the analysis are largely confirmatory of prior observations, they are of a scale and quality that make them a useful contribution to the literature.

I appreciate the authors' polite response to my comments. However, the revision and rebuttal letter do not effectively address the substance of my major concern. Specifically, the paper does not report its claims in terms of what the data actually address -- the effects of genetic variation across a protein on ligand affinity (and changes in those effects across a protein family) – instead the paper claims to reveal the nature of allostery and how it evolves. These claims are not supported: the paper provides no data or direct evidence related to allostery, allostery is not measured in the experiments, and the proteins studied are not known to be allosteric before or after mutation.

The critical issue is the definition of allostery, and I do not find the authors' rebuttal to be at all convincing on this point. In the manuscript and letter, the authors define allostery as "transmission of information spatially from one site in a protein to another," and they argue that allostery defined this way should include mutation effects on ligand affinity if the mutations occur outside the ligand binding surface. This vague definition departs from all accepted definitions of the term and from the core concept of allostery that makes it a useful concept in biochemistry. All definitions of allostery in the literature, including those cited in this manuscript and the rebuttal letter (other than the authors' own papers), are unanimous on the essence of allostery: it is a form of biological regulation by which the activity of a protein molecule (such as binding of a ligand, catalysis, etc.) is reversibly modulated by physical factors in the cellular environment that directly affect the protein but do not compete with ligand/substrate at the active site. Classically, the allosteric modulator was binding by the protein of an effector molecule, but the concept has been expanded to include other factors that can specifically and reversibly modulate a protein's activity, such as post-translational modification and sensitivity to pH, voltage, or light at sites distinct from the protein's active site. This is the sense in which allostery represents the "second secret of life" – because it allows proteins to respond to and regulate the biological state of cells – a concept the authors allude to in this manuscript. This kind of regulation of protein activity in cellular time and space is distinct from the "first secret of life," which is the genetic code and its ability to encode functional proteins and transmit that information across generations.

The authors' definition of allostery is unmoored from this foundation. Their purpose in this is to include as a form allostery when irreversible mutations (at sequence sites other than the active site or binding surface) change the protein's activity. Mutation is not a form of biological regulation and does not represent a means by which a protein can reversibly respond to changes in the cellular environment. Mutation does not affect an individual protein molecule or ensemble of molecules of a protein within a cell. Rather, the effects of mutation occur irreversibly and on a temporal and spatial scale that is irrelevant to biological regulation: the effects of a mutation are manifest only in the next generation, in different cells, in different copies of what is in fact a new and different protein.

Accepted definitions are also unanimous about the mechanisms that drive allostery: allosteric regulation arises from conformational heterogeneity or dynamics that are linked to both protein activity and the action of the effector. That is, each molecule of a protein can exist in multiple conformational states that differ in their activity, and the effector alters the activity of individual protein molecules (or the ensemble of molecules in the cell) by shifting the equilibrium towards or away from the active conformation(s). The authors' definition requires no conformational heterogeneity or dynamics in the protein either before or after the purportedly allosteric mutation; it requires only that the mutation changes the protein's structure. In this sense, too, the mutation empties the concept of allostery of a key component of its meaning.

The authors' redefinition is also confused and confusing in its implications for the relationship between the genotype/sequence of a protein and its phenotypic properties. Allostery describes a coupling between two biophysical phenomena within a protein molecule –effector binding at one location in the protein, for example, and activity at another.

Allostery is a biochemical phenotype of a protein – a complex one, defined as the relationship between two biochemical phenomena. Like any phenotype, it has a genetic basis, in that changes in a protein's sequence can affect it, altering or abolishing the coupling between stimulus and response; in turn, the phenotype of allostery can evolve via changes in a protein's sequence. This sequence-allostery relationship and its evolutionary relevance can be studied by measuring the phenotype (the coupling) in related proteins that differ in sequence. The authors' definition scrambles this conceptual structure: instead of a allostery being a phenotype that is the relationship between two biochemical phenomena, allostery becomes the relationship between one biochemical phenomenon (activity) and the protein's sequence, as revealed by a library of mutant proteins. To speak of the genetic basis of allostery then becomes meaningless: allostery is not a phenotype that has a genetic basis but is the genetic basis of a simpler and different phenotype (activity). And the idea that allostery can evolve also becomes muddled, because allostery is no longer a phenotypic property of a particular form of a protein that can potentially be acquired, lost or modified by fixation of mutations into that protein. Rather, the authors' form of allostery is a property of the "library" of a protein and all its mutants; it represents the "landscape" on which evolution takes place rather than a property that itself changes by evolution.

The authors provide citations in their manuscript and letter that they say support their redefinition of allostery as including the effects of mutations on ligand binding under the definition that allostery is "the transmission of information" from one part of the protein to another. Some of the papers cited do use the metaphor of information transfer or allosteric "communication" across a protein, but they all use this metaphor to refer to the coupling of biochemical phenomena described by the classical definition, not the effects of mutation on one phenomenon. They do not offer support to the idea that nonlocal effects of mutations on activity are a form of allostery.

• Hilser et al., which the authors cite in their manuscript for their definition of allostery, use the classical definition: "The defining aspect of allostery is the change in binding affinity [e.g., the increase in binding affinity of oxygen by hemoglobin (17) or end-product inhibition of l-Thr deaminase (4)] due to the effect of binding a second ligand at a distinct effector site. The problem of quantitative understanding arises because allostery is action-at-a-distance: The effector site can be many angstroms away from the regulated activity site (45)." Nowhere in this paper is sensitivity of activity to mutation suggested to be a form of allostery.
• Gunasekaran et al 2004 (cited in the letter) also use the classical definition: "Allosteric proteins have two identical (homotropic) or different (heterotropic) ligands. The binding of one ligand increases (or decreases) the affinity of the protein toward the second (Fig. 1)." They note that all nonfibrous proteins have "the potential to be allosteric:" all such proteins are conformationally heterogeneous/dynamic, so mutations (or introduction of a novel ligand into the cell) could cause a "nonallosteric protein to behave allosterically," causing a protein whose activity was not regulated to become reversibly regulated. This is clearly in conflict with the current authors' definition, because it implies that the protein before mutation is nonallosteric, whereas the redefinition would claim that the protein is already allosteric because mutation has the potential to change its activity.
• Reynolds et al. 2011 (cited in the letter) also define allostery in the classic way: "Allosteric regulation enables the activity of one site on a protein to modulate function at another spatially distinct site." This paper uses insertion mutagenesis of light-sensitive domains to identify sites at which insertion can confer light-sensitive allostery on a nonallosteric protein in this classic sense of the coupling of biochemical phenomena. The paper is quite clear that the protein is nonallosteric before the insertion (because its activity is not controlled by light) and allosteric afterwards (because it becomes light-regulated; this construction is inconsistent with the idea that a protein is allosteric before the insertion because introducing an insertion mutation affects function. This paper uses statistical methods to identify sites in the protein structure that may mediate this functional coupling between activity at distant parts of the protein – in this case the reception of light at the insertion and catalysis at the active site -- which it calls allosteric communication.
• Suel et al. 2003 (cited in the letter) also define allostery as a form of biological regulation, using GPCRs as an example of a modification of cytosolic protein-protein interactions when an extracellular lligand binds the receptor. They again use the term "allosteric communication" to describe "the process by which signals originating at one site in a protein propagate reliably to affect distant functional sites," and they use statistical analysis to identify covariation patterns among residues that they believe interact with each other. They find such networks not only in allosteric proteins such as GPCRs and hemoglobin but also in nonallosteric proteins such as serine proteases, but they never assert that the proteases are allosteric or suggest that mutations' effects on function represent allostery.
• Souffrant et al 2020 (cited in the letter) also use the classic definition: "Allostery is a ubiquitous phenomenon in the regulation of cellular processes. In allosteric regulations, changes from a distant site within biomolecular systems alter the conformationor dynamics of the active site via a network of inter-residue interactions. Protein dynamics play the key role in allosteric regulations in the dynamic point of view, biomolecules populate an ensemble of conformational states, and perturbations at an allosteric site cause population shifts and thereby modulate binding or catalytic activities." They report experiments on acid-beta-glucosidase, which they describe as allosteric because its catalytic activity is modified by glycosylation at a distant site. They perform structural studies to understand how glycosylation at a distant site affects conformation and therefore activity at the catalytic site. They conclude: "Our findings could help explain the allosteric effects of glycosylation on enzyme mechanisms, other disease-causing mutations in acid-β-glucosidase, and facilitate the design of drugs for the treatment of Gaucher disease." They never suggest that the protein should be considered allosteric because mutations can affect its activity or that their studies of allostery are focused on the effects of mutation on activity.
• Nusinov and Tsai 2013 (cited in the letter and the manuscript as citation #2) come closest to the authors' redefinition, but the paper does not support their view overall. This paper defines allostery in the classic way: "Allostery governs regulation and is the means through which environmental signals are communicated (del Sol et al., 2009; Kar et al., 2010; Tsai et al., 2009) within, across, and between cells…. Allostery initiates when the protein undergoes some structural disturbance. This perturbation can be the result of a change in the physical environment (Figure 1A), including exposure to light (Strickland et al., 2008); irradiation; change in pH (thus, protonation states; Martí et al., 2009); and concentration. It can also stem from noncovalent binding or a change in a covalent linkage, such as that taking place during a mutational event or when adding

