

Trade-off between flux efficiency and metabolic control shapes enzyme allocation

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28th Nov 2025

RE: Manuscript MSB-2025-13440, A universal law of thermodynamics-kinetics coupling shapes enzyme allocation and glycolytic efficiency

Dear Dr Dai,

Thank you for submitting your work to Molecular Systems Biology. We have now heard back from three reviewers who agreed to evaluate your manuscript. As you will see below, the reviewers raise substantial concerns about your work, which unfortunately preclude its publication in Molecular Systems Biology.

All three reviewers raised significant concerns about the study, including the insufficient and unclear mathematical exposition, the claim that the proposed law is universal, as well as issues with the overall validation approach. Additionally, Reviewer #2 had concerns regarding several fundamental aspects of the study. Notably, both Reviewers #2 and #3 rated the study's suitability for publication as "low."

Under these circumstances and considering the overall low level of support provided by the reviewers, I see no other choice than to return the manuscript with the message that we cannot offer to publish it. In any case, thank you for the opportunity to examine your work. I hope that the points raised in the reports will prove useful to you and that you will not be discouraged from submitting future work to Molecular Systems Biology.

Sincerely,
Jingyi

Jingyi Hou, PhD
Senior Editor
Molecular Systems Biology

Reviewer #1:

Given limited proteome resources, growth optimization requires minimization of enzyme cost. Achieving optimal enzyme efficiency is constrained by the thermodynamics-kinetics coupling, which states a relationship between rates, enzyme constants, metabolite concentrations, and thermodynamics in each reaction. Implementing this fundamental principle to pathway proteome allocation is algorithmically challenging. Here, XX et al described an analytical treatment of this question by establishing a metric called catalytic-abundance quotient (CAQ) of connected reactions and relating it to equilibrium constant at optimal enzyme allocation. Specifically, they show proteome allocation is conserved across human cells and tissues (Fig. 1). They then establish CAQ for zeroth-order, first-order, and M-M kinetics of linear pathways, and validated that CAQs closely correlate with equilibrium constants (K) of a simulated pathway. They further tested the agreement between CAQ and K using quantitative proteomics, metabolite concentrations, free energy, and kinetic constants, and concluded that glycolytic enzyme allocation follows the cost minimization principle across different organisms. Given the varying agreement between CAQ and K, they further looked for factors that might promote an optimal enzyme allocation, and found that oncogenic signaling is associated with optimal allocation.

Previous works in the field has shown that proteome cost is optimized within the constraint of thermodynamic-kinetic coupling. Overall I find that this work has a few conceptual advances. (1) defining a metric to quantify how close to optimal enzyme allocation using easily accessible data. (2) testing the proteome allocation principle across different organisms beyond microbes. (3) establishing physiological relevance of the metric to oncogenic signaling. The conclusion is interesting that oncogene drives an optimal glycolytic enzyme expression. However, some clarification is needed to fully show the utility of CAQ and validate the claims.

1. The correlation between CAQ and K is validated with glycolysis, which is well conserved across organisms. In order to claim this as a universal principle, validation using other pathways (such as TCA and PPP) is recommended. Fig 2 shows similar attempt, but it is not clear whether the parameters are drawn from distribution that mimic biologically relevant pathways.
2. Experimentally, glycolysis of mammalian cells are controlled at four steps with low relative enzyme abundance (PMID: 29960885). This is not in agreement with eq. 38, where flux control is proportional to enzyme abundance. Related to that, where does glucose transporter fall in the prediction? Glucose transporter is the classical targets of energy signaling.

3. Maybe I misinterpreted this but the correlation between CAQ and K is counterintuitive. It predicts that the more enzyme resource should be allocated to the reaction with a greater K, which means a greater thermodynamic driving force.

Points to clarify:

1. For glycolysis to operate at optimum, what are the predicted intermediate concentrations? Another evolutionary pressure for glycolysis is to avoid accumulation of toxic intermediates. Are the metabolite concentrations within a physiologically relevant range?
2. Method to generate the simulated proteome allocation under M-M kinetics is not clear. What is the formulation of objective and constraint for fmincon? How is it done while the least square optimization is also needed?

Reviewer #2:

Sun et al show a mathematical framework to connect enzyme levels and equilibrium constants in glycolysis. They derive an equation in which the equilibrium constant of a reaction equals the ratio of k_{cat} and enzyme abundances. They call this relationship a law of thermodynamics-kinetics coupling. Then, a mix of kinetic parameters, measured metabolite concentrations, and proteome data is used to show correlations between equilibrium constants and the catalytic abundance term. I have the following concerns:

1. The thermodynamics-kinetics coupling is not a feature of natural metabolic networks. It is a mathematical construct of the objective function (maximize flux per enzyme) under very hard constraints. Nothing in enzyme biology or thermodynamics requires that equilibrium constants must match ratios of k_{cat} and enzyme levels (and some of the reactions in fig 4 do strongly deviate from this law).
2. The validation relies on correlation rather than demonstrating for each reaction pair that equilibrium constants is equal to catalytic-abundance quotients. Because the "law" is an equality, a positive correlation does not show that any single reaction pair satisfies the equality within the expected error and the (large) uncertainties associated with parameters of enzymatic reactions. Without testing deviations, eg by error propagation or residual analyses, or confidence intervals for each reaction it remains unclear that glycolysis follow the "law" as implied in the text. In fact, those reaction pairs with reaction that operate far from equilibrium like PFK or HK deviate strongest from the law, which is expected.
3. The flux efficiency is probably not the only objective of a cell and the authors need to demonstrate that this objective has meaning. For example recent studies suggest that enzymes are actively kept away from saturation which would be against (even the opposite) of optimizing flux efficiency. Further this objective will probably vary between pathways. Thus the "law" is not universal.
4. The manuscript fails to explain why the results have biological relevance. If the model improves our understanding the authors need to make testable predictions that go beyond the presented correlations.
5. The work must be better embedded with current literature. The idea that thermodynamics constrains metabolic flux and that the thermodynamic driving force of reactions influences the enzyme levels is not new. Especially for glycolysis, metabolic control analysis has shown in detail the relationship of thermodynamics and enzyme kinetics and the authors need to make clear how their work relates to these findings. Especially this study (not from this reviewer): DOI 10.1038/nchembio.2077.

Reviewer #3:

The authors develop a theoretical framework for quantifying how efficiently cells allocate enzyme abundance to support metabolic flux. By combining thermodynamic rules with enzyme kinetics, they derive a necessary and sufficient condition for optimal enzyme allocation in a linear metabolic pathway: the equilibrium constant (K) of a reaction equals the catalytic-abundance quotient (CAQ), which is defined based on enzyme abundance and catalytic efficiency. They then introduce the Efficiency Optimality Index (EOI), the correlation between K and CAQ across tandem reaction pairs, and use EOI as a metric to evaluate whether glycolysis in various organisms and cellular contexts follows their predicted optimality pattern.

Understanding whether cells naturally optimize the investment in their metabolic machinery is important both for interpreting metabolic phenotypes and for improving quantitative models of metabolism. The authors provide preliminary applications to cancer data, showing that EOI correlates with expression of oncogenic pathways, and they suggest that the framework may be extendable beyond glycolysis. In practice, broader application will require accurate thermodynamic parameters, kinetic data, enzyme abundances, and metabolite concentrations. Such datasets are still limited for most pathways.

Overall, the manuscript addresses a conceptually important question and presents an intriguing theoretical result. However, as written, it is difficult to assess or verify the mathematical framework, and several aspects of the methodology are unclear. The manuscript is not yet publication-ready. Below are comments aimed at improving clarity.

Major Comment

The primary contribution of this manuscript is its theoretical framework, so mathematical clarity is essential. Unfortunately, the derivations in the Supplementary Information are difficult to follow and, in several places, incomplete. Key transitions are not justified. For example, the origin of Eq. 36 is not explained and many steps are skipped without accompanying text. The result is that central parts of the proof cannot be independently verified.

To illustrate the problem, I have listed a few specific examples in the minor comments 1-3 below; however, these are only representative. At present, the supplement contains 86 equations, but a transparent and reproducible derivation will likely require substantially more intermediate steps and explanatory text. Because the mathematical development is unclear, I was unable to follow several portions of the argument to completion.

I look forward to reviewing a revised version with a more complete and readable mathematical exposition.

Minor Comments

1) Supplementary (mathematical derivations): As noted above, there are several issues with clarity and completeness. A few examples of different types of problems include:

-Eqn 30 and 32: J as a function of $(a, e, K, \text{etc.})$ is not designated consistently and the transition in notation is not mentioned before Eqn 32.

-Eqn 36: not clear at all

-Eqn 40, 41: key substitutions are omitted, leaving significant algebraic work to the reader. Please provide a smooth, explicit derivation.

2) Similar issues in Methods:

-The first (long) Michaelis-Menten rate law is presented without any citation or pointer to the Supplement, where the derivation could be found. A reference or explicit derivation is needed; the former is acceptable but the latter is preferable.

-The sentence beginning with "Therefore" that follows this equation is confusing. The second equation is a basic mass-balance statement and does not require the first equation for justification.

3) Page 3: The statement "its apparent rate constant k , which is proportional to the enzyme abundance $[E]$," needs justification. Please explain or cite where this proportionality comes from; currently it appears as an abrupt jump.

4) Why Spearman rather than Pearson correlation for EOI? Pearson correlation would quantify proportionality directly, whereas Spearman uses only rank information. A rationale is needed.

5) Legends are generally too brief to guide interpretation. Examples include:

-Fig. 4b: the meaning of the horizontal bars is not explained.

-Fig. 5c: the meaning of the color tone is unclear.

6) Page 6, permutation tests:

The text discusses permutations of enzyme abundances and of enzyme abundances + catalytic efficiencies, but does not comment on permuting catalytic efficiencies alone. This makes interpretation difficult, since the stated logic distinguishes strategy 1 and strategy 2.

7) The sentence "For each parameter set and a given set of enzyme concentrations, the steady-state concentrations of intermediate metabolites were computed" does not specify what enzyme concentrations were used. This is essential information and should be clearly stated.

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Reviewer #1:

Given limited proteome resources, growth optimization requires minimization of enzyme cost. Achieving optimal enzyme efficiency is constrained by the thermodynamics-kinetics coupling, which states a relationship between rates, enzyme constants, metabolite concentrations, and thermodynamics in each reaction. Implementing this fundamental principle to pathway proteome allocation is algorithmically challenging. Here, XX et al described an analytical treatment of this question by establishing a metric called catalytic-abundance quotient (CAQ) of connected reactions and relating it to equilibrium constant at optimal enzyme allocation. Specifically, they show proteome allocation is conserved across human cells and tissues (Fig. 1). They then establish CAQ for zeroth-order, first-order, and M-M kinetics of linear pathways, and validated that CAQs closely correlate with equilibrium constants (K) of a simulated pathway. They further tested the agreement between CAQ and K using quantitative proteomics, metabolite concentrations, free energy, and kinetic constants, and concluded that glycolytic enzyme allocation follows the cost minimization principle across different organisms. Given the varying agreement between CAQ and K, they further looked for factors that might promote an optimal enzyme allocation, and found that oncogenic signaling is associated with optimal allocation.

Previous works in the field has shown that proteome cost is optimized within the constraint of thermodynamic-kinetic coupling. Overall I find that this work has a few conceptual advances. (1) defining a metric to quantify how close to optimal enzyme allocation using easily accessible data. (2) testing the proteome allocation principle across different organisms beyond microbes. (3) establishing physiological relevance of the metric to oncogenic signaling. The conclusion is interesting that oncogene drives an optimal glycolytic enzyme expression. However, some clarification is needed to fully show the utility of CAQ and validate the claims.

We thank the reviewer for the positive comments on the conceptual advances of our work. We will perform additional analyses and add further discussions and clarifications in the revised manuscript to address this reviewer's concerns.

1. The correlation between CAQ and K is validated with glycolysis, which is well conserved across organisms. In order to claim this as a universal principle, validation using other pathways (such as TCA and PPP) is recommended. Fig 2 shows similar attempt, but it is not clear whether the parameters are drawn from distribution that mimic biologically relevant pathways.

We thank the reviewer for the constructive advice.

We focused on glycolysis to validate the theoretical prediction of coupling between K and CAQ for two reasons: first, unlike TCA and PPP, glycolysis has a near-linear structure, which aligns with the theoretical framework we used to mathematically derive the K-CAQ relationship; second, glycolysis is the center of carbohydrate metabolism, where quantitative metabolomics data for absolute concentration of metabolites is available across several species.

We completely agree with the reviewer that successful validation of the K-CAQ relationship using pathways other than glycolysis is crucial for the relationship to become a universal law. Although TCA and PPP might not be the ideal pathway to validate the equality relationship ($K=CAQ$) for linear pathways due to their non-linear structure, the available resources of metabolite concentration data for these pathways allows validation of the branching point relationship ($K_1 < \frac{a_1[E_1]^2}{\sum_{i=2}^n a_i[E_i]^2}$ for diverging pathways and $\sum_{i=1}^{n-1} \frac{a_i[E_i]^2}{K_i} < a_n[E_n]^2$ for converging pathways). Therefore, we will perform such validation in the revised manuscript. We will also repeat the simulation in Figure 2 with more biologically relevant range of parameters to further solidify our theoretical predictions.

2. Experimentally, glycolysis of mammalian cells are controlled at four steps with low relative enzyme abundance (PMID: 29960885). This is not in agreement with eq. 38, where flux control is proportional to enzyme abundance. Related to that, where does glucose transporter fall in the prediction? Glucose transporter is the classical targets of energy signaling.

We thank the reviewer for raising these insightful questions.

Regarding the first question, the disagreement between the experimental measurements in the reference cited by this reviewer (PMID: 29960885) and theoretical results of flux control being proportional to enzyme abundance at optimal flux efficiency could arise for several reasons.

The first reason lies in the discrepancy between the mathematical definition of flux control coefficients (FCCs) and the experimental method used to quantify flux control. FCCs are defined as the derivative of pathway flux with respect to enzyme activity, requiring that changes in enzyme activity are small, while in that study, perturbation to glycolytic enzymes was done by protein over-expression, which can increase enzyme abundance by many folds. If the unperturbed cell is in the state of optimal flux efficiency (where FCCs are proportional to enzyme

abundance), such optimality will be disrupted upon the substantial over-expression of enzymes. Therefore, the experimentally measured flux control coefficients no longer reflect modes of flux control under optimal flux efficiency.

We have previously shown using enzyme inhibitors that modulate enzyme activity in a dose-dependent manner which better represents the definition of FCCs (Liberti et al, Cell Metab 2017, PMID: 28918937) that the abundant glycolytic enzyme GAPDH exerts strong control over glycolytic flux, especially in cells with enhanced glycolysis. This experimental observation supports two of our results: (1) abundant enzymes exert stronger control over pathway flux under optimal flux efficiency; (2) optimization of glycolytic efficiency is associated with oncogenic signaling, which is known to enhance the Warburg effect. We will clarify this point in the revised manuscript.

Another possible reason for the discrepancy between theoretical and experimental patterns of flux control is the existence of alternative objectives that shape enzyme profiles. We have recently developed an extended theoretical framework (not included in the original submission) about the trade-off between flux efficiency and flux control, showing that pathways maximizing flux efficiency are in general harder to control through a few reaction steps. We will include this extended analysis in the revised manuscript.

Regarding the question about glucose transporters, that step has been included in the analysis of E.coli because uptake and phosphorylation of glucose is completed in one reaction step catalyzed by the phosphotransferase system (PTS). We will add the glucose transporter step to the analysis of human and yeast in the revised manuscript.

3. Maybe I misinterpreted this but the correlation between CAQ and K is counterintuitive. It predicts that the more enzyme resource should be allocated to the reaction with a greater K, which means a greater thermodynamic driving force.

We thank the reviewer for the constructive feedback and the opportunity for us to explain the reason behind the counterintuitive result.

First, although the correlation between CAQ and K seems counterintuitive, successful validation of the relationship using cross-species omics data confirms its correctness. Second, greater equilibrium constant K for a reaction means that when the abundance of enzyme catalyzing that reaction is not very high, the net flux of this reaction is unlikely limited by

thermodynamics as it is far from thermodynamic equilibrium. Therefore, increasing enzyme amount for this reaction will be effective in enhancing the metabolic flux. This pattern reflecting a strategy of “kinetics-thermodynamics matching”: allocate more enzymes to reaction steps with strong thermodynamic driving force to boost flux as much as possible. On the other hand, inefficient pathways often suffer from mismatch between reaction kinetics and thermodynamics: too much enzyme resource is allocated to reactions where thermodynamics, not enzyme abundance, is the limiting factor. We will add discussion about this point to the revised manuscript to offer an intuitive explanation of the law of thermodynamics-kinetics coupling to the readers.

Points to clarify:

1. For glycolysis to operate at optimum, what are the predicted intermediate concentrations? Another evolutionary pressure for glycolysis is to avoid accumulation of toxic intermediates. Are the metabolite concentrations within a physiologically relevant range?

