

Evolutionary Rate Covariation is a reliable predictor of co-functional interactions but not necessarily physical interactions

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Abstract

Co-functional proteins tend to have rates of evolution that covary over time. This correlation between evolutionary rates can be measured over the branches of a phylogenetic tree through methods such as evolutionary rate covariation (ERC), and then used to construct gene networks by the identification of proteins with functional interactions. The cause of this correlation has been hypothesized to result from both compensatory coevolution at physical interfaces and non-physical forces such as shared changes in selective pressure. This study explores whether coevolution due to compensatory mutations has a measurable effect on the ERC signal. We examined the difference in ERC signal between physically interacting protein domains within complexes as compared to domains of the same proteins that do not physically interact. We found no generalizable relationship between physical interaction and high ERC, although a few complexes ranked physical interactions higher than non-physical interactions. Therefore, we conclude that coevolution due to physical interaction is weak, but present in the signal captured by ERC, and we hypothesize that the stronger signal instead comes from selective pressures on the protein as a whole and maintenance of the general function.

eLife assessment

This **useful** study seeks to address the importance of physical interaction between proteins in higher-order complexes for covariation of evolutionary rates at different sites in these interacting proteins. Following up on a previous analysis with a smaller dataset, the authors provide **compelling** evidence that the exact contribution of physical interactions, if any, remains difficult to quantify. The work will be of relevance to anyone interested in protein evolution.

Introduction

The evolutionary rate of any protein-coding gene varies over time and hence between species. It has been observed that some genes have rates that covary over time with those of other genes and that they tend to be functionally related¹. Evolutionary rate covariation (ERC) is a measure of that correlation in relative evolutionary rates² (RER), which is the gene-specific evolutionary rate on a phylogenetic tree branch normalized by the genome-wide evolutionary rate on that branch. If one considers the set of branches relating the orthologous copies of a given gene, an ERC value measures how correlated its branch-specific rates of sequence divergence are with those of another gene.

Protein pairs that have high ERC values (*i.e.* high rate covariation over time) are often found to participate in shared cellular functions, such as in a metabolic pathway^{3–5} or meiosis⁶ or being in a protein complex together. It was previously shown that physical interaction is not required for co-functional proteins to have correlated evolutionary rates, such as between the proteins of a metabolic pathway^{1,10}. However, it is still unclear whether the correlation between physically interacting proteins is strengthened by coevolution at their physical interface. Many co-functional proteins physically interact, such as those in protein complexes and enzyme-substrate interactions^{7–9}. For example, SMC5 and SMC6 form a complex and have a shared function in the spatial organization of chromatin. They also have similar variation in rates of evolution across the branches of a yeast phylogeny (**Figure 1A**). Their strong rate covariation is quantified as a Fisher transformed correlation coefficient (ftERC = 24.944). Given the statistical corrections performed, this value for SMC5-SMC6 is highly elevated because the expectation for non-correlated pairs is zero. Yet, which forces led to this high correlation? Does their physical interaction play a role? Given the strong association between physical interactions and other indicators of co-functionality, the relative contribution of their physical interaction is difficult to dissect for SMC5 and SMC6, as well as for most physically interacting proteins.

There are two hypotheses addressing the relative contributions of physical interaction versus co-function to correlated evolutionary rates. The idea that physical interactions contribute more to correlated evolutionary rates hinges on the maintenance of proper binding^{11–13}. Under this hypothesis, a mutation in one binding partner will result in a compensatory mutation in the other, *i.e.* coevolution, consistent with the “lock and key” model for maintenance of physical interactions^{11,13}. Such coincident compensatory changes over long periods of time could lead to correlated changes in rate between the two physically interacting proteins. If the physical interaction hypothesis holds, then there is great interest in using rate covariation tools, such as ERC, to predict quaternary structure and connectivity in protein complexes. However, there is a competing hypothesis that diminishes that potential utility. The second hypothesis is that rate correlations are primarily the result of parallel changes in selective pressures acting upon all genes in that function. Shared selective pressures can result in correlated rates of evolution due to several underlying causes. These causes include relaxation of constraint on a function, which results in accelerated rates for the gene set providing that function. Similarly, increased importance of a function in a species could lead to increased constraint and result in a decrease in evolutionary rate in that species for all genes involved. Adaptive change to a specific function on a branch could also create an rate acceleration for that function’s genes, specifically on that branch. Other sources of variation in selective pressure could result from changes in essentiality or expression level, network connectivity, among other forces.

For the purposes of this study, the forces that contribute to correlated evolutionary rates are grouped into two bins, physical and non-physical. The physical force is coevolution occurring at physical interaction interfaces. Non-physical forces include gene co-expression, codon adaptation, selective pressures, and gene essentiality. There is a well accepted negative relationship between gene expression and rate of protein evolution where genes that are highly expressed generally have slower rates of evolution^{14,15}. However, Cope *et al.*¹⁶ found that there is a weak

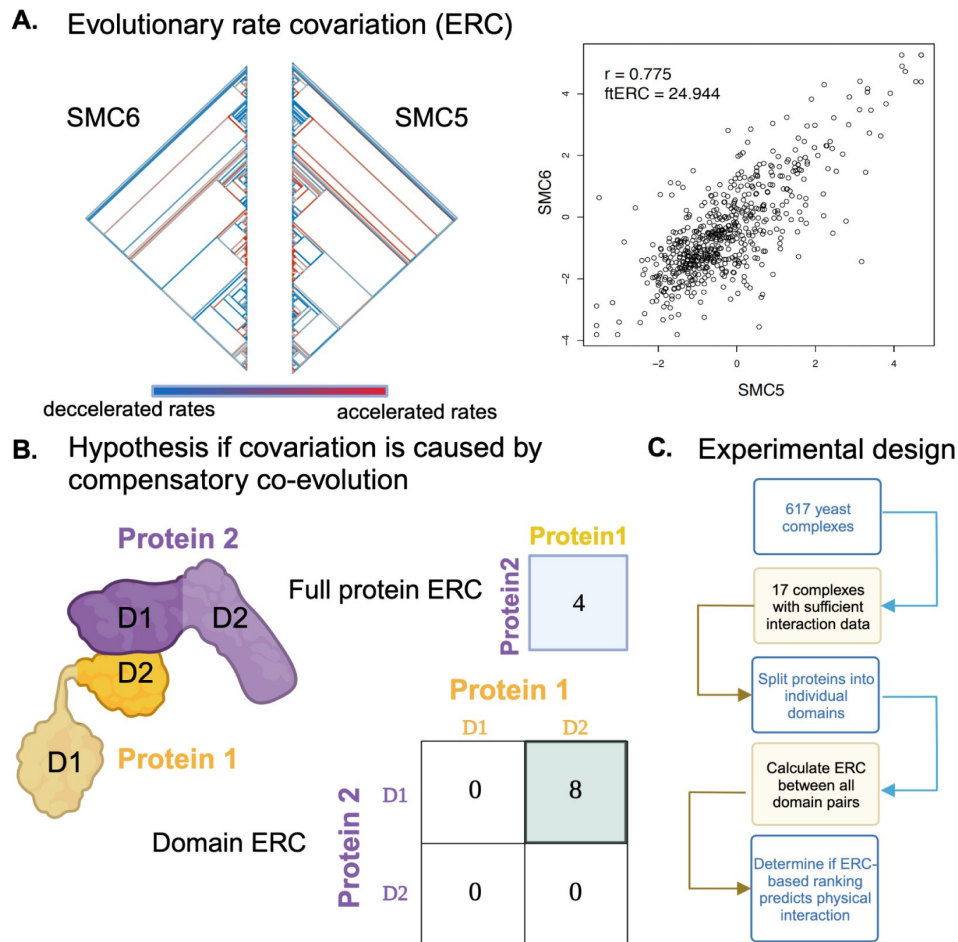


Figure 1

Overview of experimental schema and hypotheses. Proteins that share functional/physical relationships have similar relative rates of evolution across the phylogeny, as shown in (A) with SMC5 and SMC6. The color scale along the bottom indicates the relative evolutionary rate (RER) of the specific protein for that species compared to the genome-wide average. A higher (red) RER indicates that the protein is evolving at a faster rate than the genome average for that branch. Conversely, a lower (blue) RER indicates that protein is evolving at a slower rate than the genome average. The ERC (right) is a Pearson correlation of the RERs for each shared branch of the gene pair. (B) Suppose the correlation in relative evolutionary rates between two proteins is due to compensatory coevolution and physical interactions. In that case, the correlation of their rates (ie. ERC value) would be higher for just the amino acids in the physically interacting domain. (C) Outline of experimental design. Created with Biorender.com