or removing a posttranslational modification (PTM) (Nussinov et al., 2012).” This is clearly the classical definition. In another passage, the authors do include mutations under their list of activity-changing perturbations that may be considered allostery. However, this idea is never again used in the paper. When “allosteric mutations” are subsequently discussed, this term refers to mutations that affect allostery in in the traditional sense – sequence changes that alter or abolish the coupling between a reversible stimulus such as ligand binding at one site and the activity of the protein at the active site.

In their response letter, the authors claim that deep mutagenesis is a standard and broadly used approach to analyze allostery. But virtually all of the cited papers all use mutagenesis in allosteric proteins (defined in the traditional sense) to identify sites that may be involved in mediating the functional coupling between an effector site and active sites, or they use mutagenesis in non-allosteric proteins to identify sites that when mutated confer allosteric coupling between effector stimulus and activity. In each case, allostery in the traditional sense -- the coupling between effector and activity -- is measured before and after mutation. These papers do not claim that a DMS that measures only effects of mutations on activity (rather than the effect on the coupling between a reversible stimulus and activity) can provide evidence of allostery. The only clear exceptions are the papers from the current authors’ own group.

I conclude that rebuttal letter is incorrect in asserting that it is well-established or useful to define allostery to include mutations that affect a protein’s function but occur away from the active-site surface. The data reported here do not directly illuminate allostery or its evolution, and their claims are therefore confusing and misleading. The data do elucidate a truly interesting and important topic: the effects of mutation on ligand-binding, changes in these effects among related proteins, and the structural correlates of these effects and changes. The paper should report its results in those terms. I acknowledge that analysis of the effects of mutations on ligand binding can provide indirect insight into how allostery might be engineered into a nonallosteric protein or how allostery might evolve, just as was the case in the Reynolds et al. 2011 paper, because portions of a protein that when mutated affect activity might also confer true allostery, if those regions where also mutated in a way that causes the region to bind an effector or otherwise receive a reversible stimulus. I encourage the authors to speculate, interpret and discuss the meaning of their findings in relation to these topics. .

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In my view, the response to reviewers is adequate. In some places I still feel the data presentation and figures could be further simplified to get across key concepts, but this is really a matter of taste not of science - the data are very interesting. It would have helped significantly if the authors had referenced page and line numbers in the response or used highlighting to indicate the changes, it took considerable time to identify where the changes were made in some cases.

(Remarks on code availability)
I did not revisit the code during the revisions.

Reviewer #4

(Remarks to the Author)

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I think that the authors' modifications address the issues raised in my previous review and that the paper is ready for publication. I appreciate the authors' engagement with the concerns that I raised.

(Remarks on code availability)

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a large-scale experimental study in which the authors are able to distinguish the contributions to free energy from folding versus binding in a high throughput manner, allowing them to map the effect of all single and double mutations on binding free energies. The authors have previously published this map for a single PDZ domain, PSD95-PDZ3. The main new contribution in this work is to apply this to five members of the PDZ domain family with seven total binding interactions.

Although this work is incremental in the sense of not being qualitatively different, the generation of mutational sensitivity maps across multiple PDZ domains is first of its kind, allowing new insights.

We thank the referee for their enthusiasm and very constructive suggestions.

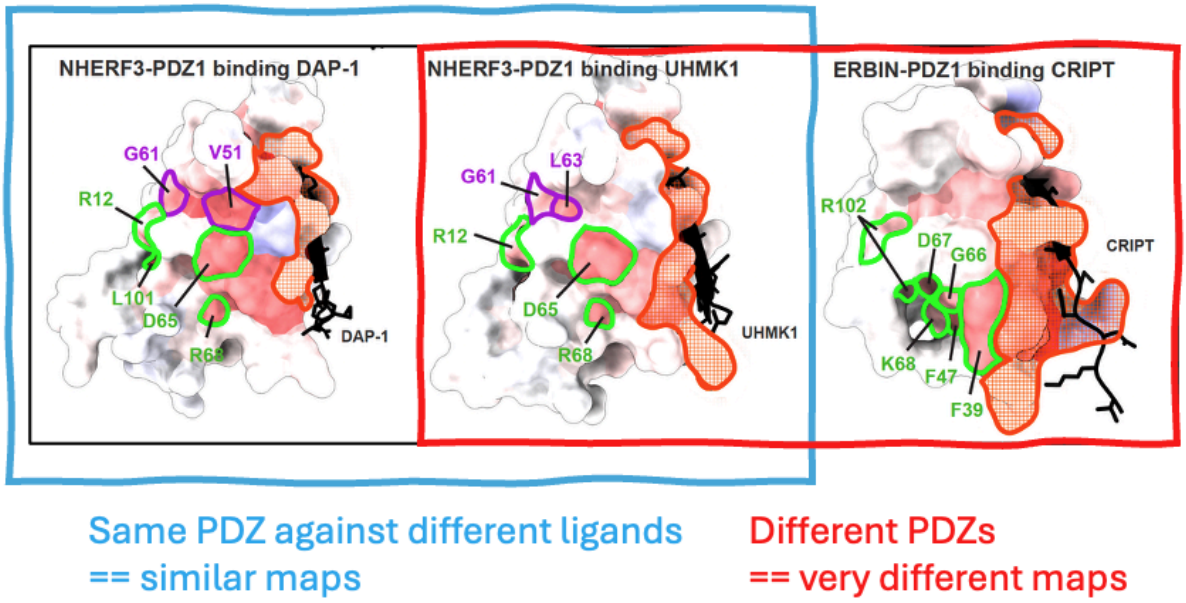
The two main contributions of this work, in my opinion, are:

1. Mapping the locations of hotspots that are more conserved versus protein specific, and showing the rarity of gain-of-function allosteric mutations contributes to our understanding of the evolutionary programmability of protein signaling within a fixed fold family. Comparing across multiple PDZ allows for the building of a consensus pattern of modular allostery in which a mostly conserved core connects to protein-specific surface hotspots. To me, the most impactful consequence of this is enabling systematic comparison with the "sectors" predicted from coevolutionary analysis of sequence alignments. To be able to systematically show that the pattern of physical coupling matches that of coevolutionary constraints would constitute a highly meaningful contribution. The authors show the general consistency of the core amongst PDZ domains in figure 2 and the occurrences of allosteric residues shared by multiple PDZ domains are listed and shown in figures 4 and 5. They also list the statistically significant overlap of hotspot residues with the core and with the sector previously identified from sequence co-evolution. However, because the sector overlaps strongly with the core, it is not clear how closely the actual geometry of the hotspots mirror that of the sector. In particular, the sector consists of a contiguous region spanning part of the core and extending to select surface sites. A plot directly comparing the allosteric hotspots with the sector would therefore greatly improve the impact of this paper.

We agree that an additional figure panel directly comparing the sector of coevolving residues with the identified allosteric hotspots would add impact to the paper. This has now been added in figure 4i and clarified in the text.

In addition, although the authors argue that the heterogeneity of allosteric sites is highest at the surface, Figure 6 seems to show a fair amount of allosteric overlap amongst the different PDZ domains. It would be helpful to show the differential conservation of the core and surface quantitatively, for example by plotting the identities of the allosteric residues of all the PDZ domains as a function of distance to the surface.

We believe this concern may arise from Figure 6 not clearly distinguishing comparisons made within the same PDZ domain across ligands from those made between different PDZ domains. As emphasized in the manuscript, allosteric and mutational effects are more conserved within a PDZ domain comparing across ligands but differ between distinct PDZs, even when binding the same ligand (Extended Data Fig. 4d,e,j,k,l). This distinction is now made clearer in Figures 5 and 6.
One example:



We agree that adding a panel to highlight differences in the distribution of conserved allosteric sites strengthens the statement. Accordingly, we re-arranged Figure 4 to include panel 4L, showing a structural view of this distribution in PDZ domains; panel 4M, a quantitative comparison of allosteric residue identities as a function of surface accessibility (RSASA); and panel S4M, illustrating the differential correlation of mutational effects between surface and core hotspots.

2. The generated data is systematic, large-scale, and of high quality. The authors took care to quantiatively compare their predicted free energies against previous work, and their method isolates the effect of mutations on binding free energy, removing confounders such as effects on folding. The authors also quantified general trends in the data, such as the distance decay of allosteric signal, which corroborates other experimental findings on other proteins as well as molecular

dynamics, NMR and evolutionary conservation studies. As the authors mention, this dataset will be valuable for future analysis. In particular, if this type of data can be more regularly produced in future for different proteins, that could potentially enable training of accurate machine-learning models for allosteric prediction and design of signaling proteins. The authors should provide a description of all the supplementary tables to facilitate future analysis of this data set.

If the authors can address the gaps in quantitative analysis of their data to support the claims, as mentioned in 1), this work will be of significance for the field of protein biophysics and molecular evolution.

We thank the referees for their enthusiasm and very constructive suggestions. We agree that the supplementary tables would benefit from a detailed description to facilitate future analysis of this dataset. This has now been added.

Reviewer #2 (Remarks to the Author):

This paper reports comparative DMS experiments of the effects of single amino acid mutations on the free energy of protein folding and peptide binding across multiple homologous PDZ proteins. This work applies the same experimental/computational pipeline developed in a number of recent papers by the Lehner group and here uses it in a comparative framework. The scope of the experiments is impressive and the data are of good quality, with more than adequate replicability and a good fit to the models used. However, I find that the way the experiments are presented and interpreted to be misleading. The meaning of the data and their contribution to the field need to be substantially reconceived for this to represent a constructive addition to the literature.

Understanding how mutations' effects change as protein families diverge is an important question in protein evolution and is also important for understanding the architecture by which protein functions – in this case, protein-protein interactions -- are physically and genetically determined. This is not the first paper to report changes in mutations' functional effects across homologous proteins using DMS – similar approaches have been applied to, for example, numerous viral proteins, TIM barrels, and bacterial and eukaryotic transcription factors (e.g., PMC9273037, PMC9429997, PMC4626756, PMC5343507, PMC9662819, PMC10515859, and others. The list includes comparative analysis of changing constraints over evolutionary divergence, the structural determinants of such changes,and the effects of mutations on allostery in allosterically regulated proteins (PMC9662819). Nevertheless, a strength of the current paper is its ability to deconvolute effects on binding from effects on stability using ddPCA, although it should be noted that a similar approach was also used in previous work on viral proteins (PMC7418704), although without the same comparative framework. This is also not the first paper to

use DMS to show how mutations across a protein's sequence affect protein-protein interactions, including at sites far from the binding interface (see, for example, several of the papers described above, as well as the authors' own PMID 35388192, PMC5896888, etc.). The work in the current manuscript therefore does not break entirely new ground. However, the data are of a scope and quality that the paper has the potential to make a contribution to the field if the paper were focused more sharply on differences among homologous proteins in the sequence and structural determinants of protein-protein binding that allow this paper to go beyond prior work.

We thank the referee for their enthusiasm and very constructive suggestions. Thank you also for highlighting these previous publications on comparative mutational scanning, which we have now cited in the introduction.

1. My major concern about this paper is that the purpose of the experiments and the interpretation of the data are described in a way that obscures rather than illuminates their meaning and their relationship to existing knowledge. The authors center their manuscript around the topic of allostery – the genetic basis of allostery, the ways in which that basis is conserved or variable during evolution, and how allostery itself evolves. This focus is the organizing principle of the manuscript, the justification for the work, and the interpretation of the data. Allostery means regulation of a protein's activity (catalysis, binding a biological ligand) by binding an effector ligand at a site on the protein distinct from the active site. This is the classic definition that goes back to Monod, whom the authors cite, and which was subsequently formalized biophysically in the classic papers of Monod-Wyman-Changeux and Koshland-Nemethy-Filmer. This definition has been reiterated many times, including in the more recent review by Motlagh-Wraby-Hilser, which is the first citation in the current manuscript. The latter papers begins, "Allostery is the process by which biological macromolecules (mostly proteins) transmit the effect of binding at one site to another, often distal, functional site, allowing for regulation of activity." This definition is in every biochemistry textbook and is central to modern understanding of how proteins work and are regulated. The PDZ proteins that the authors study are not allosteric or allosterically regulated. Their functional activity at their "active site" is to bind peptides, but this activity is not regulated by binding to a second effector ligand or by any other form of regulation (such as post-translational modification) discussed in the manuscript. The data in the current manuscript do not address how allostery evolves, how it is encoded in protein sequence, how that encoding changes over time, etc., because the proteins studied are not allosteric. (I note that some papers have used an expanded definition of allostery that refers to changes in protein conformation caused by ligand-binding, irrespective of whether activity is regulated by the binding event. This "structural" definition of allostery has been applied to PDZ domains. Whatever the merits of this definition, it is not the one the authors of the current manuscript use, and it would not accurately describe the subject addressed by the authors' data.)