We thank the reviewer for the important comment. In this study, we used experimental metabolite concentrations retrieved from several independent studies to estimate the catalytic efficiency of metabolic enzymes (Methods in the manuscript), which were then used to validate the coupling between K and CAQ. By applying this approach, physiological constraints are implicitly embedded in the experimental metabolite concentrations. We will add relevant clarification of this point to the revised manuscript. We will also compute steady state concentrations of the intermediates directly from the kinetic parameters, equilibrium constants, and enzyme abundances using a mathematical model of glycolysis to confirm that the metabolite concentrations are within a physiological relevant range.

2. Method to generate the simulated proteome allocation under M-M kinetics is not clear. What is the formulation of objective and constraint for fmincon? How is it done while the least square optimization is also needed?

We thank the reviewer for raising this concern and apologize for the confusion. We will add detailed description of the objective and constraint for fmincon, as well as the rationale of using least square optimization to solve the steady state to the revised manuscript.

Reviewer #2:

Sun et al show a mathematical framework to connect enzyme levels and equilibrium constants in glycolysis. They derive an equation in which the

equilibrium constant of a reaction equals the ratio of k_{cat} and enzyme abundances. They call this relationship a law of thermodynamics-kinetics coupling. Then, a mix of kinetic parameters, measured metabolite concentrations, and proteome data is used to show correlations between equilibrium constants and the catalytic abundance term. I have the following concerns:

We thank the reviewer for the time and effort invested in evaluating our manuscript, yet several of the concerns raised by this reviewer appear to stem from misunderstandings of the theoretical framework. We appreciate the opportunity to clarify these points, as the questions raised, together with the reference suggested by the reviewer, actually help us clarify key aspects of our theory and highlight additional evidence supporting it. We will clarify these points explicitly in the revised manuscript.

1. The thermodynamics-kinetics coupling is not a feature of natural metabolic networks. It is a mathematical construct of the objective function (maximize flux per enzyme) under very hard constraints. Nothing in enzyme biology or thermodynamics requires that equilibrium constants must match ratios of k_{cat} and enzyme levels (and some of the reactions in fig 4 do strongly deviate from this law).

We thank the reviewer for raising this concern and are happy to take the opportunity to clarify the misunderstanding regarding the nature of the thermodynamics-kinetics coupling.

First, although reaction thermodynamics and enzyme biology per se do not require enzymes catalyzing connected reactions to follow the coupling between K and CAQ , evolutionary forces could favor efficient metabolic pathways by affecting fitness of cells. As K is fixed by metabolite structures and intracellular conditions, cells cannot adjust K to match the CAQ ; instead, they adjust CAQ , by evolution of kinetic parameters and adjustment of enzyme abundances, to better match the pre-existing thermodynamic landscape of the pathway. This interpretation transforms the relationship from an abstract mathematical structure into an evolutionary plausible principle. We will explain this explicitly in the revised manuscript to avoid misunderstanding that the theory requires unrealistic biochemical assumptions.

Second, our opinion is that all theories must: (1) originate from biologically relevant hypothesis; (2) be validated by real-world experimental data to demonstrate their correctness. We have the confidence to say that our work matches both requirements. First, the

fundamental hypothesis that metabolism and many other cellular processes optimize the efficiency of resource utilization has been tested in many systems including overflow metabolism in microbes, which is closely related to glycolysis. Second, the relationship between K and CAQ has been validated by cross-species data spanning several omics layers.

Finally, we thank the reviewer for raising the question about deviation of some reaction steps from the K-CAQ coupling. We emphasize that our theoretical framework does not assume that all pathways, or even glycolysis under all conditions, must operate at maximal flux efficiency and adhere to the K-CAQ equality. This is the reason why we developed the EOI metric, which quantifies the degree to which a pathway approaches the maximal efficiency. We will also extend our theoretical framework to model the trade-off between flux efficiency and metabolic control, which we will explain in detail in the response to the next point raised by this reviewer.

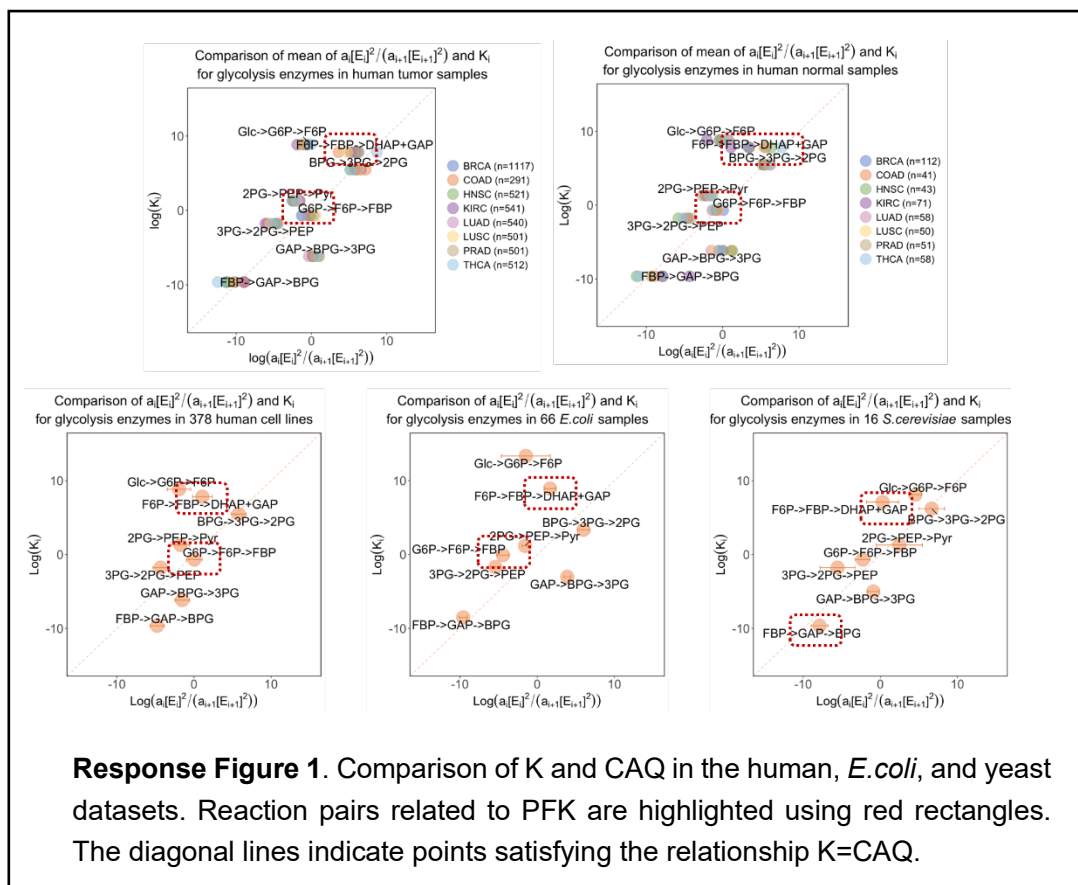
2. The validation relies on correlation rather than demonstrating for each reaction pair that equilibrium constants is equal to catalytic-abundance quotients. Because the "law" is an equality, a positive correlation does not show that any single reaction pair satisfies the equality within the expected error and the (large) uncertainties associated with parameters of enzymatic reactions. Without testing deviations, eg by error propagation or residual analyses, or confidence intervals for each reaction it remains unclear that glycolysis follow the "law" as implied in the text. In fact, those reaction pairs with reaction that operate far from equilibrium like PFK or HK deviate strongest from the law, which is expected.

We thank the reviewer for raising this concern.

First, we agree with the reviewer that validation beyond a simple positive correlation is important. However, our analyses in Figure 4 already goes beyond that. The scatter plots (Figure 4b-d,f,g) clearly show that nearly all data points lie close to the theoretical diagonal of $K=CAQ$ (Response Figure 1) rather than forming an arbitrary correlated pattern. This directly tests and strongly supports the equality relationship predicted by our theory. Given the noisy nature of biological measurements, one should not expect that all data points would fall on the theoretical line. Nevertheless, the fact that the data points cluster tightly around the theoretical diagonal and are evenly distributed on both sides provide evidence substantially stronger than the correlation alone. We will clarify this in the revised manuscript.

Second, although we agree that quantifying deviations is useful and plan

to do so in the revision, this reviewer’s statement that “reactions far from equilibrium deviate strongest from the law” is not supported by the data. The reviewer specifically cited PFK as an example. PFK is involved in two reaction pairs, (1) G6P->F6P->FBP; (2) F6P->FBP->DHAP+GAP. The two reaction pairs actually lie close to the theoretical $K=CAQ$ line in almost all datasets (Response Figure 1). These PFK-related reaction pairs in fact represent some of the points most consistent with the predicted equality, which directly contradicts the reviewer’s claim.



Finally, the deviation of the HK catalyzed reaction (Glc->G6P) from the theoretical $K=CAQ$ line in some datasets can be explained by the trade-off between maximizing flux efficiency and facilitating flux control. We have developed an extended theoretical framework capturing such trade-off: when a pathway operates at maximal efficiency, there should not be any “rate-limiting step” that constrains the pathway flux, hence the pathway will be hard to control through a few reaction steps. Reducing abundances of enzymes catalyzing reactions with strong thermodynamic driving force, such as HK, will result in a larger negative Gibbs free energy $\Delta_r G$ and stronger flux control of that reaction. Therefore, the fact that CAQ of the HK-GPI reaction pair is lower than the K of the HK reaction reflects this strategy of reducing HK abundance for stronger control over glycolytic flux through HK and is consistent with the fact that HK serves

as a key control point of glycolysis. We will add this extended analysis to the revised manuscript.

3. The flux efficiency is probably not the only objective of a cell and the authors need to demonstrate that this objective has meaning. For example recent studies suggest that enzymes are actively kept away from saturation which would be against (even the opposite) of optimizing flux efficiency. Further this objective will probably vary between pathways. Thus the "law" is not universal.

We appreciate the reviewer's concern, but as clarified above, we emphasize again that our theory does not assume that efficiency optimization is the sole objective of metabolic pathways and have developed the EOI metric to quantify its heterogeneity across conditions.

Regarding the reviewer's point about enzymes being kept away from saturation, while isolated exceptions may exist, multiple studies, including the very reference the reviewer cites in the 5th comment, have reported that most enzymes operate near substrate saturation to optimize their efficiency. Beyond the observation at single-enzyme level, numerous theoretical and experimental works have shown that optimization of efficiency explains global features of cellular systems, including the pattern of proteome partition, amino acid usage, codon systems, and many others (see the Introduction of our manuscript for details). These findings together support the biological relevance of efficiency optimization within broad biological processes including metabolism.

4. The manuscript fails to explain why the results have biological relevance. If the model improves our understanding the authors need to make testable predictions that go beyond the presented correlations.

We appreciate this reviewer's feedback, but we respectfully disagree with the assessment regarding the biological relevance of our work. The reviewer's concern appears to stem from assuming that our validation solely relies on correlation. As clarified above, evidence supporting our theory ("K=CAQ") spans several independent dimensions unfortunately not considered in the reviewer's criticism: (1) data points distribute close to the theoretical K=CAQ diagonal, which demonstrates equality beyond correlation; (2) kinetic parameters of glycolytic enzymes are pre-optimized for maintaining the K-CAQ relationship (Figure S7), which supports evolutionary adaptation favoring optimal efficiency; (3) the EOI metric reveals that the degree of efficiency optimality is biologically meaningful as it increases with enhanced oncogenic signaling, which is known to drive glycolytic reprogramming.

Regarding the reviewer's request for "testable predictions", each line of evidence directly corresponds to a testable prediction and successfully validates it. Comparison between K and CAQ tests the prediction of "K=CAQ"; the shuffling experiment tests whether enzyme parameters are pre-optimized; the EOI analyses test whether efficiency optimality varies with known biological drivers. Together, these multi-layered tests strongly support the biological relevance of the framework.

5. The work must be better embedded with current literature. The idea that thermodynamics constrains metabolic flux and that the thermodynamic driving force of reactions influences the enzyme levels is not new. Especially for glycolysis, metabolic control analysis has shown in detail the relationship of thermodynamics and enzyme kinetics and the authors need to make clear how their work relates to these findings. Especially this study (not from this reviewer): DOI 10.1038/nchembio.2077.

We thank the reviewer for suggesting this reference, which we already cite in the manuscript. Notably, the key finding of this study is fully aligned with the principle of efficiency optimization underlying our theoretical framework. Briefly, by quantifying absolute metabolite concentrations, the study shows, as its title ("Metabolite concentrations, fluxes and free energy imply efficient enzyme usage") suggests, that most metabolic enzymes operate near substrate saturation. This contradicts with the reviewer's claim and directly supports the basic hypothesis of our work.

Furthermore, regarding the criticism of novelty, two key points distinguish our work from previous literature. First, existing thermodynamic analyses of metabolic pathways (e.g. Noor et al, PLOS Comput Biol 2014, PMID: 24586134; Henry et al, Biophys J 2007, PMID: 17172310; Dai et al, JBC 2018, PMID: 30361440) constrain the feasible space of fluxes, but do not identify a unique optimal solution. Our work derives a closed-form equality that specifies the optimal enzyme allocation maximizing flux efficiency. Second, our theoretical prediction is experimentally testable because K and CAQ can be computed from experimental measurements and directly compared. Earlier theories (e.g. Noor et al, Interface Focus 2024, PMID: 38344407) do not yield testable quantitative relationships due to the inability to handle complex rate laws and isozymes. Therefore, our framework advances beyond prior work both mathematically and experimentally. We will explicitly clarify this in the revised manuscript.

Reviewer #3:

The authors develop a theoretical framework for quantifying how efficiently cells allocate enzyme abundance to support metabolic flux. By combining thermodynamic rules with enzyme kinetics, they derive a necessary and sufficient condition for optimal enzyme allocation in a linear metabolic pathway: the equilibrium constant (K) of a reaction equals the catalytic-abundance quotient (CAQ), which is defined based on enzyme abundance and catalytic efficiency. They then introduce the Efficiency Optimality Index (EOI), the correlation between K and CAQ across tandem reaction pairs, and use EOI as a metric to evaluate whether glycolysis in various organisms and cellular contexts follows their predicted optimality pattern.

Understanding whether cells naturally optimize the investment in their metabolic machinery is important both for interpreting metabolic phenotypes and for improving quantitative models of metabolism. The authors provide preliminary applications to cancer data, showing that EOI correlates with expression of oncogenic pathways, and they suggest that the framework may be extendable beyond glycolysis. In practice, broader application will require accurate thermodynamic parameters, kinetic data, enzyme abundances, and metabolite concentrations. Such datasets are still limited for most pathways.

Overall, the manuscript addresses a conceptually important question and presents an intriguing theoretical result. However, as written, it is difficult to assess or verify the mathematical framework, and several aspects of the methodology are unclear. The manuscript is not yet publication-ready. Below are comments aimed at improving clarity.

Major Comment

The primary contribution of this manuscript is its theoretical framework, so mathematical clarity is essential. Unfortunately, the derivations in the Supplementary Information are difficult to follow and, in several places, incomplete. Key transitions are not justified. For example, the origin of Eq. 36 is not explained and many steps are skipped without accompanying text. The result is that central parts of the proof cannot be independently verified.

To illustrate the problem, I have listed a few specific examples in the minor comments 1-3 below; however, these are only representative. At present, the supplement contains 86 equations, but a transparent and reproducible derivation will likely require substantially more intermediate steps and explanatory text. Because the mathematical development is unclear, I was unable to follow several portions of the argument to completion.

I look forward to reviewing a revised version with a more complete and readable mathematical exposition.

We thank the reviewer for the encouraging feedback regarding the conceptual importance of our work and the feasibility of revision. We will rewrite the mathematical derivations to ensure clarity and readability in the revised manuscript.

Minor Comments

1) Supplementary (mathematical derivations): As noted above, there are several issues with clarity and completeness. A few examples of different types of problems include:

-Eqn 30 and 32: J as a function of $(a, e, K, \text{etc.})$ is not designated consistently and the transition in notation is not mentioned before Eqn 32.

-Eqn 36: not clear at all

-Eqn 40, 41: key substitutions are omitted, leaving significant algebraic work to the reader. Please provide a smooth, explicit derivation.

We thank the reviewer for pointing out these and apologize for the confusion. We will rewrite the mathematical derivations to ensure that all necessary steps are included.

2) Similar issues in Methods:

-The first (long) Michaelis-Menten rate law is presented without any citation or pointer to the Supplement, where the derivation could be found. A reference or explicit derivation is needed; the former is acceptable but the latter is preferable.

We thank the reviewer for the helpful advice. We will add both reference and derivation of the reversible Michaelis-Menten rate law to the revised manuscript.

-The sentence beginning with "Therefore" that follows this equation is confusing. The second equation is a basic mass-balance statement and does not require the first equation for justification.

We thank the reviewer for raising this concern and apologize for the confusion. We agree that the mass-balance assumption does not require the reversible Michaelis-Menten kinetics as a prerequisite. We will move the Michaelis-Menten equation to a new section detailing its derivation to avoid confusion.