relationship between both gene expression and the number of interactions a protein has with the coevolution of expression level. Conversely, they found a strong relationship between proteins that physically interact and the coevolution of gene expression. These findings illuminate the difference between the strong relationship of gene expression level on the average evolutionary rate of a protein and the weak contribution of gene expression level to correlated evolutionary rates of proteins across branches. The finding that physically interacting proteins have strong expression level coevolution brings to question how much coevolution of physically interacting proteins contributes to overall covariation in protein evolutionary rates.

Previous studies have weighed whether physical coevolution is a strong contributor to rate covariation over time^{9,17,18}. The conclusions drawn from these studies are variable and, in some cases, contradictory. Furthermore, the studies had differing sample sizes and used different protein units to examine the physical interaction, such as surface residues⁹, the binding neighborhood¹⁸, and protein domains¹⁷. It is difficult to compare the conclusions between these different experimental designs. The different units of the protein will, by nature, give different results. For instance, surface residues are under different constraints than buried residues, making it difficult to assess whether the physical interaction is driving the change or if it instead reflects selection on stability of the protein structure. Whereas examining protein domains gives a view of how the entire three-dimensional structure of the protein is potentially affected by changes in the binding partner. Given the contradictory conclusions and lack of statistical power in previous studies, the overall question regarding the contribution of coevolution to the overall rate covariation remains unanswered.

In this study, we test the contribution of compensatory physical coevolution to rate covariation by measuring ERC on a large dataset of 343 yeast species. Specifically, we ask whether physically interacting domains have higher ERC than domains of the same proteins that do not physically interact (**Figure 1B**). To illustrate the expectation being tested under the physical coevolution hypothesis, we present a hypothetical 2-protein complex in **Fig 1B**. Only its 2 physically interacting domains would have an elevated ERC value above the null expectation of zero, or no correlation. Since domains can be analyzed separately, their ERC can be easily quantified in a practical workflow (**Figure 1C**). By looking only within complexes rather than across all proteins with annotated physical interactions, we normalize signals from functional associations, assuming that proteins in the same complex will be under the same functional constraints and, therefore, the same selective pressures and other non-physical forces. Ultimately, we show a weak contribution from physical coevolution and, therefore, poor predictability of physical interactions based on ERC scores, regardless of complex size or average complex ERC. We further show that the ranking of physically interacting domains across the complex has little generalizability in predicting which domains or proteins physically interact.

Results

Protein pathways and complexes both have elevated ERC

Protein pathways and complexes are both functional units of the cell; however, complexes are defined by their physical interactions, while pathways may contain many proteins that do not physically interact at all. To investigate the discrepancy between contributions to ERC signal from co-function and physical interaction, we used a dataset of 343 evolutionarily distant yeast species. 332 of the species are Saccharomycotina with 11 closely related outgroup species providing as much evolutionary divergence as between humans to roundworms¹⁹. This dataset started with 12,552 orthologous genes, which we parsed into annotated pathways from KEGG²⁰ and YeastPathway²¹ and protein complexes from the EMBL-EBI yeast complex portal²².

ERC was calculated for all pairs of the 12,552 genes. For each pair the correlation is Fisher transformed to normalize for the number of shared branches that contribute to the correlation. This normalization is necessary to reduce false positives that have high correlation solely due to a small number of data points. This normalization also allows for direct comparison of ERC between gene pairs that have differing numbers of branches contributing to the score.

In general, ERC values for pathways and complexes were both high and had similar distributions after accounting for their sizes (**Figure 2A**). A majority of complexes (346 of 617) and pathways (109 of 125) had mean ERC values significantly greater ($p < 0.05$) than a null distribution consisting of random size-matched protein sets (**Figure S1**). While protein complexes have higher mean ERC scores (median 5.366) than the pathways (median 4.597), the members of a given complex are also co-functional, making interpretation of the relative contribution of physical interactions to the average ERC score difficult.

To illustrate this difficulty at a more granular level, we present two examples, the SLIK protein complex and the motor protein pathway, both of which have significantly high average ERC values ($p < 0.001$) (**Figure 2B**). It is notable that when the ERC values for all physical interactions within the motor protein pathway are removed, it continues to have a significantly high average ERC. While the highest scoring protein pair in the SLIK complex, TAF5 and TAF6, physically interact and have a highly elevated ERC score (in top 1% genome-wide; **Figure S2**), the highest scoring pair in the motor protein pathway, TUB3 and TPM1, also have a highly elevated ERC score but do not physically interact (**Figure 2C**). This observation, which reflects the global pattern, makes it difficult to determine if the higher score between TAF5 and TAF6 is indicative of a stronger contribution from physical interaction to the ERC value or if co-functionality is the main driver. These observations at both the global level and in individual complexes and pathways prompted further investigation into individual complexes to determine if the physical interactions within a complex have higher average ERC scores.

ERC does not distinguish physical interactions from non-physical interactions within a given protein complex

To more directly test whether the signal contributing to the high rate covariation comes from the coevolution of physical interactions, we divided the proteins in a complex into their domains. A domain-level ERC analysis allowed us to directly contrast physically interacting domains with non-physically interacting domains. To maintain the highest level of confidence in the analyses, we only selected complexes whose members had well-defined domain boundaries and annotated physical interactions between complex members (**Methods**). This resulted in a dataset of 14 complexes spanning functions from transcription and translation to autophagosome formation. We also added three complexes from Jothi *et al.*¹⁷; Mitochondrial F1-ATPase, SEC23/24 heterodimer, and exportin CSE1 with substrate (**Table 1**).

We split the proteins from the 17 complexes into their annotated domains (**Methods**) and calculated ERC between all domains in a complex. The average ERC for the physically interacting and non-physically interacting domains for each complex were compared. Of the 17 complexes, 12 had higher average ERC for the physically interacting than the non-physically interacting. When we look at an individual complex such as the COMA complex (**Figure 3**), the physically interacting domains have some of the highest ERC values. However, other physically interacting domains have some of the lowest ERC scores. To classify how often physically interacting domains have greater ERC and the statistical significance of such results, we proceeded with a rank-based analysis.