Instead of following this well-established definition (or even the looser structural definition), the current manuscript redefines an allosteric protein as one in which mutations that are far from the active site alter the protein's function – in this case, that means that mutations away from the peptide binding surface affect binding affinity. This does not make these proteins allosteric, because allostery refers to regulation of a protein (or, by the structural definition, to conformational change). A mutation changes the protein's sequence, creating a new protein; this is not a form of regulation of the activity of an existing protein (or a change in its conformation). It is meaningless and confusing to call mutations that affect binding in a nonallosteric protein "allosteric" mutations, because the protein remains nonallosteric both before and after the mutation. Genetic perturbation of a nonallosteric protein – such as in the DMS experiments reported here – reveal the genetic basis of peptide binding, not the basis of allostery. I recognize that the authors have used their re-definition in some of their previous papers, and that there are some other papers in the literature that have also used it. But this redefinition is problematic: to conflate allosteric regulation and the effects of mutation on protein-peptide binding muddies our understanding of both topics.

The paper's claims to experimentally dissect the genetic basis of allostery and changes in that basis among homologs should therefore be removed from the paper. This will require a fundamental reframing to focus on the questions that the experiments do address – the genetic basis of peptide binding and how that basis changes among homologs.

We thank the reviewer for this comment and the opportunity to clarify our use of the term allostery. We agree that the original definition involved regulation by effector binding at a site distinct from the active site (Monod, Wyman, and Changeux), but the concept has since expanded to include regulation triggered by other perturbations (small molecule binding, macromolecular interactions, covalent modifications such as post-translational modifications and mutations can all have allosteric effects). Accordingly, we use a broader and widely-accepted updated definition of allostery - "the transmission of information spatially from one site to another in a protein" (Gunasekaran et al., 2004; Nussinov & Tsai, 2013; Reynolds et al., 2011; Souffrant et al., 2020; Süel et al., 2003) - without redefining allostery as exclusively mutation-driven.

In this study, we use deep mutational scanning as a standard and broadly used approach to identify allosteric coupling between distant sites (Carlson & Fenton, 2016; Faure et al., 2022; Guarnera & Berezovsky, 2020; Leander et al., 2020; McCormick et al., 2021; Naganathan, 2019; Reynolds et al., 2011; Tang & Fenton, 2017; Weng et al., 2023), not to imply that allostery arises solely from mutations.

PDZ domains are classic model systems to study allostery, even though most of them have no known allosteric ligands and mutations are primarily used to quantify

allosteric coupling (Stevens and He 2022; Petit et al. 2009; van den Berk et al. 2007; Peterson et al. 2004; Cowan et al. 2023; Smock and Gierasch 2009; Motlagh et al. 2014; Raman et al. 2016; Reynolds et al. 2011; Kumawat and Chakrabarty 2017)

We have revised the introduction of the manuscript to clarify these points.

2. This reframing of the paper will also require the authors to clarify what is novel in their work relative to other work on the genetic derterminants of peptide binding. Many of the results in the manuscript retread issues already shown in prior work (including that of the authors). For example, the fact that certain residues within binding interfaces – particularly those that are fully buried near the "center" of an interface rather than near its edge -- are more likely when mutated to affect ligand binding or specificity has already been established. So has the fact that mutations at sites distant from the binding interface can affect binding, as well as the fact that these couplings follow predictable trends in terms of distance from the binding surface, structural relationship to the protein core, etc. I found myself wondering frequently where the novel impact of this paper lies. Eventually in reading the manuscript we do arrive at the most novel and impactful data, which is the comparative analysis among homologs of the distribution of sites that affect binding at a distal surface. I would suggest that in their revision the authors should focus more on these topics and bring out the significance for evolution, the physical basis of protein function, and therapeutics more clearly.

Thank you. We agree that the comparative analysis among homologs of allosteric mutations (sites where mutations affect binding at a distal surface) is the most important and impactful section of the paper, as we highlight and focus on in the abstract, title, introduction and discussion of the revised manuscript.

However we also believe the comparative analyses of the binding interfaces are important and interesting and deserve space in the results section. Previous landmark studies have focussed on individual interfaces or computational analyses of very different interfaces, not analyses of structurally homologous interfaces in a protein family.

3. A reframing like this would still allow the authors to speculate in the discussion about the relevance of their findings for allostery. It is well known by now that mutations far from the binding surface can affect peptide binding in nonallosteric proteins. As the authors of this manuscript note, such findings in nonallosteric proteins can inform our understanding of how allostery works biophysically and how it evolves. For example, the fact that mutations can affect peptide binding at a distant surface implies that the mutated regions and the binding surface are conformationally coupled. It is possible that mutations in the former region, if they were to confer binding to an effector, might plausibly confer allostery on the protein –

an important finding for evolution and for engineering novel allosteric proteins. Similarly, if an effector can be engineered to bind to such a coupled surface, even without mutations, this would represent an allosteric drug that can modulate the activity of a protein that is not endogenously allosterically regulated. These points have already been made repeatedly in the literature, so if the authors of the current manuscript raise these issues, they should have something particular to say about the insights provided by their data that adds to the existing body of knowledge in this area. Since the novelty of this paper lies in the scope of its comparative analysis and the isolation of effects on binding from those on folding, I would hope that the authors would address the specifics of the apparent lability across homologs of this kind of conformational coupling and its implications for engineering, evolution, and therapeutics.

In our study, we define allosteric sites as residues distant from the binding interface that can significantly affect binding when perturbed. We—and many others—believe this is the most useful definition as it focuses on the key phenomenon, which is long-range energetic coupling. Perturbations that can impart allosteric effects include small molecules (e.g. natural ligands or drugs) but they also include protein-protein or protein-nucleic acid binding, post-translational modifications (such as phosphorylation), and mutations. The huge advantage of using mutations to quantify allostery is that they can be used as perturbations at every site. This is not possible using small molecules or post-translational modifications. Using mutations therefore provides an unbiased, systematic and comprehensive approach to quantify long-range energetic coupling between all sites and an active site in a protein. We very strongly believe this systematic approach is required to better quantify the phenomena, to better understand it (including its conservation in homologous protein), and also to generate the data that will be required to train models to predict (and engineer) it.

In the Discussion of the revised manuscript we discuss broader implications for drug design, protein engineering, and evolution.