3) Page 3: The statement "its apparent rate constant k , which is proportional to the enzyme abundance $[E]$," needs justification. Please explain or cite where

this proportionality comes from; currently it appears as an abrupt jump.

We thank the reviewer for pointing this out. The reason of considering apparent rate constant k as proportional to enzyme abundance is that the reaction rate is linear to apparent rate constant k (by its definition) and proportional to enzyme abundance regardless of the rate law. We will clarify this in the revised manuscript.

4) Why Spearman rather than Pearson correlation for EOI? Pearson correlation would quantify proportionality directly, whereas Spearman uses only rank information. A rationale is needed.

We thank the reviewer for raising this concern. We have shown in the original manuscript that using Pearson correlation instead of Spearman correlation as the EOI metric did not affect the high EOI values across datasets (Fig. S5). Yet we agree with the reviewer that Pearson correlation is a better metric quantifying proportionality and will use it instead of Spearman correlation for the EOI metric in the revised manuscript.

5) Legends are generally too brief to guide interpretation. Examples include:

-Fig. 4b: the meaning of the horizontal bars is not explained.

-Fig. 5c: the meaning of the color tone is unclear.

We thank the reviewer for pointing this out. We will revise the legends to include more information in the revised manuscript.

6) Page 6, permutation tests:

The text discusses permutations of enzyme abundances and of enzyme abundances + catalytic efficiencies, but does not comment on permuting catalytic efficiencies alone. This makes interpretation difficult, since the stated logic distinguishes strategy 1 and strategy 2.

We thank the reviewer for raising this concern. We will add discussion about permutation of catalytic efficiency to the revised manuscript.

7) The sentence "For each parameter set and a given set of enzyme concentrations, the steady-state concentrations of intermediate metabolites were computed" does not specify what enzyme concentrations were used. This is essential information and should be clearly stated.

We thank the reviewer for raising this concern. In the simulation, enzyme abundances were generated from a log-normal distribution. We will add description of the technical details in the revised manuscript.

8th Dec 2025

Manuscript Number: MSB-2025-13440R-Q

Title: A universal law of thermodynamics-kinetics coupling shapes enzyme allocation and glycolytic efficiency

Author: Ziwei Dai

Kai Sun

Weiyan Zheng

Wenchao Fan

Chuyun Ding

Dan Huang

Dear Dr Dai,

Thank you for your message requesting that we reconsider our decision regarding your manuscript MSB-2025-13440. I have now had the opportunity to review your preliminary point-by-point response. Based on your appeal letter and the outline you provided, we are willing to give you the opportunity to submit a revised manuscript, provided that the issues raised by the reviewers can be convincingly addressed.

Regarding the comments from Reviewer #2 on quantitative evidence, Reviewer#1 provided additional feedback during our pre-decision cross-commenting:

"Correlation can be misleading as it only assumes linearity but not $y=x$. Metric such as root mean squared error on a log scale is more suitable. Moreover, the authors should discuss why particular reaction pairs deviate strongly from the prediction, eg. Glc->G6P-> F6P. Lastly, given the spread of correlation from the simulation, is EOI a robust reporter? More than showing correlation between gene expression and EOI (which is itself a correlation metric), it would be necessary to show CAQ and what reaction pairs are driving EOI." We ask that you carefully consider the suggestions from Reviewer #1 when addressing this point.

All other issues raised by the reviewers need to be satisfactorily addressed. As you may already know, our editorial policy allows in principle a single round of major revision so it is essential to provide responses to the reviewers' comments that are as complete as possible. Please feel free to contact me in case you would like to discuss in further detail any of the issues raised by the reviewers.

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

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

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

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

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

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

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

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

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

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

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

Data availability

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

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

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

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

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

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

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

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

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

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

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

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

-Regarding data quantification:

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

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

Please also include scale bars in all microscopy images.

- Please provide a "standfirst text" summarizing the study in one or two sentences (approximately 250 characters, including space), three to four "bullet points" highlighting the main findings and a "synopsis image" (550px width and 400-600 px height, PNG format) to highlight the paper on our homepage.

Please refer to published papers for examples.

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

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

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees.

A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at

this stage that the eventual outcome will be favorable.

I look forward to receiving the revised manuscript soon.

Kind regards,
Jingyi

Jingyi Hou, PhD
Senior Editor
Molecular Systems Biology

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (8th Mar 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

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

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General response regarding extension of the theoretical framework

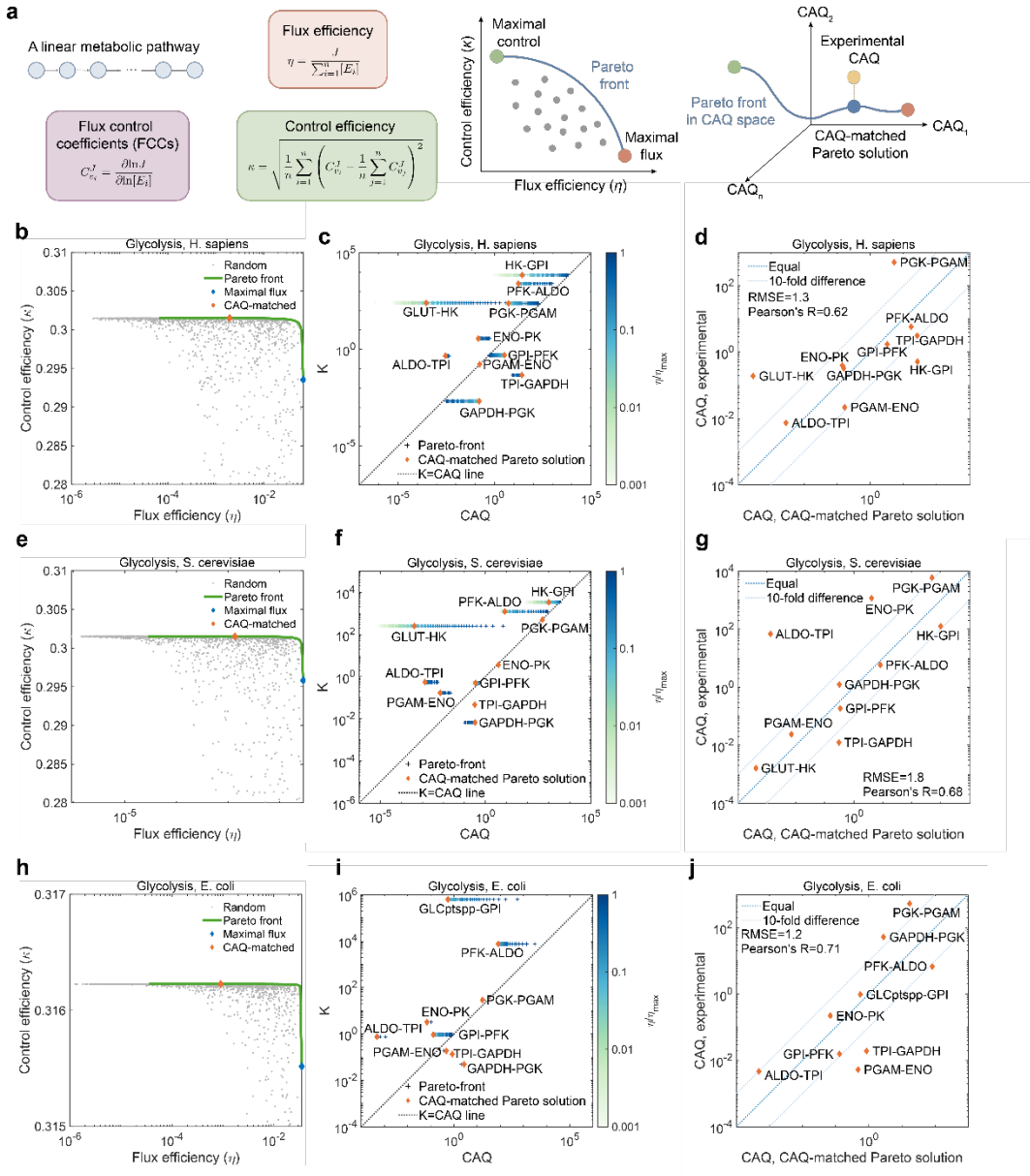
We greatly appreciate the constructive comments and suggestions from all three reviewers. After carefully considering these comments, we realized that although our original theory of flux efficiency maximization captures an important optimality principle governing metabolic enzyme allocation, it is not sufficient to fully explain physiological enzyme allocation in real metabolic pathways. These comments helped us recognize the need to extend our original theoretical framework by incorporating an additional objective of pathway optimization: the organization of flux control.

Several comments from the reviewers converge on this central point. Reviewer #1 noted a potential discrepancy between the theoretical control profile at the maximal flux state (i.e. $C_{v_i}^J \propto [E_i]$) and experimentally measured control coefficients. Reviewer #1 also suggested further validation with metabolite concentrations and additional pathways such as TCA cycle. These analyses revealed limitations of the pure flux maximization framework, but the discrepancies can be addressed by a Pareto optimality model that balances flux efficiency and flux control.

Reviewer #2 pointed out that flux maximization is unlikely to be the only objective of metabolism. Reviewer #2 also noted that the largest deviations between physiological K and CAQ occur at key control points of glycolysis, such as glucose transport and phosphorylation. At these steps, we repeatedly observed $CAQ < K$, indicating that the abundance of upstream enzymes is lower than expected value under maximal flux efficiency. From the perspective of metabolic control analysis, reducing abundance of an enzyme can increase its flux control coefficient (FCC). Therefore, the reduced abundance of enzymes such as GLUT and HK may enhance their control over pathway flux, allowing efficient regulation through a limited number of key reaction steps.

Motivated by these observations, we developed an extended theoretical framework that considers the trade-off between flux efficiency and control efficiency. We quantify control efficiency κ as the standard deviation of FCCs across all reaction steps in a pathway, with larger κ indicating more concentrated flux control at key steps. We then applied an ε -constraint algorithm to compute the Pareto front between flux efficiency and control efficiency. This analysis shows that at the maximal flux solution, flux control is relatively evenly distributed among reaction steps. To achieve more concentrated control at a few key steps, the enzyme allocation profile must deviate from the maximal flux solution ([Response](#)

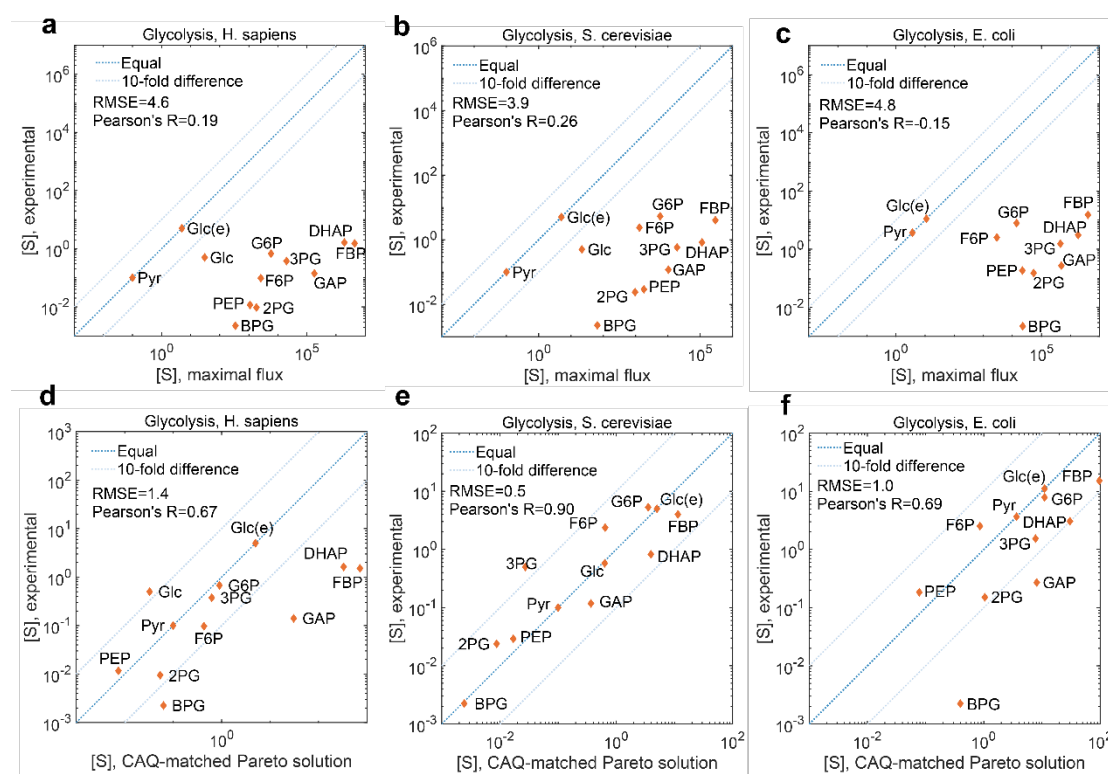
Figure 1).



Response Figure 1. Trade-off between flux efficiency and control efficiency. (a) Definition of flux efficiency and control efficiency, and the Pareto front balancing the needs to maximize these two objectives. (b) Scatter plot showing flux efficiency and control efficiency in randomly sampled enzyme allocation profiles (grey dots), Pareto front balancing these two (green curve), and the CAQ-matched Pareto solution (orange diamond) for human glycolysis. (c) Relationship between K and CAQ on the Pareto front for glycolysis in *H.sapiens*. Orange diamonds indicate the CAQ-matched Pareto solution. (d) Scatter plot comparing CAQ values between the CAQ-matched Pareto solution and experimental values computed from proteomics data for glycolysis in *H.sapiens*. (e) Same as in (b), but for *S. cerevisiae*. (f) Same as in (c), but for *S.cerevisiae*. (g) Same

as in (d) but for *S.cerevisiae*. (h) Same as in (b), but for *E.coli*. (i) Same as in (c), but for *E.coli*. (j) Same as in (d), but for *E.coli*.

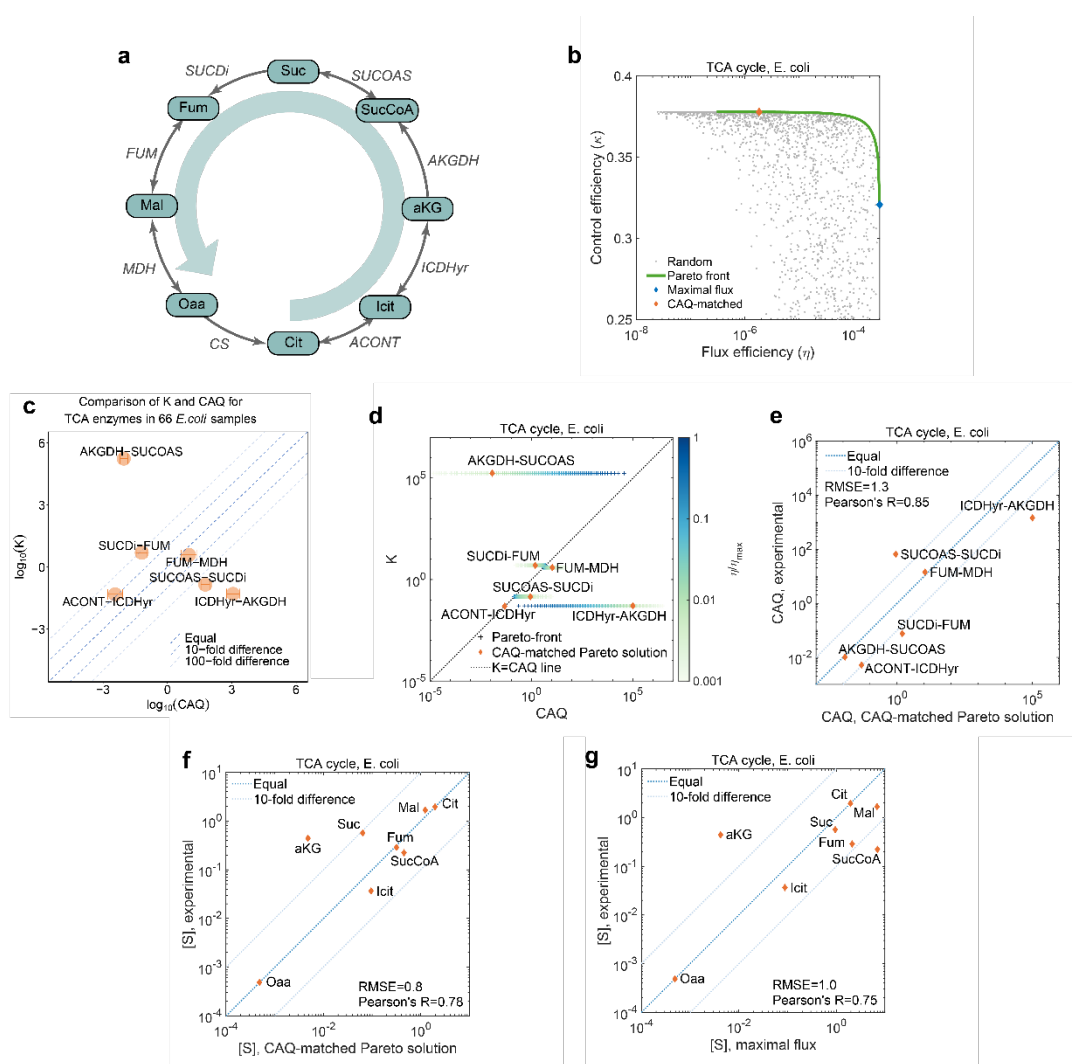
We further identified a representative solution on the Pareto front, named **CAQ-matched Pareto solution**, by selecting the solution with the smallest Euclidean distance to the physiological CAQ profile computed from the proteomics data ([Response Figure 1a](#)). Importantly, this procedure does not guarantee a close match between the CAQ-matched Pareto solution and physiological CAQ profile, unless the physiological CAQ profile lies near the Pareto front. Therefore, a close agreement between the CAQ-matched Pareto solution and physiological CAQ profiles indicates that real enzyme allocation lies near the theoretically predicted Pareto front and supports the theoretical framework of flux-control tradeoff.