The rank-based method, receiver operating characteristic (ROC) curve analysis, ranks the domain pairs based on their ERC score and calculates the true positive rate (TPR) and false positive rate (FPR) based on whether each pair is physically interacting (positive) or not (negative). The

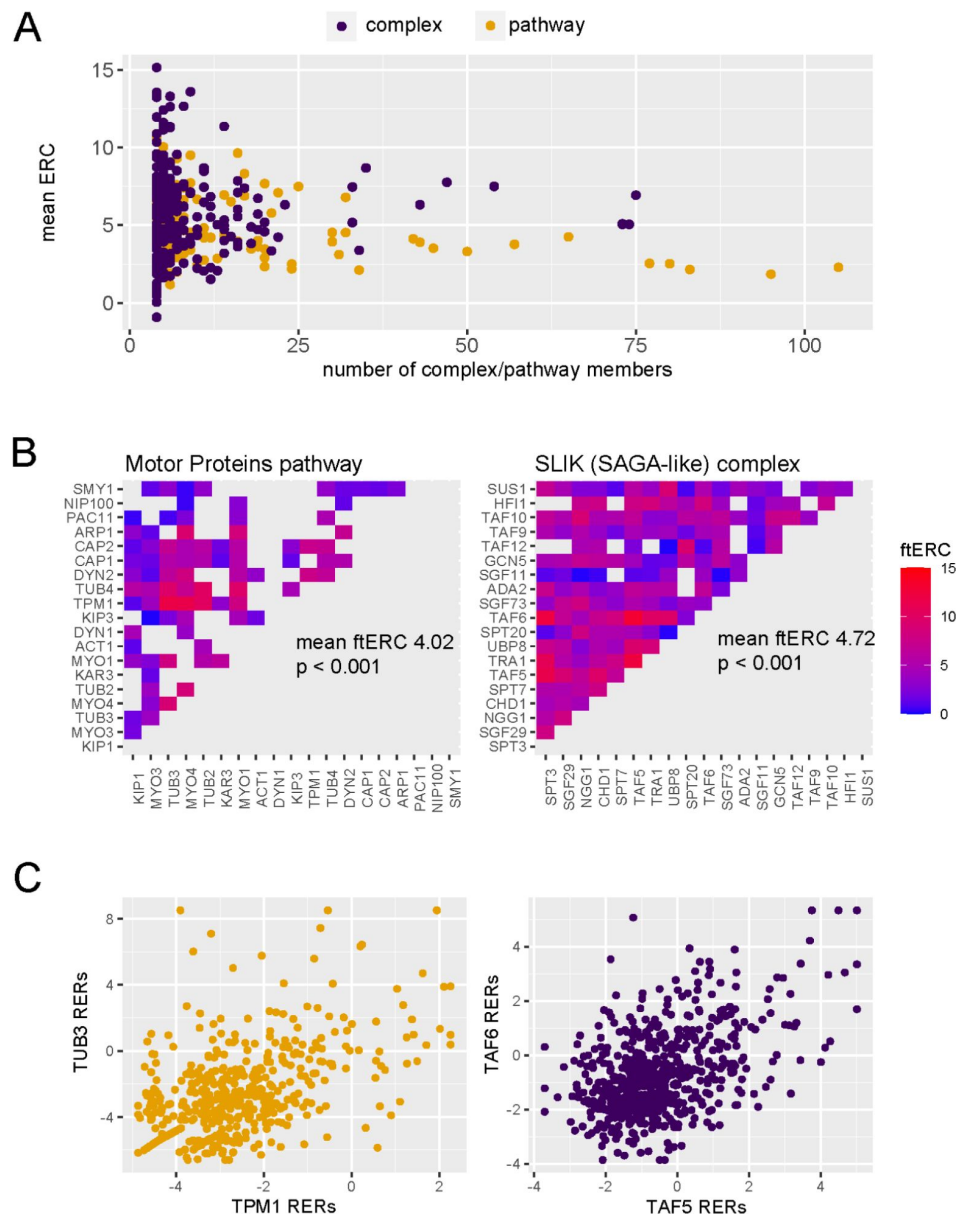


Figure 2

Protein complexes and cellular pathways have significantly high average ERC. (A) The mean ERC values for 617 protein complexes (purple, median 5.366) and 125 cellular pathways (orange, median 4.597) versus the number of members contributing to the score. (B) Heat maps of the ERC scores for each protein pair in the motor proteins pathway (left) and SLIK complex (right). ERC for members of the motor proteins pathways that physically interact was set to NA (gray). (C) Scatter plots of the relative evolutionary rates for the top scoring pair from the motor proteins pathway (orange) and SLIK complex (purple).

Table 1

Complex	Complex ERC	Complex permutation p-value	Physically interacting domain ERC	Non-physically interacting domain ERC	Number of proteins (domains)
Eukaryotic translation initiation factor 3 core complex ²⁸	11.539	<0.001	8.852	11.125	6 (14)
MCM complex ²⁹	12.116	<0.001	10.283	8.000	6 (26)
NUP84 ³⁰	3.590	0.001	1.541	4.411	5 (9)
Origin of replication complex ³¹	5.420	0.012	5.250	4.035	6 (15)
PAN1 actin cytoskeleton-regulatory complex ³²	6.140	0.014	4.963	4.716	3 (13)
SMC5-6 SUMO ligase complex ³³	8.813	0.004	5.153	4.110	4 (15)
TREX ³⁴	5.368	0.02	4.157	4.129	5 (12)
EXOCYST ³⁵	12.040	<0.001	6.221	5.974	8 (21)
COMA complex ³⁶	2.883	0.157	3.051	2.869	4 (11)
SWI/SNF chromatin remodeling complex ^{37,38}	1.853	0.291	4.121	1.783	7 (20)
CUL8-MMS1-MMS22-CTF4 E3 ubiquitin ligase complex ³⁹	0.9925	0.175	2.611	1.576	5 (14)
GET4-GET5 transmembrane domain recognition complex ⁴⁰	2.501	0.17	4.569	3.302	4 (7)
ATG17-ATG31-ATG29 complex ⁴¹	1.825	0.29	3.444	1.584	5 (9)
ESCRT-I complex ⁴²	2.752	0.137	4.041	2.402	4 (8)
Mitochondrial F1 ATPase ¹⁷	11.015	<0.001	4.883	5.654	3 (7)
SEC23/24 heterodimer ¹⁷	9.321	0.004	10.530	7.754	2 (10)
Exportin CSE1 with cargo ¹⁷	10.902	<0.001	8.871	6.326	2 (12)

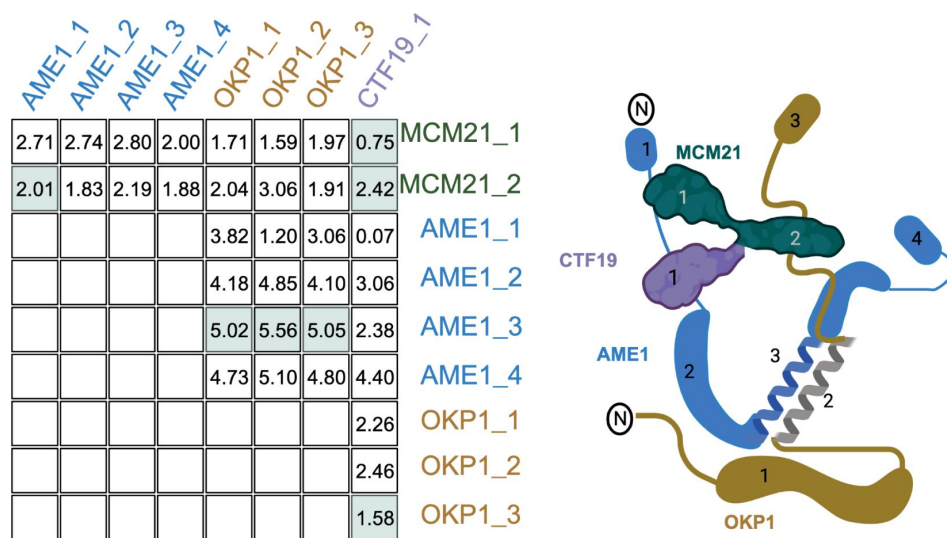


Figure 3

Recreation of **figure 1B** with the COMA complex. The table shows the ERC values for each domain pair as labeled in the cartoon on the right. The domain pairs with physical interactions are highlighted in green.

relationship between TPR and FPR is then plotted on a curve that scores the ability to rank a true positive above a false positive; the curve is summarized by the area under the curve (ROC-AUC). In our analysis, the ROC-AUC gives the proportion of times a random pair of physically interacting domains has a higher ERC score than a random pair of non-physically interacting domains where a value of 1 would indicate that all physical interactions ranked above all non-physical interactions.