4. In the discussion, the authors identify several “important insights into the evolution of allostery,” but many of these are not new insights. The authors do cite other work as if it is confirmatory of their findings, but I think it is more accurate to say that the authors’ findings on some of these topics are confirmatory of the prior work. For example, the distance-dependence decay from the active site of mutation effects on bindings is already established. Similarly, the paper claims to “reveal a modular allosteric architecture via a conserved allosteric core connecting to protein-specific extensions to surface sites.” As the authors note, this directly echoes prior work on PDZ domains and other proteins in which sets of covarying residues structurally extend from the protein core to protein surfaces. This is therefore not a new insight. The authors should identify what was uncertain in prior analyses and clearly state

the contribution made by their experiments that was not already settled by prior work.

Thank you for the suggestion, we have now rephrased our findings to make this distinction clearer.

5. The authors claim to identify “gain of function” mutations, but the definition of this term is problematic. The authors appear to use this phrase to refer to mutations that increase a protein’s affinity for a ligand that it already binds. The term normally refers not to refinement or amplification of an existing functon (as the authors use it) but to the acquisition of an entirely new function – e.g., binding to a ligand to which the protein previously had no detectable binding or affinity. I would suggest finding a different term to avoid confusion.

We thank the reviewer for this helpful clarification. To avoid confusion, we have revised our terminology and we now refer to these variants as “affinity-increasing”.

6. The final paragraph of the discussion seems out of place. It seems potentially better suited to the introduction, because it states why the type of analyses the authors are conducting is relevant to developing therapeutics, but it does not reflect on the implications of the authors’ particular findings for this area. Either the latter should be developed with specific reference to the data, moved to the introduction, or omitted.

This final paragraph discusses the implications of our findings for evolution, engineering and drug discovery. In the revised manuscript we have also added these points to the introduction.

7. The paper says that “our data suggest that allostery primarily evolves via the gain and loss of peripheral extensions to a conserved allosteric core.” Because none of their proteins or mutants are allosteric, their data do not support this statement.

As discussed above PDZ domains are allosteric and are widely used as model systems to study allostery in single domains. Mutagenesis is a well established method to quantify coupling between distant sites. Thus our analysis of this data supports this statement.

8. Given the scope of the experiments and the nature of the technology, I think that the replicability of the experiments is more than reasonable. However, some of the correlation coefficients -- especially to the biophysical measurements -- are very modest, and when squared some of them would indicate a nontrivial amount of unexplained variance attributable either to measurement noise or model misfit. Acknowledgement of the cases in which the correlations are only moderate would enhance the transparency of the paper.

We are not certain which correlations the reviewer refers to. The fitness replicate correlations (Extended Data Fig. 1a) are excellent, and all values are shown for transparency. The correlations for biophysical measurements are reported in the "From molecular phenotypes to free energy changes" section (R = 0.89 for PSD95-PDZ2–NMDAR2A and R = 0.76 for previously published PSD95-PDZ3–CRIPT data). As these values include measurement noise (indicated through error bars), perfect correlations are not expected. We have revised the text to note the slightly more moderate correlation in the latter case. The correlations between observed and model-predicted fitness values (Extended Data Fig. 2) are also mentioned in the same section, and all values are provided in the figures for transparency.

9. The authors present correlations across replicates for their experimental measurements, but there may be much greater uncertainty in the estimates of binding and folding energies, which are fit to a complex nonlinear model. As these energies are the object of interest in the paper that drive all the subsequent interpretations, it would be worth characterizing the replicability of these inferences or at least the extent to which the inferred energies predict the observations. Extended data Fig. 2c presents these fits, and some of them appear by eye to be relatively modest, although the correlation coefficients do not seem to be presented. In addition to noise, another source of mis-fitting could be epistasis: for an additive model to fit the data extremely well would imply virtually no epistasis other than that imposed by the global nonlinearity, and this would be somewhat surprising. I would therefore like to see more detailed quantification of the fits and if necessary a more cautious description in the text.

Extended Data Fig. 2a shows how well the inferred energies predict the observed fitness effects with all correlations reported by library and block for transparency. These correlations are quantified by ten-fold cross validation using held out genotypes. We have revised the text to clarify this point. Extended Data Fig. 2c provides a visual representation of the model's internal weights and their relationship to the data, rather than an evaluation of model performance.

In summary, the data in this paper show in detail how the effects of mutations on peptide binding can change among evolutionarily related proteins. The authors should reframe their paper to explore the significance of these observations without casting them as a direct investigation of allostery, which is not valid. I think that in general authors should have broad leeway in determining how to present their results; moreover, definitions of terms are not written in stone but rather represent meanings, agreed upon by the community, that allow us to think and communicate clearly with each other. After consideration, however, I feel that the authors' use of the term allostery does not accurately convey the meaning and significance of their work, and that the redefinition proposed would lead to less clarity in our

understanding of the genetics and structural foundations of protein function, regulation, and evolution.

Reviewers #3 and #4 (Remarks to the Author):

Marti-Aranda and Lehner present a comparative study of the mutations tuning binding affinity and stability across a collection of seven protein-peptide interactions. This data-rich paper enables — for the first time to our knowledge — a comprehensive examination of how allosteric networks are conserved or diverge across homologs. The methods used to measure allosteric maps are state of the art and have been sufficiently validated (Faure et al., 2022). Specifically, the ability of ddPCA + Mochi to approximate in vitro binding free energies is strong. The authors study the PDZ domain, which is a well-explored model system for ligand binding and allostery, allowing them to place their results in dialog with other previous work. The main shortcoming of the PDZ domain is that the potential for long range communication is somewhat limited by the relative small size of the domain. Key results are that: (1) allostery in the protein core is largely conserved, while surface hotspots vary and (2) allosteric maps of the same protein binding different ligands are more similar to each other than maps of different PDZ domains binding the same ligand. These results are well-supported by the presented data. From these observations, the authors propose a model in which allostery evolves via gain and loss of peripheral extensions to a conserved allosteric core. This aligns with some earlier proposals in the field, but the dataset from Marti-Aranda provides some of the most extensive and direct evidence that this model might be correct. Taken together, we anticipate the results and data will be of interest to a broad community of scientists working in molecular evolution, allosteric regulation, protein engineering and signaling. We propose a few changes to the paper that we feel would increase the impact, interpretability and utility of the work.

We thank the referees for their enthusiasm and very constructive suggestions.

Major comments:

1.Clarity of figures. In this work the authors face a considerable challenge of presenting a large and multi-dimensional data set. While the paper includes ample figure panels and supplementary materials, at times it is hard to appreciate the main conclusions from the graphic presentation. In particular, it is challenging to visually compare the results (ΔΔGb and ΔΔGf) across domains. As a few sub-points/suggestions on how to achieve this:

a.Variability in the spatial distribution of allosteric hotspots is the key result that needs to be communicated visually, but this was not conveyed satisfactorily in

Figures 2, 5, and 6. Figure 2C only shows the surface of ΔΔGb in an orientation focused on the binding site. In Figures 5 and 6, the annotations somewhat obscure the differences. We propose either representing ΔΔGb as colored VDW spheres on top of ribbon structures in different orientations with limited annotation or plotting two maps on the same structure. Positions in common could be shown in one color, while differing positions are shown in contrasting colors. Possibly one or two representative pairs could illustrate the concept simply, while the full data is presented separately. Alternatively, differences between two allosteric maps could be plotted directly using a diverging color map.