Response Figure 2. CAQ-matched Pareto solution correctly predicts metabolite concentration. (a-c). Scatter plots comparing metabolite concentrations predicted by the maximal-flux solution to experimental measurements for glycolysis in *H.sapiens* (a), *S.cerevisiae* (b), and *E.coli* (c). (d-f). Scatter plots comparing metabolite concentrations predicted by the CAQ-matched Pareto solution to experimental measurements for glycolysis in *H.sapiens* (d), *S.cerevisiae* (e), and *E.coli* (f).

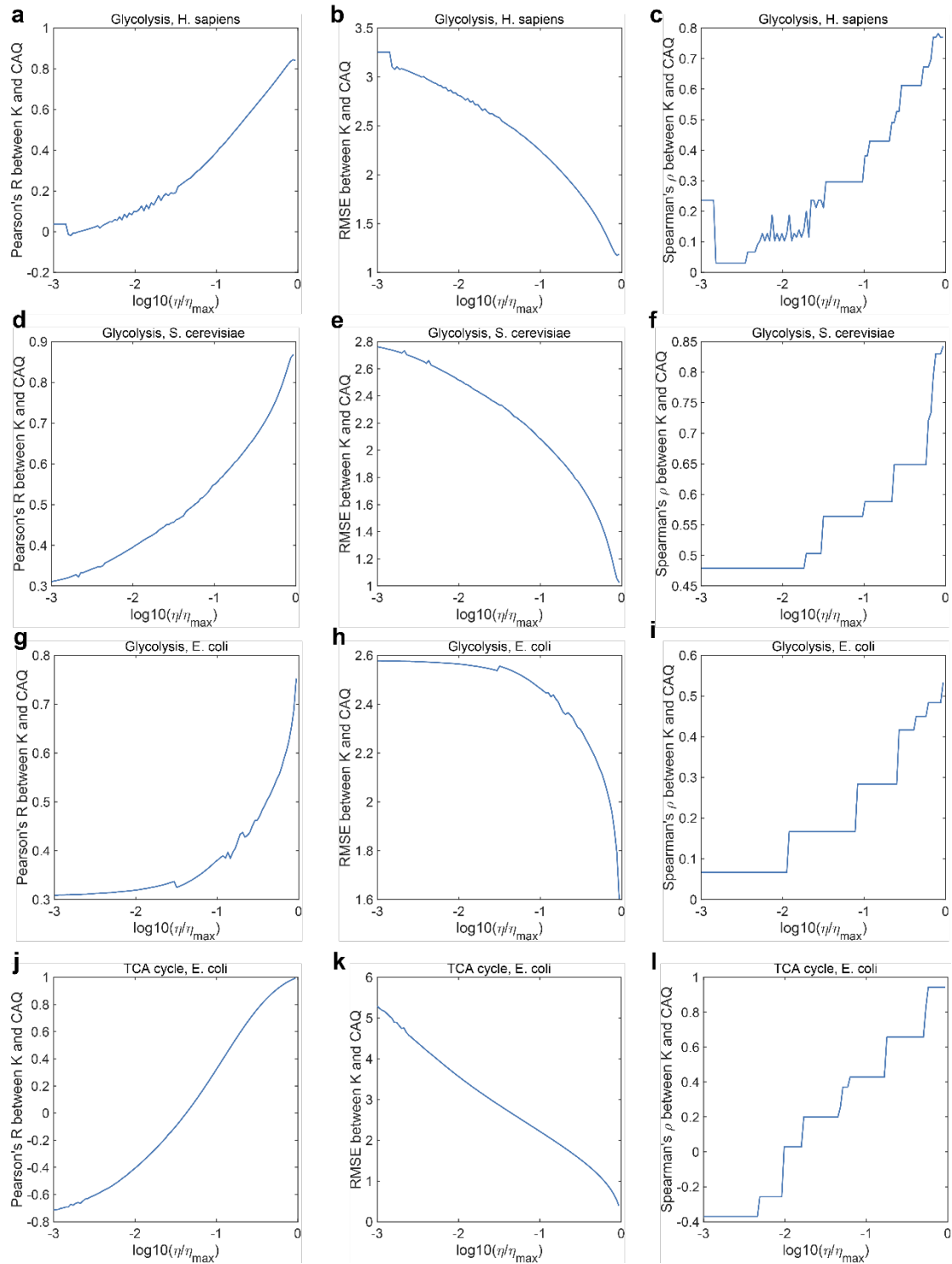
We provide several lines of evidence supporting this theoretical framework: (1) Experimental CAQ profiles lie near the theoretically predicted Pareto front, as the closest point on this independently

computed Pareto front, termed the CAQ-matched Pareto solution, closely agrees with the omics-derived physiological CAQ values across all species and pathways ([Response Figure 1d,g,j](#)); (2) unlike the solution maximizing flux efficiency, the CAQ-matched Pareto solution accurately predicts metabolite concentrations in glycolysis across human, yeast, and *E.coli* ([Response Figure 2](#)); (3) The CAQ-matched Pareto solution correctly predicts enzyme allocation and metabolite concentration for *E.coli* TCA cycle ([Response Figure 3](#)); (4) Along the Pareto front, both RMSE and Pearson's correlation coefficient between K and CAQ quantitatively capture the tendency of a solution toward either flux maximization or concentrated flux control ([Response Figure 4](#)).



Response Figure 3. Flux-control tradeoff for TCA cycle in *E. coli*. (a) Scheme of the TCA cycle in *E. coli*. (b) Scatter plot showing flux efficiency and control efficiency in randomly sampled enzyme allocation profiles (grey dots), Pareto front balancing these two (green curve), and the CAQ-matched Pareto solution (orange diamond). (c) Scatter plot comparing K and CAQ for reaction pairs. (d) Relationship between K and CAQ on the Pareto front balancing flux efficiency and control efficiency. (e) Scatter

plot comparing CAQ values between the CAQ-matched Pareto solution and experimental values computed from proteomics data. (f) Scatter plot comparing metabolite concentrations predicted by the CAQ-matched Pareto solution to experimental measurements. (g) Scatter plot comparing metabolite concentrations predicted by the maximal-flux solution to experimental measurements.



Response Figure 4. Change of Pearson's R, RMSE, and Spearman's rho

between K and CAQ with respect to flux efficiency alongside the Pareto front. K and CAQ values were log10-transformed before computing the correlation coefficients and RMSE values. (a-c) Pearson's R (a), RMSE (b), and Spearman's rho for glycolysis in H.sapiens. (d-f) Same as in (a-c) but for S.cerevisiae. (g-i) Same as in (a-c) but for E.coli. (j-l) Same as in (a-c) but for TCA cycle in E.coli.

Together, these results show that the extended theoretical framework resolves the discrepancy between cross-species multi-omics data and the original theory of flux maximization, and provides a new perspective on how metabolic pathways balance flux efficiency with regulation of fluxes. We have included the extended theoretical framework in the revised manuscript.

Reviewer #1:

Given limited proteome resources, growth optimization requires minimization of enzyme cost. Achieving optimal enzyme efficiency is constrained by the thermodynamics-kinetics coupling, which states a relationship between rates, enzyme constants, metabolite concentrations, and thermodynamics in each reaction. Implementing this fundamental principle to pathway proteome allocation is algorithmically challenging. Here, XX et al described an analytical treatment of this question by establishing a metric called catalytic-abundance quotient (CAQ) of connected reactions and relating it to equilibrium constant at optimal enzyme allocation. Specifically, they show proteome allocation is conserved across human cells and tissues (Fig. 1). They then establish CAQ for zeroth-order, first-order, and M-M kinetics of linear pathways, and validated that CAQs closely correlate with equilibrium constants (K) of a simulated pathway. They further tested the agreement between CAQ and K using quantitative proteomics, metabolite concentrations, free energy, and kinetic constants, and concluded that glycolytic enzyme allocation follows the cost minimization principle across different organisms. Given the varying agreement between CAQ and K, they further looked for factors that might promote an optimal enzyme allocation, and found that oncogenic signaling is associated with optimal allocation.

Previous works in the field has shown that proteome cost is optimized within the constraint of thermodynamic-kinetic coupling. Overall I find that this work has a few conceptual advances. (1) defining a metric to quantify how close to optimal enzyme allocation using easily accessible data. (2) testing the proteome allocation principle across different organisms beyond microbes. (3) establishing physiological relevance of the metric to oncogenic signaling. The conclusion is interesting that oncogene drives an optimal glycolytic enzyme expression. However, some clarification is needed to fully show the utility of

CAQ and validate the claims.

We thank the reviewer for the positive comments on the conceptual advances of our work. We have performed additional analyses and added further discussions and clarifications in the revised manuscript to address this reviewer's concerns.

1. The correlation between CAQ and K is validated with glycolysis, which is well conserved across organisms. In order to claim this as a universal principle, validation using other pathways (such as TCA and PPP) is recommended. Fig 2 shows similar attempt, but it is not clear whether the parameters are drawn from distribution that mimic biologically relevant pathways.

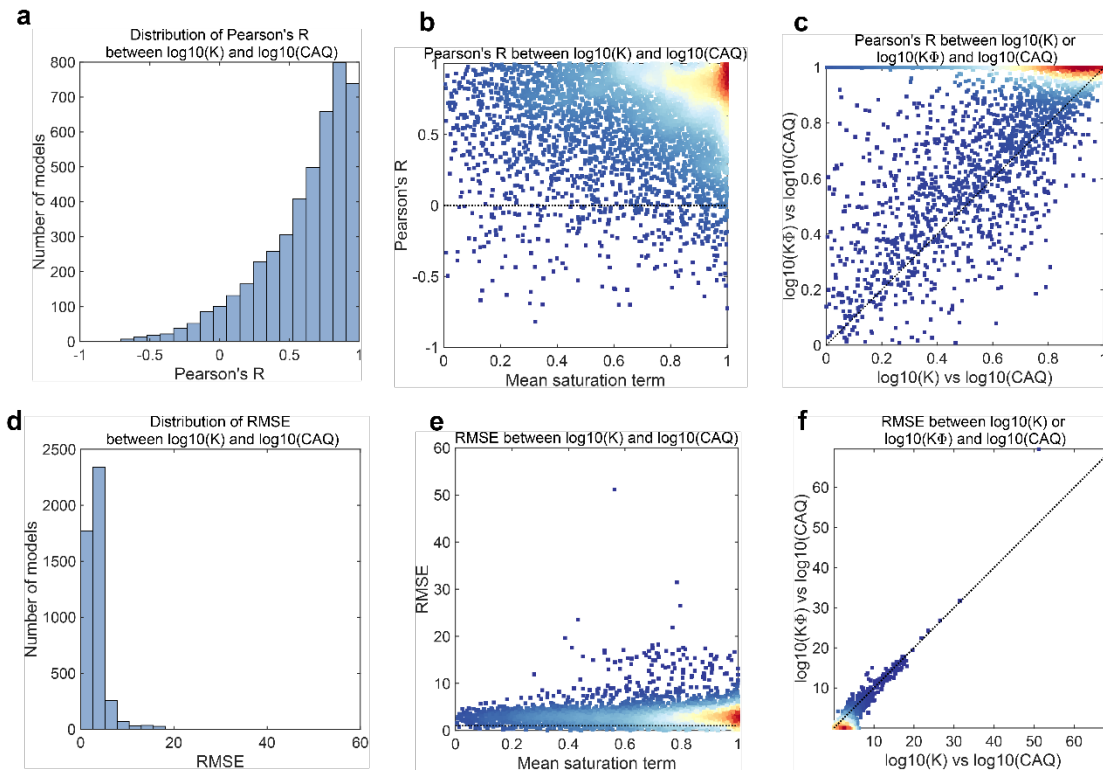
We thank the reviewer for the constructive advice.

We focused on glycolysis to validate the theoretical prediction of coupling between K and CAQ for two reasons: first, glycolysis has a near-linear structure, which aligns with the theoretical framework we used to mathematically derive the K-CAQ relationship; second, glycolysis is the center of carbohydrate metabolism, where quantitative metabolomics data for absolute concentration of metabolites is available across several species.

We completely agree with the reviewer that successful validation of the K-CAQ relationship using pathways other than glycolysis is crucial for the relationship to become a universal law. To enhance the generalization ability of our theory, we extended the analysis to TCA cycle in E.coli (Response Figure 3). We found that although the enzyme abundances in E.coli do not fall on the K=CAQ line (Response Figure 3c), it still locates close to the Pareto front for the flux-control tradeoff, as the CAQ-matched Pareto solution showed good agreement with experimental enzyme allocation profiles (Response Figure 3d,e) and metabolite concentrations (Response Figure 3f) in E.coli, therefore supporting our extended theoretical framework of flux-control tradeoff. We have added the analysis to the revised manuscript. Furthermore, because deviations from the K=CAQ line were observed in several reactions of glycolysis and TCA cycle, we have changed its name from “a universal law of thermodynamics-kinetics coupling” to “K-CAQ relationship at maximal flux efficiency” to avoid overinterpretation of the theoretical result.

For the simulation in Figure 2 of the original manuscript, we have repeated it with biologically relevant parameters to further solidify our theoretical predictions. Briefly, in the revised manuscript, the simulation was done with kinetic parameters directly sampled from enzymatic

parameters predicted by the DLKcat and Kroll's algorithm from the sequences of human enzymes, instead of the artificial log-normal distribution. The simulation showed that using realistic enzymatic kinetic parameters does not change the conclusion: (1) K and CAQ are still linearly coupled for most parameter sets under Michaelis-Menten kinetics; (2) the saturation term has little effect on the coupling between K and CAQ; (3) Compared to K , $K\Phi$ has stronger correlation (Pearson's R near 1) with and smaller deviation (RMSE near 0) from CAQ, supporting the relationship $K\Phi=CAQ$ for Michaelis-Menten kinetics. We have replaced Figure 2c and Figure 2d with this new analysis in the revised manuscript.



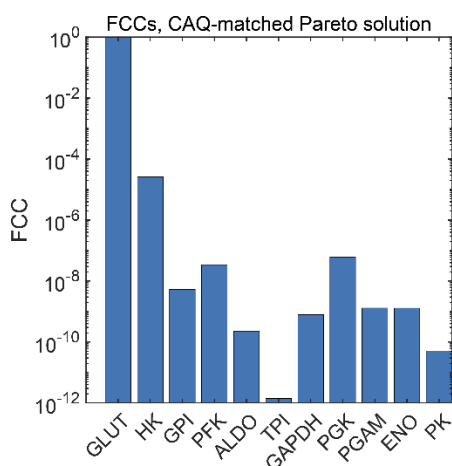
Response Figure 5. Relationship between K and CAQ for enzyme allocation profiles maximizing flux efficiency of linear metabolic pathway under Michaelis-Menten kinetics. (a) Distribution of Pearson's R between $\log_{10}(K)$ and $\log_{10}(CAQ)$. (b) Scatter plot showing relationship between enzyme saturation term and Pearson's R between $\log_{10}(K)$ and $\log_{10}(CAQ)$. (c) Scatter plot comparing Pearson's R between $\log_{10}(K)$ or $\log_{10}(K\Phi)$ and $\log_{10}(CAQ)$. (d) Same as in (a) but for RMSE. (e) Same as in (b) but for RMSE. (f) Same as in (c) but for RMSE.

2. Experimentally, glycolysis of mammalian cells are controlled at four steps with low relative enzyme abundance (PMID: 29960885). This is not in agreement with eq. 38, where flux control is proportional to enzyme abundance. Related to that, where does glucose transporter fall in the prediction? Glucose transporter is the classical targets of energy signaling.

We thank the reviewer for raising these insightful questions.

Regarding the first question, the disagreement between the experimental measurements in the reference cited by this reviewer (PMID: 29960885) and theoretical results of flux control being proportional to enzyme abundance at optimal flux efficiency could arise for several reasons.