Twelve of the seventeen complexes had a ROC-AUC greater than 0.5. Since the random expectation would be that half would exceed 0.5, this is a significant excess (Binomial test, $P = 0.0245$). Moreover, five complexes had AUCs greater than 0.7 (**Figure 4** [↗](#)). These results indicate that physically interacting domains tend to have a higher ERC than non-physically interacting domains in those complexes. Likewise, four complexes had their physically interacting domains ranked significantly higher than non-interacting domains at an alpha of 5% (one-tailed Mann-Whitney U test), which is also a significant excess of complexes overall (Binomial test, $P = 0.0012$). Once again, this indicates a significant amount of ERC signal coming from physically interacting domain pairs within these complexes.

We then applied a third test for a general pattern among all 17 complexes. We generated a null ROC-AUC distribution by permuting the location of the positive class along the ranked list for each complex (**Methods**). We compared the true average ROC-AUC from the 17 complexes (0.596) to the permuted distribution of average ROC-AUCs, which resulted in a permutation p-value of 0.08. The lack of significance in the global permutation test suggests that the global signal for the contribution of physical coevolution to ERC is weak enough that it can be masked by the majority of complexes that show no evidence in favor.

The power of ERC to detect physically interacting domains is not generalizable in single protein pairs

The wide range in ROC-AUCs from the previous analysis suggested the possibility of confounding factors masking the signal. Potential confounders could be: members of the complex moonlighting in other pathways²³ [↗](#) or core membership versus peripheral membership within the complex itself²⁴ [↗](#). To test for the effect of physical interaction free from these confounders, we broke each complex down and only compared the domains within two proteins at a time. Thus, the variation introduced by these potential confounders will be consistent across all domains between a single protein pair, and comparing only their domains pair-by-pair allows cleaner testing of the contribution of physical coevolution to the ERC signal. This analysis also allowed us to determine the significance of a complex having a physical interaction ranked first. If the ranking is significant, it would indicate a future use case of ERC to predict physical interactions.

To determine the significance of the physically interacting domain ranking within each pair of complex proteins, we calculated the proportional rank of the physically interacting domain pair versus all other domain pairs (**Methods**). This metric gave us the proportion of times the physical interaction ranked higher than non-physical interactions. The complexes had individual protein pair proportional rank values spanning the entire range of 0 to 1 without clustering at either extreme (**Figure 5** [↗](#)). These results indicate that even within the same complex, there is a wide variation in how strongly the physical interaction correlates with high ERC.

We then looked at the complex average proportional rank to determine if there was some overarching signal. We took the average for all protein pairs within a complex to get the mean complex proportional rank (**Supplementary data**). The significance of the complex proportional rank was determined by generating a null distribution of proportional rank values for each protein pair and randomly sampling from that to generate a complex-wide null distribution. The observed average for each complex was compared to the null. At a permutation p-value of 0.01, only two complexes had significantly high proportional rank, indicating a strong contribution

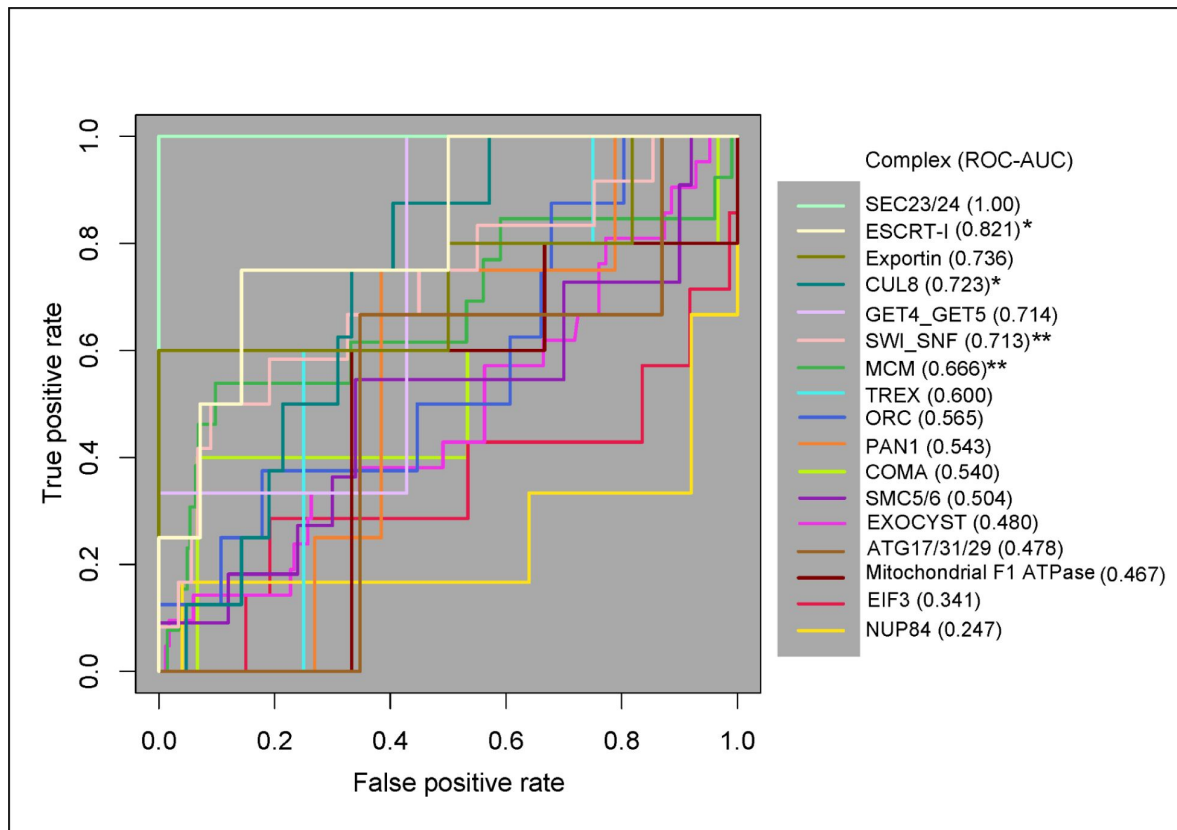


Figure 4

ROC curve analysis of all 17 protein complexes. 12 of the 17 complexes have a ROC-AUC greater than 0.5. The SEC23/24 complex (bright green) has the highest ROC-AUC at 1, and the NUP84 complex (marigold) has the lowest AUC of 0.247. One-tailed Mann-Whitney U test, $p < 0.05^*$, $p < 0.01^{**}$

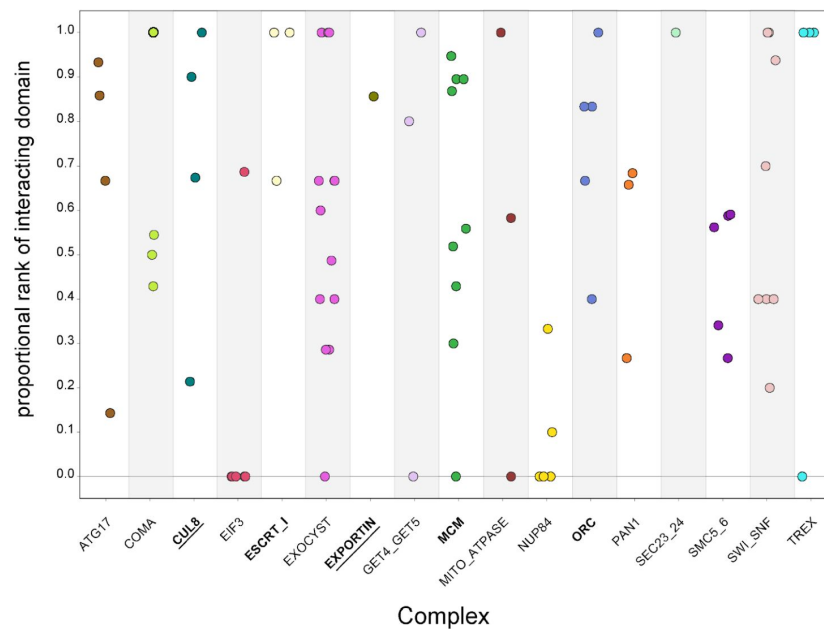


Figure 5

Protein-vs-protein physically interacting domains do not consistently rank higher than non-physically interacting domains. The domains from individual protein pairs within each complex were ranked and the proportional ranking of the physically interacting domain was calculated. Each dot represents the proportional ranking of the physically interacting domain pair for a protein pair, with colors representing the complexes. A score of 1 indicates that the physically interacting domains were ranked first. A score of 0 indicates that the physically interacting domains were ranked last. Permutation test, $p < 0.05$ (**bold**), $p < 0.01$ (**bold and underlined**)

from physical interactions: CUL8-MMS1-MMS22-CTF4 E3 ubiquitin ligase and exportin complexes (Figure 5). Three additional complexes were significant at a permutation p-value of 0.05: MCM, ORC, and ESCRT-I. This leaves over two-thirds of the complexes with no significant contribution from physical interactions to the ERC signal.