Figure 2C was not intended to depict allostery but to illustrate the distribution of detrimental mutations. We have redesigned and simplified Figures 1 and 2 to clarify this and now show only a single representative example.

We already use a two color scheme to distinguish shared versus variable allosteric sites (Figures 4-6). We believe it is now clearly represented in Figure 6, however, we could reduce it to only panel a in the main figure, moving the remaining panels to a new Supplementary Figure S6 if required. We also agree that Figure 5 would benefit from a simpler representation, and we have revised it accordingly.

b. In figure 2A the authors present all of the ΔΔGb and ΔΔGf data as heat maps. While this is very thorough, heat maps can be challenging to visually compare, and do not allow error in the measurements to be easily represented. We propose making this a supplemental figure, and instead showing a more compact comparison of the ΔΔGb and ΔΔGf between domains. One idea would be to show a heat map of the correlation coefficients between ΔΔGb (or ΔΔGf) in one protein interaction versus another to allow rapid comparison of which domains exhibited more or less similar mutational profiles. Another possibility would be to show correlation plots (with error bars) or difference maps for select pairs of domains , to emphasize which mutations are similar or divergent across homologs.

We believe it is important to retain the complete ΔΔGb and ΔΔGf maps in the main figure, as they represent the full dataset and provide detailed information on the mutational effects at each site. However, we agree that Figure 2 needed clearer organization. We have therefore simplified panel C to one representative example and added correlation boxplots (new panel 2h) to compare the similarity of mutational effects across domains.

2. Limited discussion. The brief discussion summarizes results and highlights the implications for drug discovery, but would benefit from a deeper treatment of the broader significance of the findings. The authors might speculate on mechanisms of allostery that underpin the observed differences in allosteric maps. Could the variation across homologs be explained by a single physical model, or are multiple models required? What physical differences might distinguish allosteric mechanisms

in the core versus the periphery? And what is the relationship between the conserved/non-conserved allosteric hotspots in this dataset and sequence conservation in the protein family — are conserved allosteric hotspots more conserved in sequence space as well? In general it feels like there is a little bit of a missed opportunity to synthesize the data with sequence evolution patterns in the PDZ family.
They should also discuss related work that has addressed how the distribution of hotspots and pathways changes upon mutation (Leander et al., PNAS 2020), ligand binding (Glasgow et al., Nat. Commun. 2023), across homologs (Salinas and Ranganathan, eLife 2018; Leander et al., eLife 2022), and local sequence contexts (Laursen et al., PNAS 2022). All of this relates to the same overarching problem: variability in the spatial distribution of allostery. Finally, the discussion should expand on the evolutionary implications of a modular allosteric architecture . Does such modularity accelerate evolutionary adaptation?

Thank you. We have cited all of these studies in the revised manuscript. We have also added a comparison with the previously defined sector of coevolving residues and sequence conservation (new version of Figure 4i). We summarize these points in the Discussion and have revised it to clarify and expand on these concepts. We would be happy to further extend the discussion if required (but it isn't our typical writing style).

3.Justification of allosteric hotspot definition. The authors define allosteric hotspots by comparing the inferred |ΔΔGb| to the expected effect based on distance from the active site. It is not clear why this choice was made over the simpler method of identifying allosteric sites as positions outside the binding site with median |ΔΔGb| comparable to the median effect of binding site mutations (as used in some of the authors' own prior work). Along the same lines, in the section "Allostery beyond distant dependent decay" the authors compare the ΔΔGb-residuals between domains, why compare the residuals rather than the values themselves? This implies there is some kind of difference in the scaling or dynamic range of the different datasets?

We thank the reviewer for raising this issue and for the opportunity to clarify this concept. We define allosteric hotspots as residues whose distribution of (19) ΔΔGb values are larger than expected from the distance-dependent decay model. This method allows a normalized comparison of coupling strength across domains while accounting and normalizing for structural differences between equivalent residues, particularly their distance from the ligand. In addition, it increases sensitivity for detecting distal hotspots (unusually allosteric distal sites), which are not expected to show the same effect sizes as residues closer to the ligand. We use ΔΔGb residuals rather than raw values for the same reasons. We have clarified these points in the revised manuscript.

4.Data accessibility. It would be helpful to have a table of the allosteric hotspot positions for each domain peptide interaction, either in excel or PDF format. This will be easier to parse than the raw data if someone wants to quickly map the relevant positions to a structure. Likewise, we appreciated that the measurements are provided in machine-readable (CSV) format in supplemental tables, but these tables really need some accompanying legend or description that describes what the different headers mean. While the meaning of some columns is clear, others are ambiguous (is ddg the ΔΔGb or ΔΔGf?) or unclear (what is scHAmin_ligand?)

Supplementary Table 6 lists, for each residue in every domain, whether it is an allosteric hotspot, a binding interface hotspot, and whether it is conserved across domains. We agree the column headers were ambiguous and, as also suggested by Reviewer 1, have added detailed column descriptions in the table legends for clarity.

Minor comments:
1. A more informative title would be “Variation of allostery across a protein family.” The use of the word “evolution” may lead some readers, including us, to initially expect a study on how allostery emerges through mutation and selection.

We appreciate the reviewer’s suggestion regarding the title. We have revised the title to “Conservation and divergence of allostery in a protein family” which avoids this ambiguity while accurately reflecting the scope and findings of our study.

2. Top of page 3, “More than 270 PDZ domains in 155 different proteins…” How are the 155 proteins defined? Are these in the human genome or across some broader definition of the protein family?

Thank you. We have edited the sentence to clarify that these refer to human proteins.

3. Results section, “Generating seven complete allosteric and energetic maps” – the experimental “blocks” are referenced throughout the figures and explained in the methods, but need some succinct description / explanation in the results so that the reader can better interpret the figures as they go (without having to skip to the methods section to understand the blocks definition).

Thank you, we agree that the “blocks” concept should have been described before in the text. We have now added a brief introduction to this concept.

4. When the authors reference Fig. 3A, they state that “The data for the seven PDZ domain interactions is consistent with the hotspot concept, with a bimodal distribution of ΔΔGb values.” However, the presence of bimodality is less obvious for the PSD95-PDZ2 CRIPT and NHERF-PDZ1 DAP1 interactions. Is this an artifact of how the data are visualized (and if so can the figure be improved), or do these

domains/interactions have different mutational distributions? Was there some statistical fitting to establish bimodality for these distributions?

Thank you, we agree that the two-peaked distribution is less evident in some interactions, such as PSD95-PDZ2 CRIPT. We have rephrased the sentence accordingly to clarify this point in the text.

5. Results section, “A conserved allosteric core”. It is interesting that the authors compare to the previously calculated PDZ sector, however this concept (what is the sector/how is it calculated?) likely needs a little more introduction for a broad audience.

We agree that this concept could use a brief introduction for clarity, and we have added this to the text.

6. The presentation in figure 3b is somewhat confusing, this figure might benefit from a more comprehensive legend. We might have missed this in the text, but why do some positions depict only a few bars, but others include all the interactions? Does this reflect missing measurements? Or only significant measurements are shown?