The first reason lies in the discrepancy between the mathematical definition of flux control coefficients (FCCs) and the experimental method used to quantify flux control. FCCs are defined as the derivative of pathway flux with respect to enzyme activity, requiring that changes in enzyme activity are small, while in that study, perturbation to glycolytic enzymes was done by protein over-expression, which can increase enzyme abundance by many folds. If the unperturbed cell is in the state of optimal flux efficiency (where FCCs are proportional to enzyme abundance), such optimality will be disrupted upon the substantial over-expression of enzymes. Therefore, the experimentally measured flux control coefficients could deviate from modes of flux control under maximal flux efficiency.



Response Figure 6. Flux control coefficients (FCCs) for the CAQ-matched Pareto solution of human glycolysis.

Another possible reason for the discrepancy between theoretical and experimental patterns of flux control is the existence of alternative objectives that shape enzyme profiles. As we have noted in “General response regarding extension of the theoretical framework”, the tradeoff between flux and control can better explain the enzyme allocation and metabolite concentration profiles compared to the single objective of flux maximization. To enhance control of pathway flux through a few key steps, abundances of enzymes with larger FCC in the maximal flux state need to be reduced to further enhance their FCC, facilitating flux control through these reaction steps. This explains why the well-known control points of glycolysis, such as GLUT and HK, deviate the most from the K=CAQ line. To further support this theory, we computed flux control coefficients for the CAQ-matched Pareto solution in human glycolysis and found that the

experimentally determined key control steps (GLUT, HK, PFK) also exhibited highest FCCs ([Response Figure 6](#)). We have added this analysis to the revised manuscript.

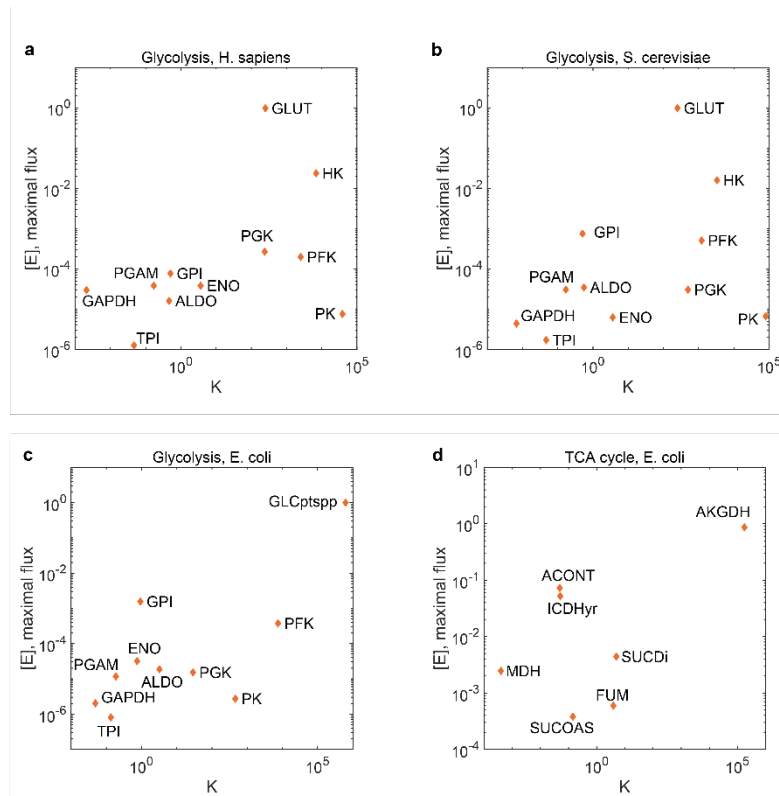
Regarding the question about glucose transporters, that step has been included in the analysis of *E.coli* because uptake and phosphorylation of glucose is completed in one reaction step catalyzed by the phosphotransferase system (PTS). We have also added the glucose transport step to the glycolysis pathway in human and yeast, and shown that inclusion of this reaction step does not impair the consistency between the theory of flux-control tradeoff and experimental data.

3. Maybe I misinterpreted this but the correlation between CAQ and K is counterintuitive. It predicts that the more enzyme resource should be allocated to the reaction with a greater K , which means a greater thermodynamic driving force.

We thank the reviewer for the constructive feedback and the opportunity for us to explain the reason behind the counterintuitive result.

First, greater equilibrium constant K for a reaction means that when the abundance of enzyme catalyzing that reaction is not very high, the net flux of this reaction is unlikely limited by thermodynamics as it is far from thermodynamic equilibrium. Therefore, increasing enzyme amount for this reaction will be effective in enhancing the metabolic flux. This pattern reflecting a strategy of “kinetics-thermodynamics matching”: allocate more enzymes to reaction steps with strong thermodynamic driving force to boost flux as much as possible. On the other hand, inefficient pathways often suffer from mismatch between reaction kinetics and thermodynamics: too much enzyme resource is allocated to reactions where thermodynamics, not enzyme abundance, is the limiting factor.

Second, as CAQ depends on enzyme abundance and kinetic parameters of two reactions in the tandem reaction pair, equilibrium constants alone do not determine the amount of enzyme resource allocated into a reaction step. As an example, we compared the theoretically predicted enzyme abundances and equilibrium constants of reactions in glycolysis and TCA cycle, and showed that reactions with greater K do not necessarily have higher enzyme abundance although a positive correlation between these two exists ([Response Figure 7](#)). We have added this figure and related discussion in the revised manuscript.



Response Figure 7. Relationship between K and enzyme allocation under maximal flux in glycolysis (a-c) and TCA cycle (d). (a). Glycolysis in *H.sapiens*. (b) Glycolysis in *S.cerevisiae*. (c) Glycolysis in *E.coli*. (d) TCA cycle in *E.coli*.

We have added discussion about this point to the revised manuscript to offer an intuitive explanation of the law of thermodynamics-kinetics coupling to the readers.

Points to clarify:

1. For glycolysis to operate at optimum, what are the predicted intermediate concentrations? Another evolutionary pressure for glycolysis is to avoid accumulation of toxic intermediates. Are the metabolite concentrations within a physiologically relevant range?

We thank the reviewer for the important comment. To assess whether the model-predicted intermediate concentrations are close to experimental measurements, we computed metabolite concentrations in the maximal flux solution and CAQ-matched Pareto solution (see “General response regarding extension of the theoretical framework” for details), and compared these model-predicted metabolite concentrations to experimental values available in literature. The comparison shows that while the maximal flux solutions tend to overestimate concentrations of intermediates, the CAQ-matched Pareto optimal solution accurately reproduce experimental metabolite concentrations across the three

species for glycolysis ([Response Figure 2](#)) and in *E.coli* for TCA cycle ([Response Figure 3](#)). We have added these results in the revised manuscript.

2. Method to generate the simulated proteome allocation under M-M kinetics is not clear. What is the formulation of objective and constraint for fmincon? How is it done while the least square optimization is also needed?

We thank the reviewer for raising this concern and apologize for the confusion.

To simulate the enzyme allocation profile maximizing pathway flux under Michaelis-Menten kinetics, we solved a constrained optimization problem with fmincon. The objective function is the steady-state flux through the pathway, which is computed by solving the steady-state equation:

$$v_i(k_{cat}, K_S, K_P, K, E, S) = v_{i+1}(k_{cat}, K_S, K_P, K, E, S)$$

In which $v_i(k_{cat}, K_S, K_P, K, E, S)$ is the rate of the i -th reaction under Michaelis-Menten kinetics. Because directly solving the nonlinear steady state equations using the MATLAB function “fsolve” has numerical issues, we converted the problem into a least square optimization problem:

$$\min_S \|v_i(k_{cat}, K_S, K_P, K, E, S) - v_{i+1}(k_{cat}, K_S, K_P, K, E, S)\|_2$$

This problem was solved using the MATLAB built-in function “lsqnonlin” to compute steady state metabolite concentrations and the corresponding pathway flux J .

The constraints for fmincon include lower and upper bounds for each enzyme abundance $[E_i]$, and an upper bound for the total abundance of enzymes:

$$\begin{cases} 0 \leq [E_i] \leq 1 \\ \sum_{i=1}^n [E_i] \leq 1 \end{cases}$$

We have added detailed descriptions of this procedure in the revised manuscript.

Additional comment from Reviewer #1: Correlation can be misleading as it only assumes linearity but not $y=x$. Metric such as root mean squared error on a log scale is more suitable. Moreover, the authors should discuss why particular reaction pairs deviate strongly from the prediction, eg. Glc->G6P-> F6P. Lastly, given the spread of correlation from the simulation, is EOI a robust reporter? More than showing correlation between gene expression and EOI (which is itself a correlation metric), it would be necessary to show CAQ and what

reaction pairs are driving EOI.

We thank the reviewer for the helpful suggestions. Following these suggestions, we made the following revisions: (1) We redefined the Efficiency Optimality Index (EOI) from Spearman's correlation to the root mean squared error (RMSE) between $\log_{10}(K)$ and $\log_{10}(\text{CAQ})$, and clarified why RMSE is more appropriate than correlation-based metrics for evaluating the theoretical prediction $K = \text{CAQ}$; (2) We developed a framework of flux-control trade-off, which explains why specific reaction pairs have large deviations from the $K=\text{CAQ}$ relationship and contribute most strongly to the EOI (see "General response regarding extension of the theoretical framework" for details); (3) To test the robustness of the EOI metric, we assessed the behavior of several candidate EOI metrics (RMSE, Pearson's R , Spearman's ρ) alongside the Pareto front and showed that RMSE, the currently used metric for EOI, exhibited monotonic and continuous change when the pathway switches from maximizing flux efficiency to concentrating flux control (Response Figure 4). These revisions explain the discrepancy between the theoretical flux efficiency maximum and experimental enzyme allocation profiles and offer a robust EOI metric quantifying the position of a metabolic pathway in the Pareto front balancing flux efficiency and concentrated flux control.

Reviewer #2:

Sun et al show a mathematical framework to connect enzyme levels and equilibrium constants in glycolysis. They derive an equation in which the equilibrium constant of a reaction equals the ratio of k_{cat} and enzyme abundances. They call this relationship a law of thermodynamics-kinetics coupling. Then, a mix of kinetic parameters, measured metabolite concentrations, and proteome data is used to show correlations between equilibrium constants and the catalytic abundance term. I have the following concerns:

We thank the reviewer for the time and effort invested in evaluating our manuscript, and apologize that several aspects of the theory of flux efficiency optimization and K -CAQ relationship were insufficiently clarified in the original manuscript. We appreciate the opportunity to further clarify these points, as the comments raised by the reviewer helped us better explain key aspects of the framework and motivated the development of the extended theory describing the trade-off between flux efficiency and flux control.

1. The thermodynamics-kinetics coupling is not a feature of natural metabolic networks. It is a mathematical construct of the objective function (maximize flux per enzyme) an under very hard constraints. Nothing in enzyme biology or

thermodynamics requires that equilibrium constants must match ratios of k_{cat} and enzyme levels (and some of the reactions in fig 4 do strongly deviate from this law).

We thank the reviewer for raising this important point and appreciate the opportunity to clarify the nature of the thermodynamics-kinetics coupling.

First, we agree with the reviewer that the K-CAQ relationship is not a biochemical necessity imposed directly by thermodynamics or enzyme biology. Rather, it emerges as the optimal solution with the objective of maximizing flux efficiency under enzyme resource constraints. In this sense, the relationship should be interpreted as an optimality principle shaped by evolutionary selection for efficient operation of metabolism, instead of a mandatory physical law that all pathways must satisfy under all conditions.

Moreover, alignment of in vivo metabolic pathways to this relationship is feasible: as equilibrium constants are pre-determined by reaction thermodynamics and intracellular physicochemical conditions, cells cannot directly tune K values, but they can adjust CAQ values through evolution of enzyme kinetic parameters and regulation of enzyme abundances. Therefore, cells can adapt enzyme allocation profiles to the thermodynamic landscape of the pathway. We have clarified this interpretation explicitly in the revised manuscript. Furthermore, to avoid misunderstanding of this relationship, we no longer refer to it as “law of thermodynamics-kinetics coupling”. Instead, we have changed it to “K-CAQ relationship at maximal flux efficiency” in the revised manuscript.

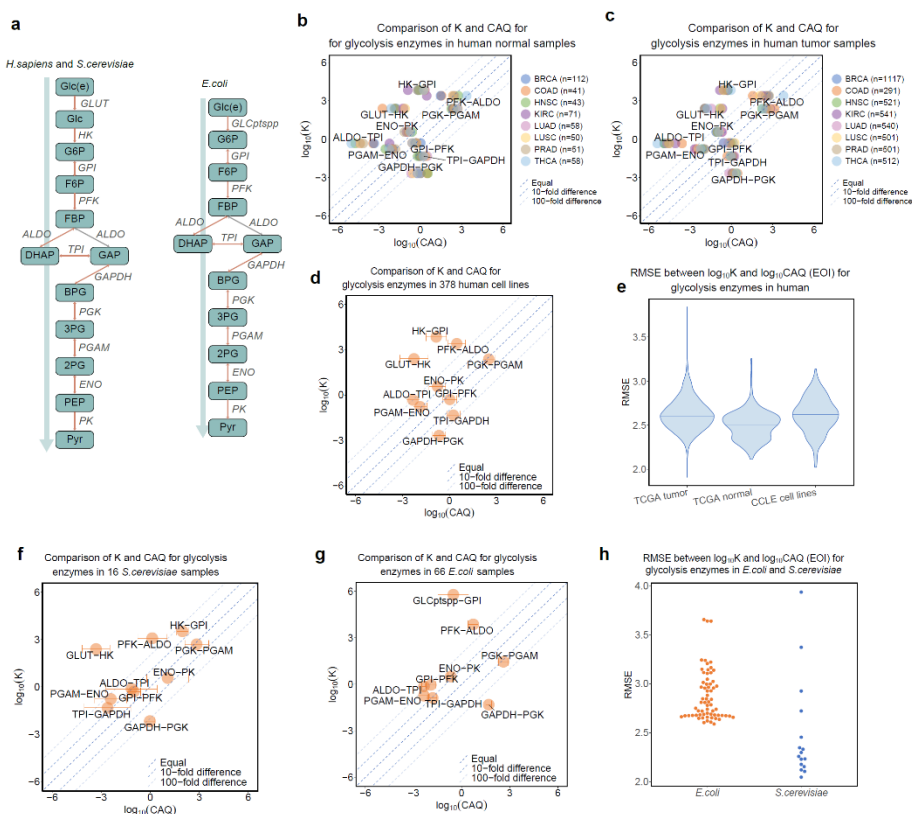
Second, we thank the reviewer for raising the question about deviation of some reaction steps from the $K=CAQ$ relationship. Our theoretical framework does not assume that all pathways, or even glycolysis under all conditions, must operate at maximal flux efficiency and adhere to the K-CAQ equality. To quantify the extent to which real metabolic pathways approach this optimality state, we developed the EOI metric. Furthermore, motivated by the systematic deviations observed at key control points of glycolysis, we extended our theoretical framework to model the trade-off between flux efficiency and metabolic control, as discussed in detail in “General response regarding extension of the theoretical framework”.

2. The validation relies on correlation rather than demonstrating for each reaction pair that equilibrium constants is equal to catalytic-abundance quotients. Because the "law" is an equality, a positive correlation does not show that any single reaction pair satisfies the equality within the expected error and the (large) uncertainties associated with parameters of enzymatic reactions.

Without testing deviations, eg by error propagation or residual analyses, or confidence intervals for each reaction it remains unclear that glycolysis follow the "law" as implied in the text. In fact, those reaction pairs with reaction that operate far from equilibrium like PFK or HK deviate strongest from the law, which is expected.

We thank the reviewer for raising this concern.