Given that some of the complexes ranked the physically interacting domains significantly higher in the proportional rank test suggests that compensatory co-evolution does contribute to the ERC signal. However, the inconsistency of the ranking indicates that there is not a consistent enough signal to confidently call an interaction physical or not and would be of little value to an experimentalist wanting to infer interacting domains. Ultimately, the contribution from physical interactions on the ERC signal is not strong enough to determine if a high-ranking protein pair is associated due to physical interaction instead of non-physical forces.

Discussion

Given the differing conclusions reached in previous studies about the strength of contribution from physical interaction to correlated evolutionary rates^{9,17,18} we aimed to provide a robust conclusion with improved experimental design and sample size. Upon the addition of hundreds of species to the analysis, we were able to look at evidence of compensatory coevolution with increased power at a number of scales: whole protein, domains within complexes, and domains between individual protein pairs. We propose that using entire domains as a unit of study, rather than amino acids at physical interfaces, captures structural changes that could still impact the binding and would not be captured by just examining the binding residues themselves. These changes would still be selected for through compensatory coevolution, and relegating them to the non-interacting category could potentially mask correlated signals.

We found that compensatory coevolution due to physical interaction contributes to evolutionary rate covariation but only in some complexes. Looking across all complexes in our study, a proportion higher than random chance showed elevated ERC between interacting domains, this result was reflected in 12 of the 17 complexes with ROC-AUCs over 0.5 and the 4 complexes with individually significant rankings. Moreover, when we examined only single protein pairs we found a similar excess of high scoring physically interacting domains. This indicates that there is a non-negligible signal from physical interaction contributing to evolutionary rate covariation. Evidence for physical coevolution however was tempered by a global permutation test, which did not reach significance, indicating that this inference is sensitive to approach and further underlines the weak contribution of physical coevolution. In light of this weak contribution and inconsistency between individual protein complexes, the practical application of a tool such as ERC to provide actionable hypotheses for physically interacting domains is not advisable, given the uncertainty as to which complexes would have high ERC between physically interacting domains.

Previous studies attributed varying degrees of evolutionary rate covariation signal to physical interactions between proteins. On one extreme, Hakes *et al.*⁹ found no evidence that the physical interaction interface between two proteins had a greater correlation of evolutionary rates than the whole protein or just surface residues. They concluded this after examining surface and potentially interacting residues across 32 complexes in at least 12 eukaryotic species for each complex using the *S. cerevisiae* sequence as the BLAST query. On the other extreme, Jothi *et al.*¹⁷ concluded that there was a strong enough signal to predict which domains of a protein complex interact with significant accuracy, however their analysis was limited to just 3 protein complexes with orthologs in at least 10 of 93 eukaryotic genomes. Because of their conclusions, we included their complexes in our own study and achieved similar rankings of the physically interacting domains. However, only one of their complexes was statistically significant in our analysis which we attribute to a lack of power given that one of the complexes only had one physically interacting domain pair out of twenty-four pairs. Kann *et al.*¹⁸ reached a similar conclusion to our study

using 70 bacterial species. They found that binding neighborhoods from 26 interacting pairs have a higher evolutionary rate covariation on average than 1291 random non-binding sequences of the same length, ultimately concluding that while physical interactions are not the sole contributor to evolutionary covariation, they still contribute strongly enough to be detected.

These previous studies agreed that compensatory coevolution is not the sole force behind evolutionary rate covariation but they came to differing conclusions as to how much it contributes. This study concludes that there is a detectable but weak contribution from physical interactions that is not strong enough to confidently predict which domains physically interact. This study also found that the contribution of physical interaction was limited to a minority of complexes despite the high power lent from using hundreds of species to calculate ERC; this inconsistent effect across complexes might explain why previous studies and this one found varying degrees of contribution, since results could have depended on which complexes were chosen and which species were used. Among the complexes we found to have a physical coevolution component, there is no obvious reason why these specific complexes would exhibit stronger coevolution between physically interacting domains when compared to other complexes. We hypothesize that while compensatory coevolution is a known phenomenon^{7,10}, amino acid changes resulting from it are relatively rare and are unlikely to contribute greatly to the general ERC signal between co-functional proteins. In contrast, previous work finds evidence that relaxation of selective constraint can lead to drastic rate variation and hence a strong covariation signal (ERC)⁶. Over all, variations in selective constraint, and other non-physical forces, such as essentiality, expression level, codon adaptation, and network connectivity, seem to be the primary contributors to ERC. Indeed, the results of these forces are visible in the elevated ERC values of genetic pathways that do not physically interact (**Figure 2**).

Methods

Calculating evolutionary rate covariation (ERC)

Evolutionary rate covariation is calculated by correlating relative evolutionary rates (RERs) between two gene trees using a Pearson correlation. The relative evolutionary rate is the rate at which a branch on a gene tree changes compared to the genome-wide average and is calculated as described by Kowalczyk *et al.*². The method limits comparisons to gene trees that share at least 15 species and requires that all trees have the same topology. Prior to correlation the RERs are Winsorized, taking the three most extreme values and condensing them to the fourth. This reduces false positives that are due to single outliers skewing the correlation. After calculating the correlation between RERs for each gene pair, the correlation values are Fisher transformed using the equation:

$$ftERC = \arctan(\text{correlation}) * \sqrt{\text{number of branches} - 3}$$

This allows for a direct comparison between gene pairs that don't have the same number of branches contributing.

The full ERC pipeline can be found at: (https://github.com/nclark-lab/erc/tree/main/physical_interaction_paper).

Complex and pathway ERC permutation test

We took the entire yeast complexome from the EMBL complex portal²² and curated yeast pathways from KEGG²⁰ and YeastPathway²¹. These lists were then compared to our dataset and pared down to complexes/pathways that had greater than 4 members. This resulted in 617 protein complexes and 125 pathways.

We then ran 1000 permutations to find the significance of the ERC scores between complex/pathway members. The null distribution was generated by sampling the same number of random genes in the complex/pathway from the entire dataset. The average ERC from the complex/pathway was then compared to the null distribution to get a p-value.

Preparing the protein complexes

14 complexes were chosen by searching for protein complexes within the EMBL yeast complex portal with crosslink, cross-link/mass spectrometry, or crystallographic data. Three additional complexes were added based on Jothi *et al.*¹⁷ Mitochondrial F1 ATPase, SEC23/24 heterodimer, and the exportin CSE1P complexed with cargo. The physical interactions were collected from the literature sources for each complex ([Supplementary data](#)).

Each protein was subdivided into domains by running the amino acid sequence through interproscan²⁵. New gene trees were generated for each domain using phangorn²⁶, and ERC was run to calculate an all-domain-by-all-domain matrix. We generated domain-vs-domain ERC matrices for each of the seventeen complexes ([Supplementary data](#)) that were used for all analyses.

Generating ROC curves

ROC curve analysis was performed using the PRROC package²⁷. First, the ERC matrices for each complex were turned into pairwise edge lists with columns [GENEA, GENEB, ERC, class] and ranked by ERC value. The positive class was defined as the physical interactions found in the primary reference for each complex and denoted with a “1”. The negative class was any protein domain pair without annotated physical interactions, denoted with a “0”.