We thank the reviewer for this helpful comment. In Figure 3b, black outlined boxplots represent significant ΔΔGb distributions, while grey ones indicate non-significant ones. Positions with fewer bars correspond to residues contacting the ligand in only some PDZ domains. There are no missing measurements in this data. To avoid this confusion, we have now included all bars, marking non-contact residues with transparency, and clarified the figure legend.

7. Unusual usage of “energy landscape.” An energy landscape is typically defined as a function describing the energy (or free energy) along collective variables or other coordinates. Since no alternative definition is provided here, the meaning is unclear.

In this context, we use the term “energy landscape” to refer to the distribution of mutational effects on binding and folding energy across sequence space, that is, the energy variation resulting from all possible single amino acid substitutions in each PDZ domain. In this case, the coordinates of the landscape correspond to sequence space rather than conformational coordinates. We have clarified this in the text.

8. Typo: “Plotting absolute ΔΔGb against. the Euclidean distance” → remove the period after against.

Done, thank you!

9. Typo: page 6, “Interface hotspot evolution” results section, line 8 → hostposts instead of hotspots.

Thank you! This was not a typo, we are indeed referring to the evolution of hotspots in the binding interface, so we will keep it as “Interface hotspot evolution”

10. Typo: page 6, “Seven comparative allosteric maps” → “focussed” instead of “focused”

Done, thank you!

11. Reference check: Allosteric drugs and mutations: chances, challenges, and necessity. Current Opinion in Structural Biology 62, 149–157 (2020). Please confirm whether this reference includes authors.

Apologies, it was missed by the automatic referencing tool. This has now been corrected, thank you!

Reviewer #3 (Remarks on code availability):

We took a cursory look at the code, it appears reasonably well documented but we did not try running though all of the analyses ourselves.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments and concerns in their revised draft. I recommend publication of the article.

Thank you.

Reviewer #2 (Remarks to the Author):

As I noted previously, this paper reports data on an interesting topic: the effects of all possible single amino acid replacements across a PDZ domain on binding affinity for peptide ligands and how those effects change among evolutionarily related PDZ domains. The data are comprehensive and quantitative, and they isolate effects on binding energy from those on protein stability. Although the results of the analysis are largely confirmatory of prior observations, they are of a scale and quality that make them a useful contribution to the literature.

I appreciate the authors' polite response to my comments. However, the revision and rebuttal letter do not effectively address the substance of my major concern. Specifically, the paper does not report its claims in terms of what the data actually address -- the effects of genetic variation across a protein on ligand affinity (and changes in those effects across a protein family) – instead the paper claims to reveal the nature of allostery and how it evolves. These claims are not supported: the paper provides no data or direct evidence related to allostery, allostery is not measured in the experiments, and the proteins studied are not known to be allosteric before or after mutation.

The critical issue is the definition of allostery, and I do not find the authors' rebuttal to be at all convincing on this point. In the manuscript and letter, the authors define allostery as "transmission of information spatially from one site in a protein to another," and they argue that allostery defined this way should include mutation effects on ligand affinity if the mutations occur outside the ligand binding surface. This vague definition departs from all accepted definitions of the term and from the core concept of allostery that makes it a useful concept in biochemistry. All definitions of allostery in the literature, including those cited in this manuscript and the rebuttal letter (other than the authors' own papers), are unanimous on the essence of allostery: it is a form of biological regulation by which the activity of a protein molecule (such as binding of a ligand, catalysis, etc.) is reversibly modulated by physical factors in the cellular environment that directly affect the protein but do not compete with ligand/substrate at the active site. Classically, the allosteric modulator was binding by the protein of an effector molecule, but the concept has been expanded to include other factors that can specifically and reversibly modulate a protein's activity, such as post-translational modification and sensitivity to pH, voltage, or light at sites distinct from the protein's active site. This is the sense in which allostery represents the "second secret of life" – because it allows proteins to respond to and regulate the biological state of cells – a concept the authors allude to in this manuscript. This kind of regulation of protein activity in cellular time and space is distinct from the "first secret of life," which is the genetic code and its ability to encode functional proteins and transmit that information across generations.

The authors' definition of allostery is unmoored from this foundation. Their purpose in this is to include as a form allostery when irreversible mutations (at sequence sites other than the active site or binding surface) change the protein's activity. Mutation is not a form of biological regulation and does not represent a means by which a protein can reversibly respond to changes in the cellular environment. Mutation does not affect an individual protein molecule or ensemble of molecules of a protein within a cell. Rather, the effects of mutation occur irreversibly and on a temporal and spatial scale that is irrelevant to biological regulation: the effects of a mutation are manifest only in the next generation, in different cells, in different copies of what is in fact a new and different protein.

Accepted definitions are also unanimous about the mechanisms that drive allostery: allosteric regulation arises from conformational heterogeneity or dynamics that are linked to both protein activity and the action of the effector. That is, each molecule of a protein can exist in multiple conformational states that differ in their activity, and the effector alters the activity of individual protein molecules (or the ensemble of molecules in the cell) by shifting the equilibrium towards or away from the active conformation(s). The authors' definition requires no conformational heterogeneity or dynamics in the protein either before or after the purportedly allosteric mutation; it requires only that the mutation changes the protein's structure. In this sense, too, the mutation empties the concept of allostery of a key component of its meaning.

The authors' redefinition is also confused and confusing in its implications for the relationship between the genotype/sequence of a protein and its phenotypic properties. Allostery describes a coupling between two biophysical phenomena within a protein molecule –effector binding at one location in the protein, for example, and activity at another. Allostery is a biochemical phenotype of a protein – a complex one, defined as the relationship between two biochemical phenomena. Like any phenotype, it has a genetic basis, in that changes in a protein's sequence can affect it, altering or abolishing the coupling between stimulus and response; in turn, the phenotype of allostery can evolve via changes in a protein's sequence. This sequence-allostery relationship and its evolutionary relevance can be studied by measuring the phenotype (the coupling) in related proteins that differ in sequence. The authors' definition scrambles this conceptual structure: instead of a allostery being a phenotype that is the relationship between two biochemical phenomena, allostery becomes the relationship between one biochemical phenomenon (activity) and the protein's sequence, as revealed by a library of mutant proteins. To speak of the genetic basis of allostery then becomes meaningless: allostery is not a phenotype that has a genetic basis but is the genetic basis of a simpler and different phenotype (activity). And the idea that allostery can evolve also becomes muddled, because allostery is no longer a phenotypic property of a particular form of a protein that can potentially be acquired, lost or modified by fixation of mutations into that protein. Rather, the authors' form of allostery is a property of the "library" of a protein and all its mutants; it represents the "landscape" on which evolution takes place rather than a property that itself changes by evolution.

The authors provide citations in their manuscript and letter that they say support their redefinition of allostery as including the effects of mutations on ligand binding under the definition that allostery is "the transmission of information" from one part of the protein to another. Some of the papers cited do use the metaphor of information transfer or allosteric

“communication” across a protein, but they all use this metaphor to refer to the coupling of biochemical phenomena described by the classical definition, not the effects of mutation on one phenomenon. They do not offer support to the idea that nonlocal effects of mutations on activity are a form of allostery.