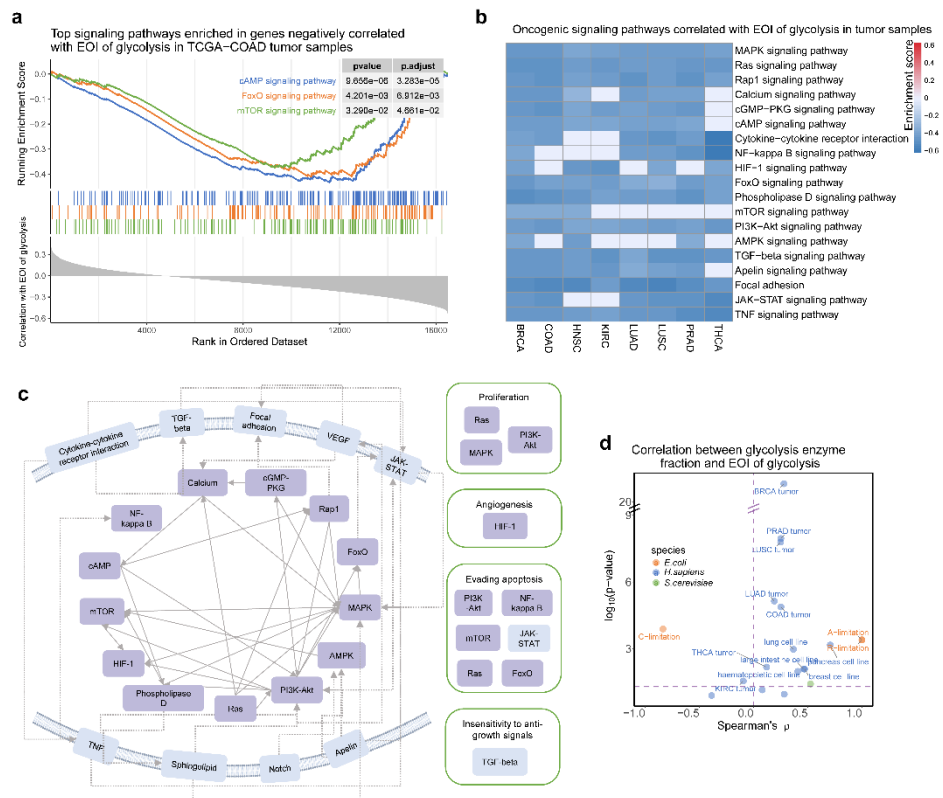
First, we agree with the reviewer that validation beyond a simple positive correlation is important. However, our analyses already go beyond that. The scatter plots in the original manuscript (Figure 4b-d,f,g) show that nearly all data points lie around the theoretical diagonal of $K=CAQ$ rather than forming an arbitrary correlated pattern. This provides quantitative support for the predicted $K=CAQ$ relationship beyond correlation alone. Given the noisy nature of biological measurements, perfect alignment with the theoretical line for every individual reaction is biologically and experimentally impractical. Nevertheless, the fact that the data points cluster around the theoretical diagonal and are distributed on both sides provide information beyond correlation alone. To better illustrate the alignment between real data and the $K=CAQ$ relationship, we have added lines indicating 10-fold and 100-fold deviations from the $K=CAQ$ relationship to the K -CAQ plots and shown that the majority of reaction pairs except for the GLUT, HK, and PFK-related ones fell within this range (Response Figure 8).



Response Figure 8. Quantification of deviation from the $K=CAQ$ relationship. (a) The glycolysis pathway in *H.sapiens*, *S.cerevisiae*, and *E.coli*. (b) Comparison of K and CAQ for glycolysis in human normal samples. (c) Comparison of K and CAQ for glycolysis in human tumor samples. (d) Comparison of K and CAQ for glycolysis in human cell lines. (e) Distribution of RMSE between K and CAQ for glycolysis in *H.sapiens*. (f) Comparison of K and CAQ for glycolysis in *S.cerevisiae*. (g) Comparison of K and CAQ for glycolysis in *E.coli*. (h) Distribution of RMSE between K and CAQ for glycolysis in *E.coli* and *S.cerevisiae*.

To further quantify the alignment between theory and real data, we used RMSE in $\log_{10}$ space (i.e. RMSE between $\log_{10}(K)$ and $\log_{10}(CAQ)$) instead of Spearman's correlation between K and CAQ as the EOI metric in the revised manuscript. Using RMSE instead of correlation for the EOI metric did not change key conclusions of the EOI analysis, such as the association between oncogenic signaling and tendency to maximize flux efficiency ([Response Figure 9](#)).

Second, the deviation of the HK- and PFK-catalyzed reactions ($Glc \rightarrow G6P$ and $F6P \rightarrow FBP$) from the theoretical $K=CAQ$ line can be explained by the trade-off between maximizing flux efficiency and concentrating flux control. After adding the glucose transport reaction ($Glc(e) \rightarrow Glc$) to the analysis (suggested by Reviewer #1), we repeatedly observed that reactions known to be key control points of glycolysis deviate the most from the theoretical $K=CAQ$ line, and the enzymes with strong flux control (GLUT, HK, PFK) have lower abundance than theoretically predicted. Therefore, we hypothesized that reducing expression of these enzymes could facilitate efficient flux control through a few reaction steps, and developed an extended theoretical framework capturing the trade-off between flux control: when a pathway operates at maximal efficiency, flux control tends to be more evenly distributed across reaction steps, hence the pathway will be hard to control through a few reaction steps. Reducing abundances of enzymes catalyzing reactions with strong thermodynamic driving force, such as GLUT, HK, and PFK, can lead to a larger negative Gibbs free energy $\Delta_r G$ and stronger flux control of that reaction. Therefore, the fact that CAQ of these reaction pairs is lower than the corresponding K is consistent with this interpretation of concentrated flux control, and is consistent with the fact that GLUT, HK, and PFK are key control steps of glycolysis. We have included discussion about this point, together with the extended framework of flux-control tradeoff in the revised manuscript.



Response Figure 9. Determinants of EOI (RMSE between $\log_{10}(K)$ and $\log_{10}(CAQ)$). (a) GSEA plot showing top three pathways enriched in genes negatively correlated with EOI of glycolysis in TCGA-COAD. (b) Oncogenic signaling pathways correlated with EOI of glycolysis in human tumors. (c) Regulatory links between oncogenic signaling pathways associated with EOI of glycolysis. (d) Spearman's rank correlation between fraction of glycolytic enzyme in total cellular proteome and EOI of glycolysis.

3. The flux efficiency is probably not the only objective of a cell and the authors need to demonstrate that this objective has meaning. For example recent studies suggest that enzymes are actively kept away from saturation which would be against (even the opposite) of optimizing flux efficiency. Further this objective will probably vary between pathways. Thus the "law" is not universal.

We thank the reviewer for raising this concern. We completely agree that flux efficiency is not the only objective of metabolism. In the original manuscript, we developed the EOI metric to quantify its heterogeneity across conditions; in the revised manuscript, to explain the systematic deviation observed in key reactions, we have further developed the extended theory of flux-control tradeoff, which acknowledges the existence of an additional objective (i.e. efficient control through a few steps). Together, these revisions explicitly acknowledge that metabolic pathways can operate at different positions along a multi-objective

tradeoff landscape, rather than assuming a single universal optimum.

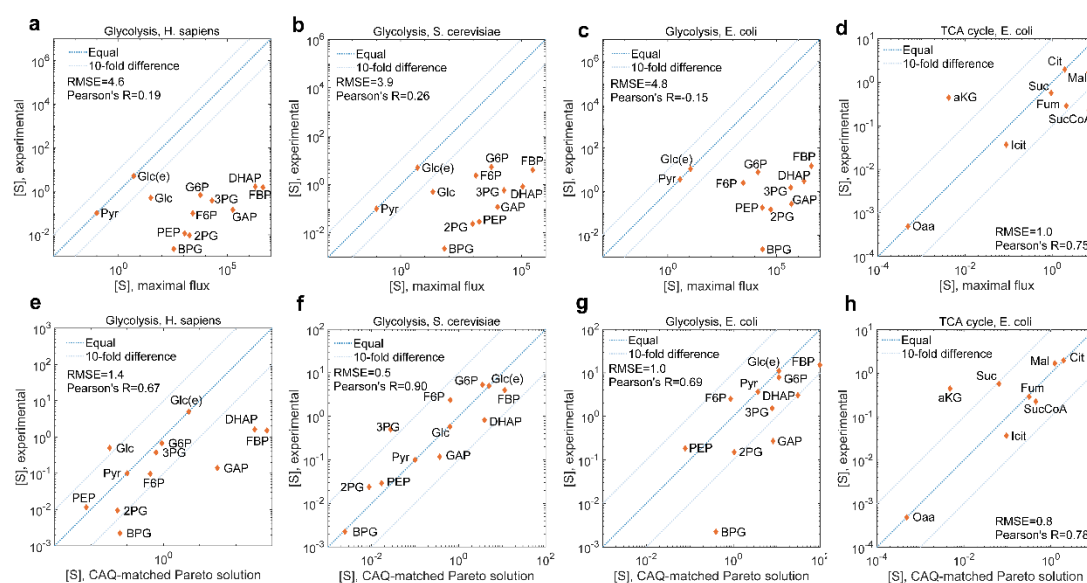
To avoid overinterpretation of our theory and better reflect its current components, we no longer refer to the theoretically predicted K-CAQ coupling as “a universal law of kinetics-thermodynamics coupling”, and use “K-CAQ relationship at maximal flux efficiency” instead in the revised manuscript.

Regarding the reviewer’s point about the relationship between enzyme saturation and optimization of flux efficiency, we agree that enzyme saturation states can vary across conditions. However, multiple experimental and theoretical studies have reported that (1) many enzymes operate near substrate saturation (e.g. Park et al, Nat Chem Biol 2016, PMID: 27159581); (2) unsaturated enzymes can also maximize efficiency in some conditions (e.g. Sahin et al, Nat Commun 2023, PMID: 37147292). Furthermore, some enzymes involved in metabolite sensing, such as chromatin modifiers, are often kept away from saturation because they need to maintain sensitivity to changes in substrate concentration, which is an alternative objective beyond efficiency optimization. Nevertheless, the heterogeneity in enzyme saturation supports the interpretation that multiple objectives might co-exist in metabolism, but does not exclude efficiency optimization as one of the objectives.

4. The manuscript fails to explain why the results have biological relevance. If the model improves our understanding the authors need to make testable predictions that go beyond the presented correlations.

We thank this reviewer for emphasizing the importance of biological relevance and validation with testable predictions. In the revised manuscript, we used experimentally measured metabolite concentrations across the three species as an additional dataset for validation. Using mathematical models for pathways representing glycolysis and TCA cycle, we computed intermediate metabolite concentrations for enzyme allocation profiles maximizing flux efficiency (i.e. maximal flux solution) and those CAQ-matched Pareto solutions balancing flux and control (see “General response regarding extension of the theoretical framework” for more information), and compared them with experimental data. The comparison demonstrated that the CAQ-matched Pareto solution showed substantially improved agreement with experimentally measured metabolite concentrations ([Response Figure 10](#)), consistent with the

revised framework describing the trade-off between flux efficiency and flux control.



Response Figure 10. Comparison between model-predicted and real metabolite concentrations for the maximal flux solution (a-d) and CAQ-matched Pareto solution (e-h). (a) Maximal flux solution for glycolysis in *H.sapiens*. (b) Maximal flux solution for glycolysis in *S.cerevisiae*. (c) Maximal flux solution for glycolysis in *E.coli*. (d) Maximal flux solution for TCA cycle in *E.coli*. (e-h) Same as in (a-d) but for CAQ-matched Pareto solution.

5. The work must be better embedded with current literature. The idea that thermodynamics constrains metabolic flux and that the thermodynamic driving force of reactions influences the enzyme levels is not new. Especially for glycolysis, metabolic control analysis has shown in detail the relationship of thermodynamics and enzyme kinetics and the authors need to make clear how their work relates to these findings. Especially this study (not from this reviewer): DOI 10.1038/nchembio.2077.

We thank the reviewer for raising this concern and suggesting the reference. We agree that it is important to compare our work to relevant previous studies. Briefly, this work (Park et al, Nat Chem Biol 2016, PMID: 27159581) studied the partition of thermodynamic driving force within glycolysis by measuring absolute concentrations of metabolites and computing $\Delta_r G$ accordingly. They found that $\Delta_r G$ were relatively evenly distributed within different steps of glycolysis and interpreted this as a strategy to avoid wasting enzyme resource in catalyzing nonproductive backward fluxes. Therefore, although this study did not attempt to derive an optimal enzyme allocation profile maximizing flux efficiency of a pathway, its observations were consistent with the hypothesis of flux efficiency optimization. Another theoretical work also linked

maximization of flux efficiency to uniform partition of thermodynamic driving force into reaction steps but did not derive a general closed-form relationship underlying optimal enzyme allocation profiles across heterogeneous rate laws (Noor et al, PLOS Comput Biol 2014, PMID: 24586134). We have added discussion about these studies in the revised manuscript.

Nevertheless, while these previous studies and our theory all acknowledge maximization of flux efficiency as an important objective of metabolism, two key points distinguish our work from the previous literature. While previous studies progressively established the conceptual connection between thermodynamics, enzyme cost, and metabolic control, our work extends this line of research by deriving an explicit, closed-form optimality condition for enzyme allocation. Importantly, this optimality condition is experimentally testable because K and CAQ can both be computed from experimental measurements and directly compared. Earlier attempts in analytically deriving enzyme allocation profiles maximizing flux efficiency (e.g. Noor et al, Interface Focus 2024, PMID: 38344407) rely on simplified assumptions such as the absence of isozymes, which complicates direct comparison with experimental data in real metabolic pathways. In addition, the revised manuscript further extends the framework beyond the maximal-flux solution by considering the tradeoff between flux and control, which explains why experimental data deviate from the theoretical solution maximizing flux efficiency. We have explicitly clarified these points in the revised manuscript.

Reviewer #3:

The authors develop a theoretical framework for quantifying how efficiently cells allocate enzyme abundance to support metabolic flux. By combining thermodynamic rules with enzyme kinetics, they derive a necessary and sufficient condition for optimal enzyme allocation in a linear metabolic pathway: the equilibrium constant (K) of a reaction equals the catalytic-abundance quotient (CAQ), which is defined based on enzyme abundance and catalytic efficiency. They then introduce the Efficiency Optimality Index (EOI), the correlation between K and CAQ across tandem reaction pairs, and use EOI as a metric to evaluate whether glycolysis in various organisms and cellular contexts follows their predicted optimality pattern.

Understanding whether cells naturally optimize the investment in their metabolic machinery is important both for interpreting metabolic phenotypes and for improving quantitative models of metabolism. The authors provide preliminary applications to cancer data, showing that EOI correlates with

expression of oncogenic pathways, and they suggest that the framework may be extendable beyond glycolysis. In practice, broader application will require accurate thermodynamic parameters, kinetic data, enzyme abundances, and metabolite concentrations. Such datasets are still limited for most pathways.

Overall, the manuscript addresses a conceptually important question and presents an intriguing theoretical result. However, as written, it is difficult to assess or verify the mathematical framework, and several aspects of the methodology are unclear. The manuscript is not yet publication-ready. Below are comments aimed at improving clarity.

We thank the reviewer for positive remarks on the conceptual importance of our work and apologize for the confusion with mathematical derivation and methodology. We have completely rewritten the Supplementary Information (now named “Appendix mathematical derivations”) to enhance its clarity. In addition to expanding intermediate derivation steps, we have also reorganized the structure of the Appendix mathematical derivations to improve readability, including clearer notation definitions, separated sections for different rate laws, and additional explanatory text connecting major derivation steps.

Major Comment

The primary contribution of this manuscript is its theoretical framework, so mathematical clarity is essential. Unfortunately, the derivations in the Supplementary Information are difficult to follow and, in several places, incomplete. Key transitions are not justified. For example, the origin of Eq. 36 is not explained and many steps are skipped without accompanying text. The result is that central parts of the proof cannot be independently verified.

To illustrate the problem, I have listed a few specific examples in the minor comments 1-3 below; however, these are only representative. At present, the supplement contains 86 equations, but a transparent and reproducible derivation will likely require substantially more intermediate steps and explanatory text. Because the mathematical development is unclear, I was unable to follow several portions of the argument to completion.

I look forward to reviewing a revised version with a more complete and readable mathematical exposition.

We thank the reviewer for the constructive feedback on the clarity of mathematical derivations. We have completely rewritten the SI note, reorganized its structure and included more detailed mathematical derivations to ensure clarity and readability. We hope that these revisions

substantially improve the transparency and reproducibility of the theoretical framework.

Minor Comments

1) Supplementary (mathematical derivations): As noted above, there are several issues with clarity and completeness. A few examples of different types of problems include:

-Eqn 30 and 32: J as a function of (a , e , K , etc.) is not designated consistently and the transition in notation is not mentioned before Eqn 32.

-Eqn 36: not clear at all

-Eqn 40, 41: key substitutions are omitted, leaving significant algebraic work to the reader. Please provide a smooth, explicit derivation.

We thank the reviewer for pointing out these and apologize for the confusion. In the revised manuscript, we have rewritten the mathematical derivations to ensure that all necessary steps are included.

For the first comment, we apologize that the distinction between these notations was not clearly explained in the previous version. The reason for using the first notation $J(a, E, K, [S_{in}], [S_{out}])$ is that when the parameters (a, K) and concentrations ($E, [S_{in}], [S_{out}])$ are known, the steady state pathway flux J can be uniquely computed by solving steady-state equations, therefore can be written as a function of

$a, E, K, [S_{in}], [S_{out}]$. The second notation $J_{a,K,[S_{in}],[S_{out}]}(E)$ means that when all other parameters and concentrations except for the enzyme abundances E are fixed, the pathway flux J can be uniquely determined by E , hence the maximization of flux efficiency can be achieved by optimizing E . We have added explanation of the notations in the Appendix mathematical derivations of the revised manuscript.

For the second comment, we apologize that several steps were skipped in the previous version of manuscript. That expression was obtained by calculating the partial derivative of the Lagrange function $L(x, \lambda)$ to the variable x_i , which is the square root of the abundance of i -th enzyme:

$x_i = \sqrt{[E_i]}$. We have expanded the derivation to include all intermediate algebraic steps (eqn 86-90 in the Appendix mathematical derivations) so that the result can now be independently verified more easily.

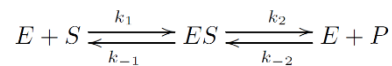
For the third comment, we have now expanded the mathematical derivation of this relationship and provided a general form of

relationship linking K and CAQ at maximal flux efficiency. Detailed information of the derivation is in the section “Optimal solution under different rate laws” of the revised Appendix mathematical derivations.