The statistical significance of the ROC-AUC was determined by a one-tailed Mann-Whitney U test, using the relationship between AUC and U defined by

$$AUC = \frac{U}{n_0 * n_1}$$

Where n_0 is the number of non-physically interacting domains and n_1 is the number of physically interacting domains.

ROC-AUC permutations were calculated by randomly shuffling the order of physical interactions within each complex ranked list 1000 times. The permuted AUC was calculated using the same pipeline as described above. The full study analysis was performed by taking the average from each of the 1000 permutations across all complexes.

Calculating proportional rank of physically interacting domain pairs versus all other domain pairs

To test how often ERC ranks the physical interactions higher than non-physical we compared each pair of proteins individually, where each protein's domains were only compared to one other protein's domains using the scores in [Supplementary data](#) if they shared a physical interaction somewhere along the full protein. For example, in the COMA complex, the domains for OKP1 were only compared to the domains of AME1 because OKP1 does not share a physical interaction with either CTF19 or MCM21. The matrix was then ranked by ERC score. The proportional ranking of the physically interacting domain pair was calculated by:

$$1 - \frac{1 - (\text{rank of physical interaction})}{1 - (\text{total number of pairs})}$$

A score of 1 indicates the physically interacting domain pair is ranked first. A score of 0 indicates the domain pair with a physical interaction was ranked last. If the protein pair had more than one domain pair that had a physical interaction, the average proportional rank score was taken. The

observed complex proportional rank score is the average of each protein's score ([Supplementary data](#)).

The permutation p-value for the complex proportional rank score was calculated by randomly shuffling the ranking of the physical interaction(s) between two proteins 1000 times and calculating the proportional rank each time to generate a null distribution. The null distribution for the entire complex was calculated by randomly selecting a proportional rank value from the null distribution of each protein pair from the complex and averaging it. The observed complex average proportional rank was then compared to this null distribution to calculate the p-value.

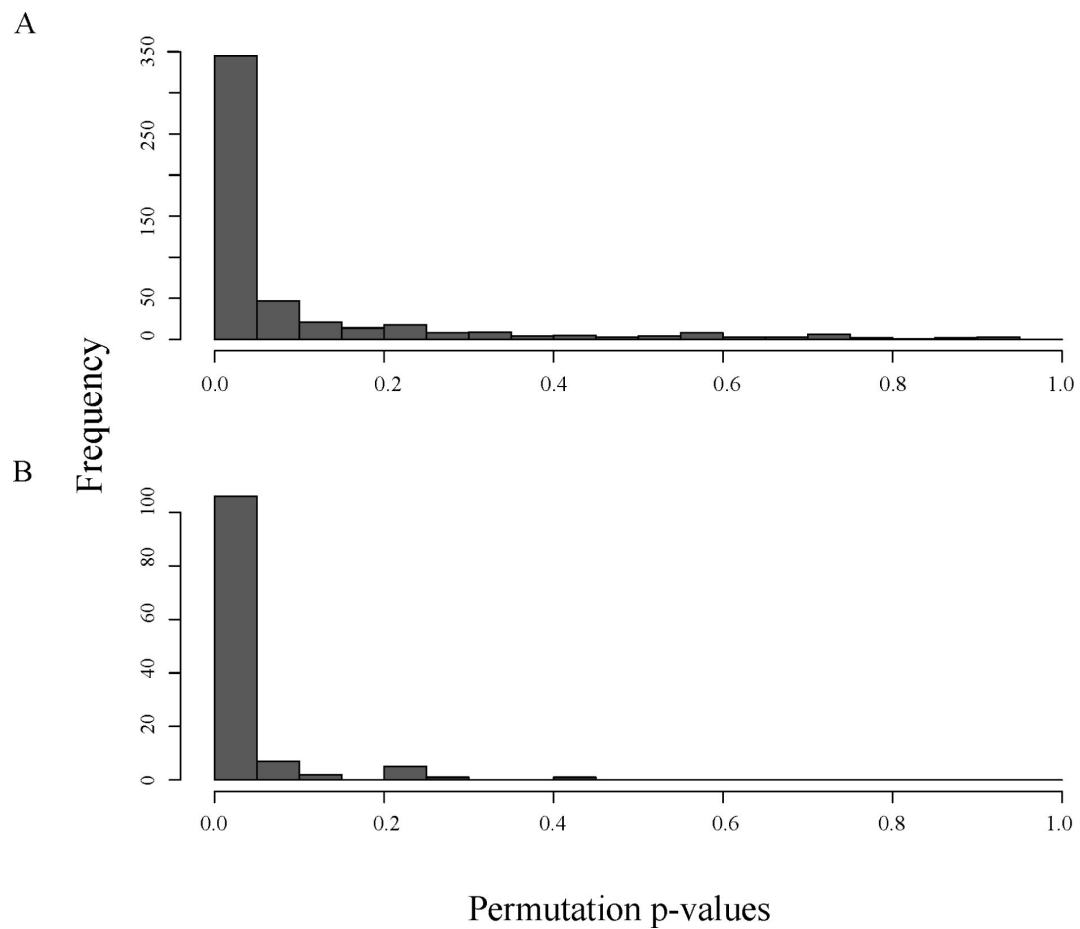
Data Availability

All code and supplementary data can be accessed in the 'erc' GitHub repository: https://github.com/nclark-lab/erc/tree/main/physical_interaction_paper 

Acknowledgements

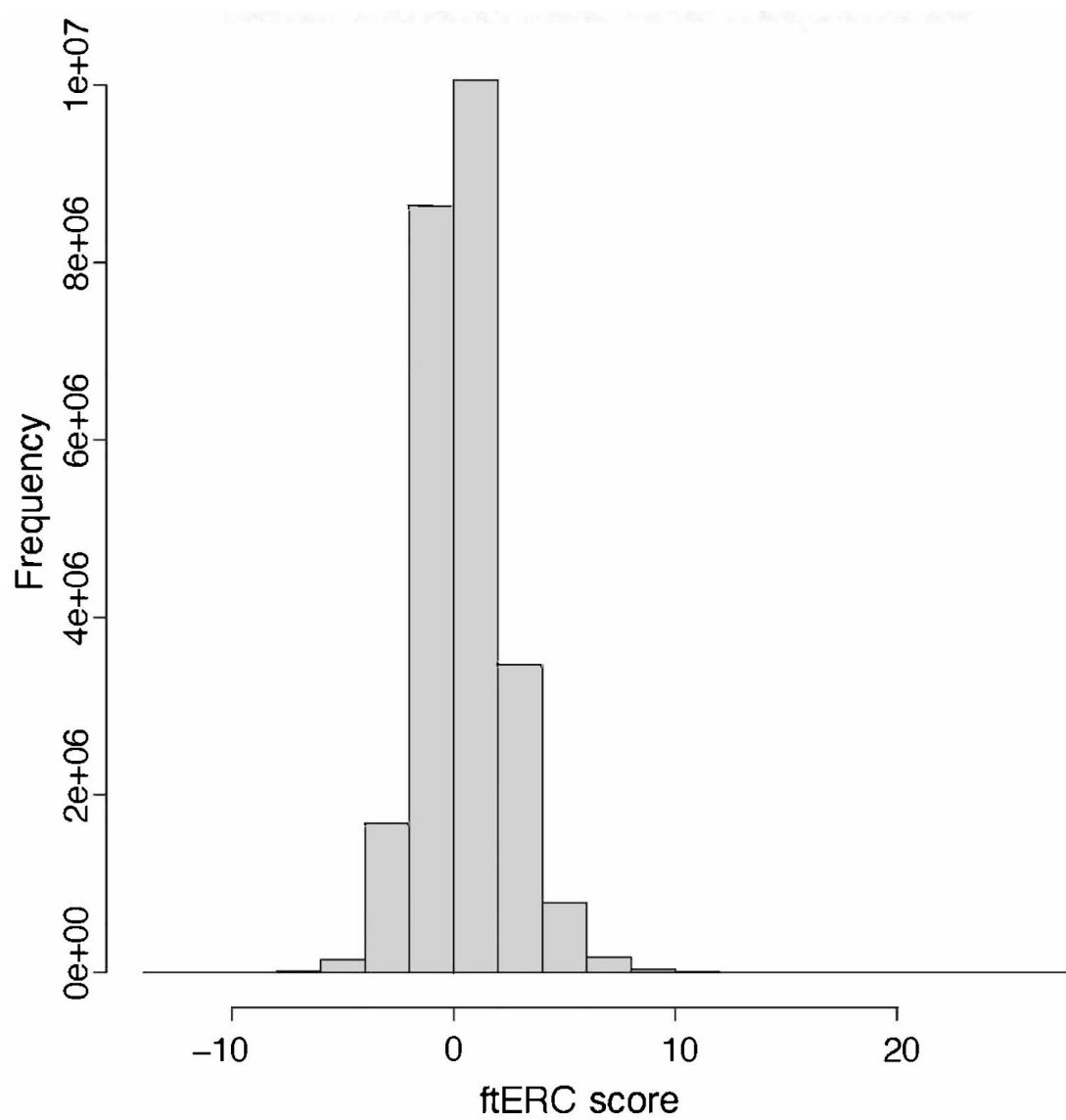
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Supplementary figure 1