• Hilser et al., which the authors cite in their manuscript for their definition of allostery, use the classical definition: “The defining aspect of allostery is the change in binding affinity [e.g., the increase in binding affinity of oxygen by hemoglobin (17) or end-product inhibition of l-Thr deaminase (4)] due to the effect of binding a second ligand at a distinct effector site. The problem of quantitative understanding arises because allostery is action-at-a-distance: The effector site can be many angstroms away from the regulated activity site (45).” Nowhere in this paper is sensitivity of activity to mutation suggested to be a form of allostery.
• Gunasekaran et al 2004 (cited in the letter) also use the classical definition: “Allosteric proteins have two identical (homotropic) or different (heterotropic) ligands. The binding of one ligand increases (or decreases) the affinity of the protein toward the second (Fig. 1).” They note that all nonfibrous proteins have “the potential to be allosteric:” all such proteins are conformationally heterogeneous/dynamic, so mutations (or introduction of a novel ligand into the cell) could cause a “nonallosteric protein to behave allosterically,” causing a protein whose activity was not regulated to become reversibly regulated. This is clearly in conflict with the current authors’ definition, because it implies that the protein before mutation is nonallosteric, whereas the redefinition would claim that the protein is already allosteric because mutation has the potential to change its activity.
• Reynolds et al. 2011 (cited in the letter) also define allostery in the classic way: “Allosteric regulation enables the activity of one site on a protein to modulate function at another spatially distinct site.” This paper uses insertion mutagenesis of light-sensitive domains to identify sites at which insertion can confer light-sensitive allostery on a nonallosteric protein in this classic sense of the coupling of biochemical phenomena. The paper is quite clear that the protein is nonallosteric before the insertion (because its activity is not controlled by light) and allosteric afterwards (because it becomes light-regulated; this construction is inconsistent with the idea that a protein is allosteric before the insertion because introducing an insertion mutation affects function. This paper uses statistical methods to identify sites in the protein structure that may mediate this functional coupling between activity at distant parts of the protein – in this case the reception of light at the insertion and catalysis at the active site -- which it calls allosteric communication.
• Suel et al. 2003 (cited in the letter) also define allostery as a form of biological regulation, using GPCRs as an example of a modification of cytosolic protein-protein interactions when an extracellular lligand binds the receptor. They again use the term “allosteric communication” to describe “the process by which signals originating at one site in a protein propagate reliably to affect distant functional sites,” and they use statistical analysis to identify covariation patterns among residues that they believe interact with each other. They find such networks not only in allosteric proteins such as GPCRs and hemoglobin but also in nonallosteric proteins such as serine proteases, but they never assert that the proteases are allosteric or suggest that mutations’ effects on function represent allostery.
• Souffrant et al 2020 (cited in the letter) also use the classic definition: “Allostery is a ubiquitous phenomenon in the regulation of cellular processes. In allosteric regulations, changes from a distant site within biomolecular systems alter the conformationor dynamics of the active site via a network of inter-residue interactions. Protein dynamics play the key role in allosteric regulations in the dynamic point of view, biomolecules populate an

ensemble of conformational states, and perturbations at an allosteric site cause population shifts and thereby modulate binding or catalytic activities." They report experiments on acid-beta-glucosidase, which they describe as allosteric because its catalytic activity is modified by glycosylation at a distant site. They perform structural studies to understand how glycosylation at a distant site affects conformation and therefore activity at the catalytic site. They conclude: "Our findings could help explain the allosteric effects of glycosylation on enzyme mechanisms, other disease-causing mutations in acid-β-glucosidase, and facilitate the design of drugs for the treatment of Gaucher disease." They never suggest that the protein should be considered allosteric because mutations can affect its activity or that their studies of allostery are focused on the effects of mutation on activity.
• Nusinov and Tsai 2013 (cited in the letter and the manuscript as citation #2) come closest to the authors' redefinition, but the paper does not support their view overall. This paper defines allostery in the classic way: "Allostery governs regulation and is the means through which environmental signals are communicated (del Sol et al., 2009; Kar et al., 2010; Tsai et al., 2009) within, across, and between cells…. Allostery initiates when the protein undergoes some structural disturbance. This perturbation can be the result of a change in the physical environment (Figure 1A), including exposure to light (Strickland et al., 2008); irradiation; change in pH (thus, protonation states; Martí et al., 2009); and concentration. It can also stem from noncovalent binding or a change in a covalent linkage, such as that taking place during a mutational event or when adding or removing a posttranslational modification (PTM) (Nussinov et al., 2012)." This is clearly the classical definition. In another passage, the authors do include mutations under their list of activity-changing perturbations that may be considered allostery. However, this idea is never again used in the paper. When "allosteric mutations" are subsequently discussed, this term refers to mutations that affect allostery in in the traditional sense – sequence changes that alter or abolish the coupling between a reversible stimulus such as ligand binding at one site and the activity of the protein at the active site.

In their response letter, the authors claim that deep mutagenesis is a standard and broadly used approach to analyze allostery. But virtually all of the cited papers all use mutagenesis in allosteric proteins (defined in the traditional sense) to identify sites that may be involved in mediating the functional coupling between an effector site and active sites, or they use mutagenesis in non-allosteric proteins to identify sites that when mutated confer allosteric coupling between effector stimulus and activity. In each case, allostery in the traditional sense -- the coupling between effector and activity -- is measured before and after mutation. These papers do not claim that a DMS that measures only effects of mutations on activity (rather than the effect on the coupling between a reversible stimulus and activity) can provide evidence of allostery. The only clear exceptions are the papers from the current authors' own group.

I conclude that rebuttal letter is incorrect in asserting that it is well-established or useful to define allostery to include mutations that affect a protein's function but occur away from the active-site surface. The data reported here do not directly illuminate allostery or its evolution, and their claims are therefore confusing and misleading. The data do elucidate a truly interesting and important topic: the effects of mutation on ligand-binding, changes in these effects among related proteins, and the structural correlates of these effects and changes. The paper should report its results in those terms. I acknowledge that analysis of the effects of mutations on ligand binding can provide indirect insight into how allostery might be

engineered into a nonallosteric protein or how allostery might evolve, just as was the case in the Reynolds et al. 2011 paper, because portions of a protein that when mutated affect activity might also confer true allostery, if those regions where also mutated in a way that causes the region to bind an effector or otherwise receive a reversible stimulus. I encourage the authors to speculate, interpret and discuss the meaning of their findings in relation to these topics.

We understand. We have extensively edited the revised manuscript to avoid any ambiguity and to communicate throughout that we are comparing the energetic effects of mutations. We have, in a few places, retained the use of the term 'allosteric mutation' to refer to mutations outside of binding sites that change the binding energy. This term avoids ambiguity and it is used across multiple fields, including to describe cancer driver mutations, drug resistance mutations, and mutations outside of the active sites of enzymes selected during directed evolution and protein engineering campaigns. Please see the accompanying version of the manuscript with all changes marked.

Reviewer #3 (Remarks to the Author):

In my view, the response to reviewers is adequate. In some places I still feel the data presentation and figures could be further simplified to get across key concepts, but this is really a matter of taste not of science - the data are very interesting. It would have helped significantly if the authors had referenced page and line numbers in the response or used highlighting to indicate the changes, it took considerable time to identify where the changes were made in some cases.

Reviewer #3 (Remarks on code availability):

I did not revisit the code during the revisions.

Thank you. Apologies for not including a version of the revised text with the changes marked!