2) Similar issues in Methods:

-The first (long) Michaelis-Menten rate law is presented without any citation or pointer to the Supplement, where the derivation could be found. A reference or explicit derivation is needed; the former is acceptable but the latter is preferable.

We thank the reviewer for the helpful advice. The derivation of this reversible Michaelis-Menten rate law is provided in the textbook “Fundamentals of Enzyme Kinetics” by Athel Cornish-Bowden. Briefly, the reversible Michaelis-Menten rate law can be derived based on the reaction scheme below:



In which each step is assumed to be reversible, and concentration of the intermediate complex ES is assumed to be at steady state. Under the assumption that each single step in this scheme follows the law of mass action, we have the steady state equation below:

$$\frac{d[ES]}{dt} = k_1[E][S] + k_{-2}[E][P] - (k_{-1} + k_2)[ES] = 0$$

Combining it with the relationship $[E] + [ES] = E_t$, in which E_t is the total abundance of enzyme in substrate-bound (ES) and unbound (E) states, both $[E]$ and $[ES]$ can be computed from the concentrations E_t , $[S]$, $[P]$, and the rate constants k_1, k_2, k_{-1}, k_{-2} :

$$\begin{cases} [E] = \frac{(k_{-1} + k_2)E_t}{k_{-1} + k_2 + k_1[S] + k_{-2}[P]} \\ [ES] = \frac{(k_1[S] + k_{-2}[P])E_t}{k_{-1} + k_2 + k_1[S] + k_{-2}[P]} \end{cases}$$

Because the net reaction rate v can be computed from $v = k_1[E][S] - k_{-1}[ES]$, we can subject the expressions of $[E]$ and $[ES]$ into it to obtain the rate law:

$$v = \frac{E_t(k_1k_2[S] - k_{-1}k_{-2}[P])}{k_{-1} + k_2 + k_1[S] + k_{-2}[P]}$$

This is the reversible Michaelis-Menten rate law mentioned in the main text, in which the kinetic and thermodynamic parameters can be computed from the rate constants k_1, k_2, k_{-1}, k_{-2} :

$$\left\{ \begin{array}{l} K = \frac{k_1 k_2}{k_{-1} k_{-2}} \\ k_{cat} = k_2 \\ K_S = \frac{k_{-1} + k_2}{k_1} \\ K_P = \frac{k_{-1} + k_2}{k_{-2}} \end{array} \right.$$

To clarify the derivation of this rate law and its relationship with other simplified rate laws (e.g. first-order and zero-order kinetics), we have added a section named “2.1 Reaction kinetics” in the Appendix mathematical derivations of the revised manuscript. We have also cited the textbook in the main text for the readers’ reference.

-The sentence beginning with "Therefore" that follows this equation is confusing. The second equation is a basic mass-balance statement and does not require the first equation for justification.

We thank the reviewer for raising this concern and apologize for the confusion. We agree that the general form of ordinary differential equations describing the dynamics of the linear metabolic pathway does not require the reversible Michaelis-Menten kinetics as a prerequisite. We have rewritten the section “Linear pathway model with Michaelis-Menten kinetics” to avoid such confusion.

3) Page 3: The statement "its apparent rate constant k , which is proportional to the enzyme abundance $[E]$," needs justification. Please explain or cite where this proportionality comes from; currently it appears as an abrupt jump.

We thank the reviewer for pointing this out. We apologize that we didn’t provide a clear definition of apparent rate constant and justification of its relationship with enzyme abundance $[E]$ in the previous version of manuscript. We introduce the apparent rate constant k as an effective quantity that allows different rate laws to be expressed in a common mathematical form:

$$v = k[S]^n \left(1 - \frac{[P]}{K[S]} \right)$$

In which n is the reaction order (0 for zero-order rate law and 1 for first-order rate law). In this sense, the reaction rate can be expressed as the product of three terms: (1) the apparent rate constant k ; (2) the

thermodynamic term $1 - \frac{[P]}{K[S]}$ describing the distance from

thermodynamic equilibrium; and (3) the substrate term $[S]^n$ that depends on substrate concentration and reaction order. The first order

and zero order rate laws can both be derived from the Michaelis-Menten rate law (Appendix mathematical derivations, section 2.1), and under Michaelis-Menten kinetics (1) the reaction rate is proportional to enzyme abundance $[E]$; (2) the thermodynamic term and substrate term are both independent of the enzyme abundance $[E]$. Hence, because the reaction rate is the product of apparent rate constant, thermodynamic term and substrate term, the apparent rate constant k must be proportional to enzyme abundance $[E]$.

We have included definition of the catalytic efficiency and apparent rate constants under reversible Michaelis-Menten, first-order, and zero-order kinetics in the Appendix mathematical derivations (Table 1: Catalytic efficiency and apparent rate constant under different rate laws) of the revised manuscript.

4) Why Spearman rather than Pearson correlation for EOI? Pearson correlation would quantify proportionality directly, whereas Spearman uses only rank information. A rationale is needed.

We thank the reviewer for raising this concern. We have shown in the original manuscript that using Pearson correlation instead of Spearman correlation as the EOI metric did not affect the high EOI values across datasets (Fig. S5). Yet we agree with the reviewer that Pearson correlation is a better metric quantifying proportionality. Furthermore, as other reviewers have also raised concerns regarding the suitability of Spearman's correlation as a metric for EOI, we have changed its definition to root mean squared error (RMSE) to assess the deviation of real data from the $K=CAQ$ equality. The reason for using RMSE instead of correlation is that our theory predicts $K=CAQ$ at maximal flux efficiency, but correlation-based metrics cannot distinguish it from other proportional relationships such as $K=10*CAQ$. We have also discussed the rationale underlying the selection of EOI metric in the revised manuscript.

5) Legends are generally too brief to guide interpretation. Examples include:
-Fig. 4b: the meaning of the horizontal bars is not explained.
-Fig. 5c: the meaning of the color tone is unclear.

We thank the reviewer for pointing this out. The horizontal bars in Figure 4b indicate standard deviation of CAQ across different cell lines. In Figure 5c, different colors were used to distinguish membrane-associated (blue) and membrane-unassociated (purple) pathways. We have added this information to the figure legends in the revised manuscript.

6) Page 6, permutation tests:

The text discusses permutations of enzyme abundances and of enzyme abundances + catalytic efficiencies, but does not comment on permuting catalytic efficiencies alone. This makes interpretation difficult, since the stated logic distinguishes strategy 1 and strategy 2.

We thank the reviewer for raising this concern. We agree that the fact that permuting catalytic efficiencies alone did not completely impair the coupling between K and CAQ could also indicate strategy of cells to robustly optimize efficiency of metabolic pathways. It suggests that, for enzyme allocation profiles observed in real cells, their tendency to optimize flux efficiency persists upon fluctuations in kinetic parameters of these enzymes, which could be caused by variations of the intracellular environment such as pH and allosteric regulators. Such interpretation is consistent with the idea that strategy 1 and strategy 2 coexist in real metabolic pathways. We have included discussion about this in the revised manuscript.

7) The sentence "For each parameter set and a given set of enzyme concentrations, the steady-state concentrations of intermediate metabolites were computed" does not specify what enzyme concentrations were used. This is essential information and should be clearly stated.

We thank the reviewer for raising this concern and apologize for the confusion. For simulation under Michaelis-Menten kinetics, enzyme abundances were not specified a priori. Instead, for each candidate enzyme allocation profile proposed by the constrained optimization algorithm, we first computed steady-state metabolite concentrations by solving the steady-state equations, which allowed evaluation of the corresponding pathway flux. The optimization algorithm then iteratively updated the enzyme allocation profile until the flux was maximized under the constraint that the total enzyme abundance did not exceed 1. Therefore, the "given set of enzyme concentrations" refers to a candidate enzyme allocation profile evaluated during this optimization procedure. We have clarified this point in the revised manuscript.

3rd Aug 2026

Manuscript Number: MSB-2025-13440RR

Title: Trade-off between flux efficiency and metabolic control shapes enzyme allocation

Author: Kai Sun

Weiyan Zheng

Wenchao Fan

Chuyun Ding

Dan Huang

Ziwei Dai

Dear Dr. Dai,

Thank you again for submitting your revised manuscript. We have now received reports from the two reviewers who were asked to evaluate your revised study. We asked the original Reviewer #1 to assess your responses to the comments from both Reviewers #1 and #2. As the original Reviewer #3 was unavailable in this round, we invited a new reviewer (Reviewer #4) to evaluate your response to the points previously raised by Reviewer #3.

As you will see from the reports below, Reviewer #1 continues to raise important concerns regarding the revised manuscript. In principle, our editorial policy allows only a single round of major revision. However, we believe that Reviewer #1's remaining concerns are sufficiently important to warrant further consideration. Given the potential relevance of your study, we are willing to allow you to address these issues in an exceptional second round of major revision. Please note that this will be the final revision round, so it is essential that all remaining concerns be fully addressed.

On a more editorial level:

1. Please remove the "Authors' contribution" section from the manuscript text.
2. "Competing interests" should be renamed to "Disclosure and Competing Interests Statement".
3. Please upload the main figures as individual high-resolution figure files rather than as a single PDF.
4. The references need to be formatted according to the Molecular Systems Biology reference style. Please list up to 10 co-authors of a paper before adding et al. in the reference list. Citations should be listed in alphabetical order.
5. "Code availability" should be included in "Data Availability". A separate section title "code availability" should be removed.
6. Appendix
 - EV dataset legends should be removed from Appendix PDF
 - change the Appendix reference list from numbered to ordered-by-author with 10 authors + et al. according to our guidelines
7. Please provide a "standfirst text" in a separate DOCX file summarizing the study in one or two sentences (approximately 250 characters, including space), three to four "bullet points" highlighting the main findings. Please also provide a visual abstract as a separate PNG file with dimensions of exactly 550 px (width) × 300 px (height) to highlight the paper on our homepage. Please refer to published papers for examples.
8. Reagents and Tools table: Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines: <https://link.springer.com/journal/44320/submission-guidelines#structuredmethods>. When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but upload it as a separate file choosing the file type "Reagent Table".
9. Please add missing callouts for Figure 6F.
10. Please address the following issues in figure legends:
 - Please note that the exact p values are not provided in the legends of figure 1B.
 - Please note that the measure of center for the error bars needs to be defined in the legends of figures 4D,F,G; 7C.
11. Sections need to be named and the order should be corrected: Title page - Abstract - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure Legends - Table(s) - Expanded View Figure Legends.

Please attach a covering letter giving details of the way in which you have handled each of the points raised by the reviewer. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

I look forward to receiving the revised manuscript soon.

Kind regards,
Jingyi

Jingyi Hou, PhD
Senior Editor
Molecular Systems Biology

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (1st Nov 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

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

*** PLEASE NOTE *** As part of the EMBO Press transparent editorial process initiative (see our Editorial at <https://dx.doi.org/10.1038/msb.2010.72>), Molecular Systems Biology publishes online a Review Process File with each accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. If you do NOT want this File to be published, please inform the editorial office at contact@molsystbiol.org within 14 days upon receipt of the present letter.

Reviewer #1:

I appreciate the authors reconsidering the flux control and formulating a new framework of efficiency-control tradeoff. The new results show a good agreement in enzyme allocation and metabolite concentrations to experimental measurements. However, the reviewer is not fully convinced that the optimization principles alone are driving the prediction.

Major points:

1. CAQ-matched Pareto solution. The authors tested the framework of flux-control tradeoff by first finding a Pareto solution that is proximal to experimental CAQ profile, and then comparing it to the experimental CAQ profile. While they justified that "this procedure does not guarantee a close match between the CAQ-matched Pareto solution and physiological CAQ profile, unless the physiological CAQ profile lies near the Pareto front", this procedure may mask the real mismatch between theoretical prediction and experiment. This is because the Pareto front is rather flat across a large range of flux efficiency (Fig. 1, b, e, h), and most experimental systems have sparsely distributed control. Therefore the point of smallest Euclidean distance from the experimental CAQ profile are most likely to be found by moving the point upward to arrive at the Pareto front, i.e. same flux efficiency as experimental value. The final optimized enzyme allocation may just reflect the initial value from the experiment. Therefore this procedure has limited power to test the optimization of flux efficiency, or the matching between predicted and experimental enzyme allocation. A critical question is, without relying on experimental proteomics data, can the Pareto front predict the CAQ profile?

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Reviewer #4:

I have reviewed the authors' response to Reviewer 3's comments, as requested. My assessment is as follows:

Overall, I find that the authors have adequately addressed all of Reviewer 3's concerns. Specifically, they have:

Refined the derivations of the key mathematical formulas, improving their clarity and completeness.

Supplemented the figure captions with necessary details for better comprehension.

Provided additional explanations and justifications for the computational setup.

Based on my review, I consider the response to be satisfactory and no further revision of the full manuscript is required from my side.

Beyond confirming that the authors have satisfactorily responded to the specific concerns, I would like to offer a brief evaluation of the manuscript's broader contribution. The core contribution of this study lies in the derivation and validation of a quantitative, concise, and universal optimality principle-the K-CAQ coupling relationship-which reveals a "Pareto optimality" economic principle governing enzyme allocation in metabolic pathways. Specifically, it translates the abstract theory of metabolic optimality into a definitive mathematical formula that can be directly tested with modern omics data. Furthermore, the theory introduces the concept of a "flux-control trade-off," providing a compelling explanation for why real cells deviate from absolute optimal efficiency, thereby achieving a balance between high-flux operation and concentrated regulation. Although the authors have primarily validated their framework in central carbon pathways such as glycolysis and the TCA cycle, the concepts proposed in this manuscript hold promise for application to constraint-based genome-scale metabolic network models, and may offer new principles for the construction of digital cells.

Reviewer #1:

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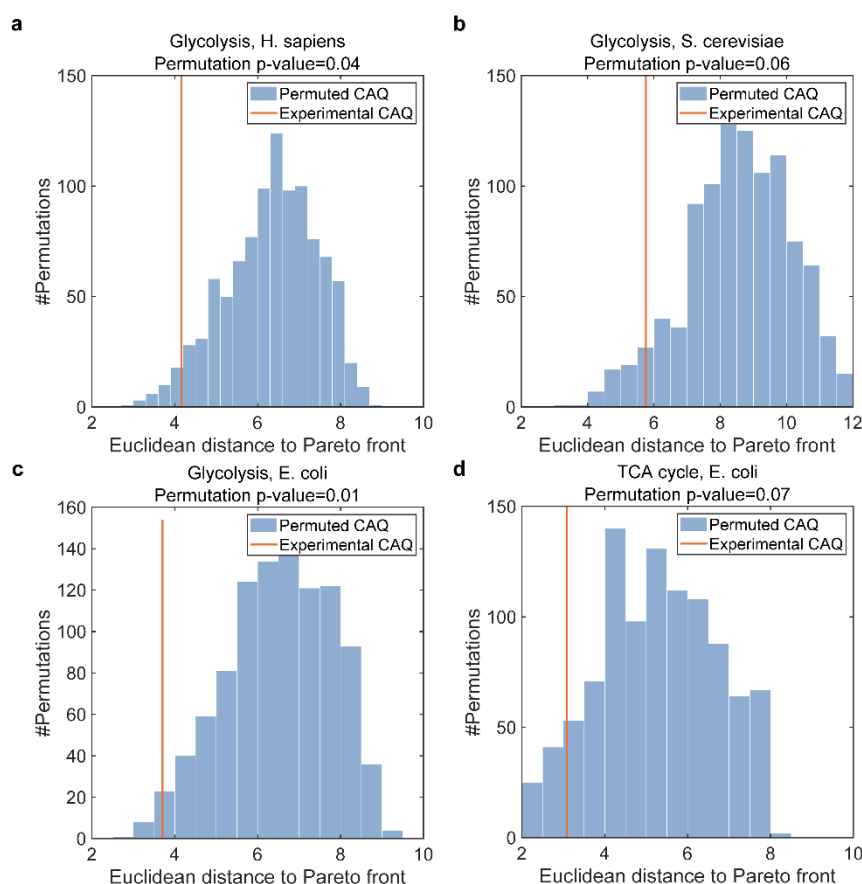
We thank the reviewer for the positive assessment of our previous revisions. To address the remaining concerns, we have performed additional analyses to demonstrate that (1) the agreement between the Pareto front and experimental CAQ profiles is not an artifact of the CAQ-matching procedure, and (2) the proportionality between flux control coefficients and enzyme abundance at maximal flux efficiency is not specific to first-order kinetics. We have also clarified why physiological enzyme allocation and flux control can deviate from the state of maximal flux efficiency predicted by the theory.