Permutation p-value distribution of 617 protein complexes (top) and 125 pathways (bottom) when compared to a null distribution of 1000 samples.



Supplementary figure 2

Histogram of the Fisher transformed ERC values for all 12,552 orthologous genes in the 343 yeast dataset. Median = 0.953.

References

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Reviewer #1 (Public Review):

Summary:

In the manuscript titled "Coevolution due to physical interactions is not a major driving force behind evolutionary rate covariation" by Little et al., explores the potential contribution of physical interaction between correlated evolutionary rates among gene pairs. They find that physical interaction is not the main driving of evolutionary rate covariation (ECR). This finding is similar to a previous report by Clark et al. (2012), *Genome Research*, wherein the authors stated that "direct physical interaction is not required to produce ERC." The previous study used 18 *Saccharomycotina* yeast species, whereas the present study used 332 *Saccharomycotina* yeast species and 11 outgroup taxa. As a result, the present study is better positioned to evaluate the interplay between physical interaction and ECR more robustly.

Strengths & Weaknesses:

Various analyses nicely support the authors' claims.

<https://doi.org/10.7554/eLife.93333.2.sa1>

Reviewer #2 (Public Review):

Summary:

The authors address an important outstanding question: what forces are the primary drivers of evolutionary rate covariation? Exploration of this topic is important because it is currently difficult to interpret the functional/mechanistic implications of evolutionary covariation. These analyses also speak to the predictive power (and limits) of evolutionary rate covariation. This study reinforces the existing paradigm that covariation is driven by a varied/mixed set of interaction-types that all fall under the umbrella explanation of 'co-functional interactions'.

Strengths:

Very smart experimental design that leverages individual protein domains for increased resolution.

Weaknesses:

Nuanced and sometimes inconclusive results that are difficult to capture in a short title/abstract statement.

EDIT: The authors have done a satisfactory job of honing their language to get the nuanced ideas across clearly. The added scholarship and theoretical discussion they added strengthen the impact of the manuscript. The revised edition addresses my concerns.

<https://doi.org/10.7554/eLife.93333.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

The manuscript titled "Coevolution due to physical interactions is not a major driving force behind evolutionary rate covariation" by Little et al., explores the potential contribution of physical interaction between correlated evolutionary rates among gene pairs. The authors find that physical interaction is not the main driving of evolutionary rate covariation (ERC). This finding is similar to a previous report by Clark et al. (2012), Genome Research, wherein the authors stated that "direct physical interaction is not required to produce ERC." The previous study used 18 Saccharomycotina yeast species, whereas the present study used 332 Saccharomycotina yeast species and 11 outgroup taxa. As a result, the present study is better positioned to evaluate the interplay between physical interaction and ERC more robustly.

Strengths & Weaknesses:

Various analyses nicely support the authors' claims. Accordingly, I have only one significant comment and several minor comments that focus on wordsmithing - e.g., clarifying the interpretation of statistical results and requesting additional citations to support claims in the introduction.

We are pleased the reviewer found the analyses to support the claims. We have addressed comments related to clarifying interpretations as suggested in the Recommendations to the Authors. For example, we have added discussion and clarification on the other parameters that could affect the strength of ERC correlations.

Reviewer #2 (Public Review):

Summary:

The authors address an important outstanding question: what forces are the primary drivers of evolutionary rate covariation? Exploration of this topic is important because it is currently difficult to interpret the functional/mechanistic implications of evolutionary covariation. These analyses also speak to the predictive power (and limits) of evolutionary rate covariation. This study reinforces the existing paradigm that covariation is driven by a varied/mixed set of interaction types that all fall under the umbrella explanation of 'co-functional interactions'.

Strengths:

Very smart experimental design that leverages individual protein domains for increased resolution.

Weaknesses:

Nuanced and sometimes inconclusive results that are difficult to capture in a short title/abstract statement.

We appreciate the reviewer's acknowledgement of the experimental design. We have addressed the nuance of the results by changing the title and clarifying other statements

throughout the manuscript as suggested in the reviewer's recommendations. We have also addressed reviewer comments asking for further explanation on using Fisher transformations when normalizing the Pearson correlations for branch counts.

Reviewer #3 (Public Review):

Summary:

The paper makes a convincing argument that physical interactions of proteins do not cause substantial evolutionary co-variation.

Strengths:

The presented analyses are reasonable and look correct and the conclusions make sense.

Weaknesses:

The overall problem of the analysis is that nobody who has followed the literature on evolutionary rate variation over the last 20 years would think that physical interactions are a major cause of evolutionary rate variation. First, there have been probably hundreds of studies showing that gene expression level is the primary driver of evolutionary rate variation (see, for example, [1]). The present study doesn't mention this once. People can argue the causes or the strength of the effect, but entirely ignoring this body of literature is a serious lack of scholarship. Second, interacting proteins will likely be co-expressed, so the obvious null hypothesis would be to ask whether their observed rates are higher or lower than expected given their respective gene expression levels. Third, protein-protein interfaces exert a relatively weak selection pressure so I wouldn't expect them to play much role in the overall evolutionary rate of a protein.

We thank the reviewer for their comments and suggestions. A point to immediately clarify is that the methods studied in this manuscript deal with rate variation of individual proteins over time, and if that variation correlates with that of another protein.. The numerous studies the reviewer refers to deal with explaining the differences in average rate between proteins. These are different sources of variation. It has not, to our knowledge, been shown that variation in the expression level of a single protein over time is responsible for its variation in evolutionary rate over time, let alone to a degree that allows its variation to correlate with that of a functionally related protein. That question interests us, but it is not the focus of this study.

In our study, we sought to test for a contribution of physical interaction to the correlation of evolutionary rate changes as they vary over time, i.e. between branches. We made many changes to clarify this distinction in our revisions.

We agree that the manuscript would be more clear to define the forces proposed to lead to difference in rate in general, which includes expression levels. We had generally considered expression level as one of the many potential non-physical forces, but failed to make that explicit and instead focused on selection pressure. In our revision we describe expression level as another potential driver of evolutionary rate variation over time. References to previous literature have been made in the introduction. We also added a more explicit explanation of the rate covariation over time that we are measuring in contrast with the association between expression level and rate differences between proteins that was studied in previous literature.

On point 3, the authors seem confused though, as they claim a co-evolving interface would evolve faster than the rest of the protein (Figure 1, caption). Instead, the observation is they evolve slower (see, for example, [2]). This makes sense: A binding

interface adds additional constraint that reduces the rate at which mutations accumulate. However, the effect is rather weak.