Major points:

1. CAQ-matched Pareto solution. The authors tested the framework of flux-control tradeoff by first finding a Pareto solution that is proximal to experimental CAQ profile, and then comparing it to the experimental CAQ profile. While they justified that "this procedure does not guarantee a close match between the CAQ-matched Pareto solution and physiological CAQ profile, unless the physiological CAQ profile lies near the Pareto front", this procedure may mask the real mismatch between theoretical prediction and experiment. This is because the Pareto front is rather flat across a large range of flux efficiency (Fig. 1, b, e, h), and most experimental systems have sparsely distributed control. Therefore the point of smallest Euclidean distance from the experimental CAQ profile are most likely to be found by moving the point upward to arrive at the Pareto front, i.e. same flux efficiency as experimental value. The final optimized enzyme allocation may just reflect the initial value from the experiment. Therefore this procedure has limited power to test the optimization of flux efficiency, or the matching between predicted and experimental enzyme allocation. A critical question is, without relying on experimental proteomics data, can the Pareto front predict the CAQ profile?

We thank the reviewer for raising this concern. We agree that the comparison between the CAQ-matched Pareto solution and the same experimental CAQ profile used to identify this solution does not, by itself, constitute an independent test of predictive power. In the revised manuscript, we retain the CAQ-matched Pareto solution only to infer the physiological position of enzyme allocation along the Pareto front, and

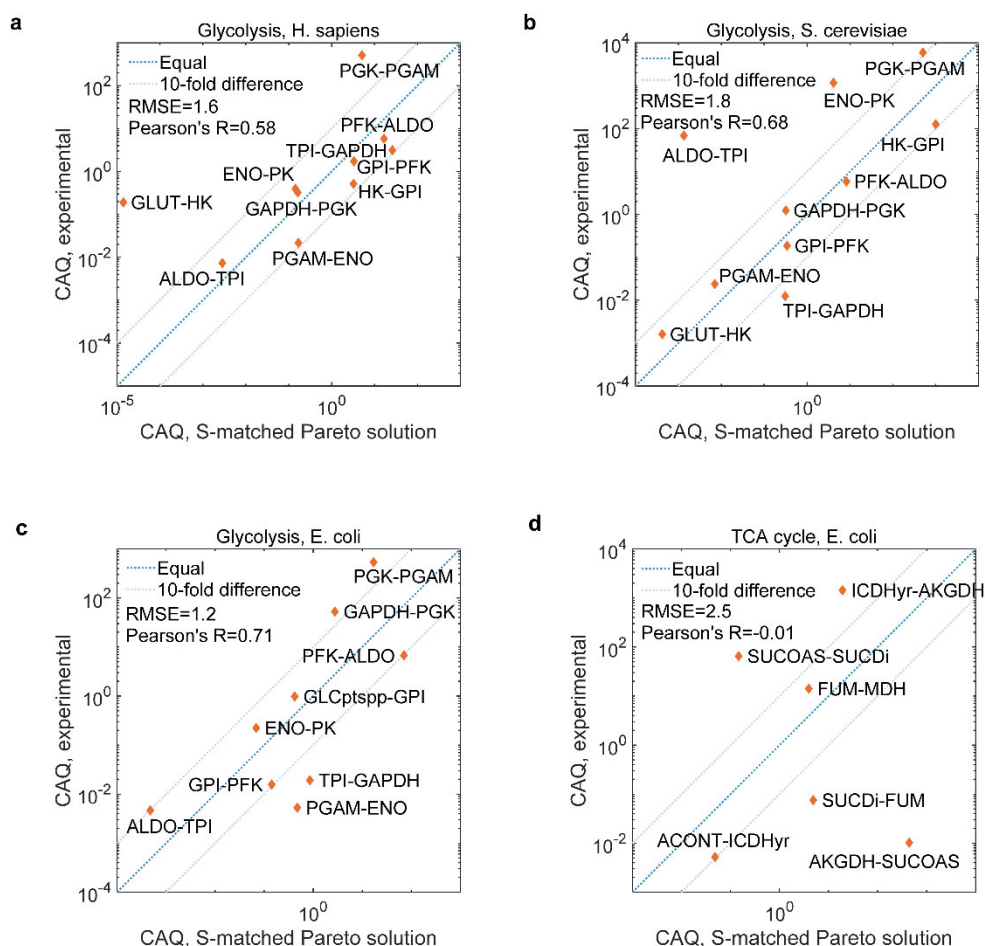
performed three additional analyses to test the predictive power of the Pareto front independently of this calibration procedure.



Response Figure 1. Euclidean distance to Pareto front for the experimental and permuted CAQ profiles in log10 space. (a) Glycolysis in *H. sapiens*. (b) Glycolysis in *S. cerevisiae*. (c) Glycolysis in *E. coli*. (d) TCA cycle in *E. coli*.

First, to test whether the close agreement between the experimental CAQ profiles and the Pareto front could arise simply from the proteomics-based calibration procedure, we performed a permutation test. For each pathway-species pair, we randomly permuted the experimental CAQ profiles 1,000 times and calculated the Euclidean distance between each permuted profile and the Pareto front in log10 space. We then compared the resulting null distribution with the corresponding distance for the unpermuted experimental CAQ profile. We defined a permutation p-value as the fraction of permuted CAQ profiles with a smaller distance to the Pareto front than the experimental CAQ profile. Across all four pathway-species pairs, the experimental CAQ profiles were consistently closer to the Pareto front than the randomly permuted profiles, with permutation p-values ranging from 0.01 to 0.07 (0.04 for glycolysis in *H. sapiens*, 0.06 for glycolysis in *S.*

cerevisiae, 0.01 for glycolysis in *E. coli*, and 0.07 for TCA cycle in *E. coli*, [Response Figure 1](#)). This analysis indicates that experimental CAQ profiles tend to lie closer to the Pareto front, supporting the alignment between physiological enzyme allocation and the predicted Pareto front.

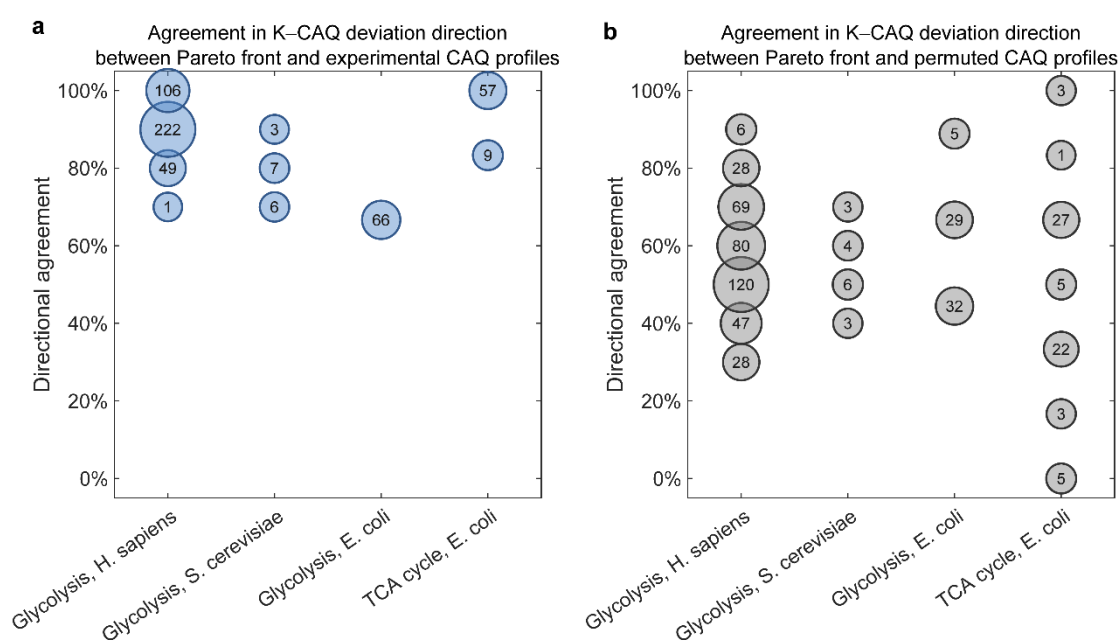


Response Figure 2. Comparison between the experimental CAQ profile and CAQ for the metabolite (S) -matched Pareto solution. (a) Glycolysis in *H. sapiens*. (b) Glycolysis in *S. cerevisiae*. (c) Glycolysis in *E. coli*. (d) TCA cycle in *E. coli*.

Second, to test whether the Pareto front can predict experimental CAQ profiles without using proteomics data to select a physiological Pareto solution a priori, we identified a metabolite-matched Pareto solution (S-matched Pareto solution) by selecting the Pareto solution whose predicted metabolite concentrations showed the smallest Euclidean distance from the experimental measurements in log10 space. The metabolite-matched Pareto solutions showed moderate-to-strong agreement with the experimental CAQ profiles for glycolysis across all three species (Pearson's R ranging from 0.58 to 0.71, [Response Figure 2](#)). The correlation was weaker for the *E. coli* TCA cycle, largely due to

the discrepancy for the AKGDH-SUCOAS reaction pair, whose CAQ varies substantially along the Pareto front. Nevertheless, 21 of 35 reaction pairs showed less than 10-fold difference between the predicted and experimental CAQ values, supporting the general agreement between the Pareto front and experimental CAQ profiles. Importantly, because the metabolomics and proteomics datasets were obtained from independent studies and were not sample-matched, this analysis provides a cross-dataset validation to show that these two independent omics modalities converge on a shared underlying region of the Pareto front.

Third, to show that the Pareto front captures properties of K-CAQ deviation when a pathway moves away from the maximal flux efficiency state, we quantified the agreement in the direction of K-CAQ deviation between the Pareto front and experimental CAQ profiles. For each reaction pair, the deviation between K and CAQ is unidirectional in the Pareto front: for reaction pairs with key control points such as GLUT, PFK, and HK as the upstream reaction, $CAQ \leq K$ holds within the Pareto front; while for some other reaction pairs such as GAPDH-PGK, $CAQ \geq K$ holds within the Pareto front. Therefore, for each experimental CAQ profile, we can examine its direction of deviation between K and CAQ for each reaction pair and check whether these directions align with predictions by the Pareto front. We computed the percentage of K-CAQ deviation directions consistent between the proteomics data and Pareto front predictions for each sample, and compared these directional agreement values between experimental and permuted CAQ profiles. Strong agreement was observed for the experimental CAQ profiles, but not for the permuted CAQ profiles ([Response Figure 3](#)).



Response Figure 3. Directional agreement in K-CAQ deviation between

Pareto front and (a) experimental CAQ profiles; (b) permuted CAQ profiles. Numbers in the circles indicate numbers of experimental samples or permuted profiles.

Taken together, these additional results show that the agreement between experimental CAQ profiles and the Pareto front is not an artifact of the CAQ-matching procedure, and that the predictive power of the Pareto front can be tested without using proteomics data to select a physiological Pareto solution. We have included these additional analyses and relevant discussions in the revised manuscript.

2. The result of FCC profile in Fig. S8 is an important argument for why flux control optimization is also implemented in addition to flux efficiency optimization. However, per the theory, FCC should be proportional to enzyme abundance at maximal flux efficiency, which does not really agree with the experimental glycolysis enzyme allocation. GLUT1 and HK are usually at low abundance, whereas GAPDH is usually the most abundant glycolytic enzyme. It is unclear where this discrepancy arises, but it questions the assumptions made in the max flux efficiency model in page 8, such as using first order kinetics and linear pathway summation rule.

We thank the reviewer for this comment and apologize for the confusion here.

The reviewer is correct that physiological flux control coefficients are not proportional to physiological enzyme abundances. Importantly, our theory does not predict that the proportionality relationship holds at physiological state, instead, it applies specifically to the maximal flux efficiency endpoint. Physiological metabolic pathways can move away from this endpoint along the flux-control Pareto front to achieve more concentrated flux control. Therefore, deviation from the $C_{v_i}^J \propto [E_i]$ proportionality in real glycolysis is expected under the flux-control tradeoff framework. To achieve concentrated flux control, the pathway tends to reduce abundances of enzymes at reactions with the potential to become key control points, such as GLUT, HK, and PFK, to further increase their flux control coefficients. This explains why GLUT1 and HK are usually expressed at low abundance in physiological states as the reviewer notes.

Moreover, it is worth noting that the theoretical prediction that flux control coefficients are proportional to enzyme abundances at maximal flux efficiency does not require first-order kinetics. Derivation of this relationship (see Section 3.2 “Maximization of flux efficiency” of the

Appendix Mathematical Derivations for details) only requires the reaction rate to be linear to enzyme abundance, which applies to most rate laws including first-order, zero-order, and Michaelis-Menten kinetics. The rate law affects the mathematical form of elasticity coefficients and flux control coefficients, but does not change the proportionality between flux control coefficients and enzyme abundances when the pathway achieves maximal flux efficiency.

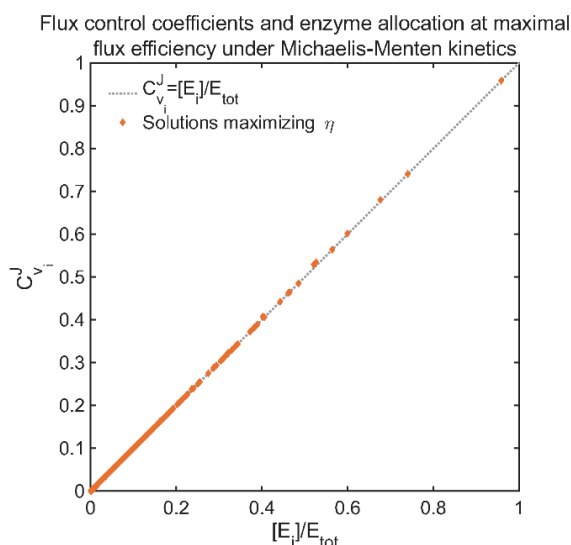
To better clarify this point, we performed a numerical simulation with a linear metabolic pathway under Michaelis-Menten kinetics. At maximal flux efficiency, since our theory predicts $C_{v_i}^J \propto [E_i]$ at maximal flux efficiency, and according to the summation theorem in metabolic control analysis (which also only requires the reaction rate to be linear to enzyme abundance), $\sum_{i=1}^n C_{v_i}^J = 1$, each flux control coefficient equals the fraction of metabolic enzyme catalyzing this reaction in all enzymes within that pathway:

$$C_{v_i}^J = \frac{[E_i]}{E_{tot}}$$

In which E_{tot} is the total abundance of enzymes in that pathway. We performed the simulation 100 times with random parameters, and compared the flux control coefficients $C_{v_i}^J$ and enzyme abundance

fractions $\frac{[E_i]}{E_{tot}}$. The simulated values fell perfectly on the $C_{v_i}^J = \frac{[E_i]}{E_{tot}}$ line

(Response Figure 4), confirming that the proportional relationship between flux control coefficients and enzyme abundances is conserved under Michaelis-Menten kinetics. We have included this result in the revised manuscript.



Response Figure 4.
Relationship between flux control coefficients and enzyme allocation at maximal flux efficiency for a linear metabolic pathway with Michaelis-Menten kinetics.

Minor point:

3. minimize Enzyme cost for converging pathways, the inequality Eq126 takes a different form. In this case, what is the form of CAQ? how does it compare to K?

We thank the reviewer for raising this question. For converging pathways, the inequality linking K and CAQ (Eqn.126 in Appendix Mathematical Derivations) takes the form below:

$$a_n[E_n]^2 > \sum_{i=1}^{n-1} \frac{a_i[E_i]^2}{K_i}$$

Because the converging pathway has multiple upstream branches, we can define CAQ_i for each of these branches:

$$CAQ_i = \frac{a_i[E_i]^2}{a_n[E_n]^2}$$

With this definition, the inequality can be rewritten to the form below that only contains K and CAQ:

$$\sum_{i=1}^{n-1} \frac{CAQ_i}{K_i} < 1$$

Therefore, for each branch, we also have $\frac{CAQ_i}{K_i} < 1$, which means $K_i >$

CAQ_i. We have revised the manuscript to clarify the definition of CAQ and their relationship with K for diverging and converging pathways.

Reviewer #4:

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We thank the reviewer for the encouraging comments on our work.

19th Sep 2026

Manuscript number: MSB-2025-13440RRR

Title: Trade-off between flux efficiency and metabolic control shapes enzyme allocation

Dear Dr. Dai,

Thank you again for sending us your revised manuscript. We have now heard back from the two reviewers who agreed to evaluate your revised study. As you will see below, they are both satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to Molecular Systems Biology.

Kind regards,
Jingyi

Jingyi Hou, PhD
Senior Editor
Molecular Systems Biology

Reviewer #1:

The authors have provided additional independent analysis to address my previous concern about predictability of CAQ profile, and clarified the confusion around flux control at maximal efficiency. I have no further requests or questions.

Reviewer #4:

In this revised manuscript, the authors have presented their work more clearly. I have no further concerns and recommend acceptance for publication.

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Please note that a copy of this checklist will be published alongside your article.

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The data shown in figures should satisfy the following conditions:

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.

Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Include a statement about sample size estimate even if no statistical methods were used.	Yes	Figure legends: 1b-e, 2c-d, 4b-h, 5b-c, 5e-f, 5h-i, 7b-d, 8d
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For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends: 1b,8a

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For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

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Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	Data availability section
If publicly available data were reused, provide the respective data citations in the reference list .	Yes	Materials and Methods