The values in Fig 1B are a measure of correlation, specifically a Fisher transformed correlation coefficient. They are not evolutionary rates, so they are not reflecting faster or slower evolution, rather more or less covariation of evolutionary rates over time. We are not predicting that physically interacting interfaces evolve faster than the rest of the protein, but rather that if physical interaction drives covariation in evolutionary rates over time, their correlation would be stronger between pairs of physically interacting domains. In response, we have used clearer language in the figure caption and reorganized labels in Figure 1B to clearly show that the values are correlations. Revised Figure 1 Legend:

“Overview of experimental schema and hypotheses. Proteins that share functional/physical relationships have similar relative rates of evolution across the phylogeny, as shown in (A) with SMC5 and SMC6. The color scale along the bottom indicates the relative evolutionary rate (RER) of the specific protein for that species compared to the genome-wide average. A higher (red) RER indicates that the protein is evolving at a faster rate than the genome average for that branch. Conversely, a lower (blue) RER indicates that protein is evolving at a slower rate than the genome average. The ERC (right) is a Pearson correlation of the RERs for each shared branch of the gene pair. (B) Suppose the correlation in relative evolutionary rates between two proteins is due to compensatory coevolution and physical interactions. In that case, the correlation of their rates (ie. ERC value) would be higher for just the amino acids in the physically interacting domain. (C) Outline of experimental design. Created with Biorender.com”

All in all, I'm fine with the analysis the authors perform, and I think the conclusions make sense, but the authors have to put some serious effort into reading the relevant literature and then reassess whether they are actually asking a meaningful question and, if so, whether they're doing the best analysis they could do or whether alternative hypotheses or analyses would make more sense.

[1] <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4523088/>

[2] <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4854464/>

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major comments

(1) Numerous parameters influence ECR calculation. The authors note that their use of a large dataset of budding yeast provides sufficient statistical power to calculate ECR. I agree with that. However, a discussion of other parameters needs to be improved, especially when comparing the present study to others like Kann et al., Hakes et al., and Jothi et al.. For example, what is the evolutionary breadth and depth used in the Kann, Hakes, Jothi and other studies? How does that compare to the present study? Budding yeast evolve rapidly with gene presence/absence polymorphisms observed in genes otherwise considered universally conserved. Is there any reason to expect different results in a younger, slower-evolving clade such as mammals? There is potential to acknowledge and discuss other parameters that may influence ECR, such as codon optimization and gene/complex "essentiality," among others.

More discussion of these parameters is a good idea. We have added the number and phylogeny of species used in the previous studies in the discussion paragraph starting with “Previous studies attributed varying degrees of evolutionary rate covariation signal to

physical interactions between proteins.” We also like the idea of studying the effect of younger and more slowly evolving clades as opposed to the contrary, but currently we lack the required number of datasets to do this.

We have also added more discussion and clarification of potential non-physical forces leading to ERC correlations in the introduction.

Minor comments

(1) It would be good to add a citation to the second sentence of the first paragraph, which reads, “It has been observed that some genes have rates that covary with those of other genes and that they tend to be functionally related.”

Added citation to Clark et al. 2012

(2) In the last sentence of the first paragraph of the introduction, ERC is discussed in the context of only amino acid divergence, however, there is no reason that DNA sequences can't be used, especially if ERC is being calculated among species that are less ancient than, for example, *Saccharomycotina* yeasts. Thus, it may be more accurate to suggest that ERC measures how correlated branch-specific rates of sequence divergence are with those of another gene.

Nice suggestion to generalize. We have made this change.

(3) ERC was not calculated in reference #2. For the sentence “Protein pairs that have high ERC values (i.e., high rate covariation) are often found to participate in shared cellular functions, such as in a metabolic pathway² or meiosis³ or being in a protein complex together,” I think more appropriate citations (including inspiring work by the corresponding author) would be

a) Coevolution of Interacting Fertilization Proteins (<https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1000570>)

b) Evolutionary rate covariation analysis of E-cadherin identifies Raskol as a regulator of cell adhesion and actin dynamics in *Drosophila* (<https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1007720>)

c) An orthologous gene coevolution network provides insight into eukaryotic cellular and genomic structure and function (<https://www.science.org/doi/10.1126/sciadv.abn0105>)

d) PhyKIT: a broadly applicable UNIX shell toolkit for processing and analyzing phylogenomic data (<https://academic.oup.com/bioinformatics/article/37/16/2325/6131675>)

Thank you for pointing out these works. We agree that there are more appropriate citations and we have referenced your suggested b-d.

(4) The dataset of 343 yeast species also includes outgroup taxa. Therefore, indicating that 332 species are *Saccharomycotina* yeast and 11 are closely related outgroup taxa may be more accurate.

Thank you for the suggestion, the following sentence has been added, citing the Shen et. al 2018 paper that the dataset was derived from:

“To investigate the discrepancy between contributions to ERC signal from co-function and physical interaction, we used a dataset of 343 evolutionarily distant yeast species. 332 of the

species are Saccharomycotina with 11 closely related outgroup species providing as much evolutionary divergence as humans to roundworms³

(5) Are there statistics/figures to support the claim that "Almost all complexes and pathways had mean ERC values significantly greater than a null distribution consisting of random protein pairs"?

This is shown in supplementary figure 1. A reference to this figure was added as well as quantification within the text.

(6) Similar to the previous comment, can quantitative values be added to the statement "While protein complexes appear to have higher mean ERC scores than the pathways..."?

The median of the mean ERC scores for protein complexes is 5.366 while the median for the mean ERC score in pathways is 4.597. This quantification has been included in the text: "While protein complexes have higher mean ERC scores (median 5.366) than the pathways (median 4.597), the members of a given complex are also co-functional, making interpretation of the relative contribution of physical interactions to the average ERC score difficult"

These quantifications are were also added to the figure caption for figure 2A

(7) A semantic point: In the sentence "The lack of significance in the global permutation test shows that the...", I recommend saying that the analysis suggests, not shows, because there is potential for a type II error.

Good suggestion, we have made this change.

(8) The authors suggest that shared evolutionary pressures, "and hence shared levels of constraint," drive signatures of coevolution. The manuscript does not delve into selection measures (e.g., dN/dS). Perhaps it would be more representative to remove any implication of selection.

We have added better language to clarify that discussion of selection is purely a hypothesis and that selection is not probed in our analyses.

"Previous work finds evidence that relaxation of selective constraint can lead to drastic rate variation and hence covariation⁶. Rather, the greater and consistent contribution comes from non-physical interaction drivers that could include variation in essentiality, expression level, codon adaptation, and network connectivity. These non-physical forces would be under shared selective pressures and hence shared levels of constraint, the result of which was elevated ERC between non-interacting proteins, as visible in our study of genetic pathways that do not physically interact (Figure 2)."

Reviewer #2 (Recommendations For The Authors):

Major comments:

-Title: In my opinion, the title of the manuscript is a somewhat misleading summary of the results of this paper. In the majority of the analyses in this paper, physical interactions do account for a significantly outsized portion of the ERC signature. The current title downplays the consistent (although sometimes small effect-sized) result that physically interacting domains do show higher ERC than non-physically interacting domains by every statistical measure employed in this paper to compare physical vs non-physical interactions. The authors' interpretation of their results within the manuscript

body is that the effect of physical interactions is an inconsistent, weak, and non-generalizable driver of ERC. I generally agree with the authors' interpretations, but the nuance of these interpretations is lost in the title of the paper. I would suggest rewording the title to try to capture the nuance or at least be subjectively accurate. For example, stating that "...physical interactions are not the sole driving force.." is inarguably accurate based on these results.

As an alternative title, I would suggest focusing on an important takeaway from the paper: ERC is a reliable predictor of co-functional interactions but not necessarily physical interactions. I agree with the statement that "there is not a strong enough signal to confidently call an interaction physical or not and would be of little value to an experimentalist wanting to infer interacting domains" and I think that a title that emphasizes this idea would be more accurate and impactful.

Great suggestion. We agree that the current title is downplaying the minutiae of the method and the signal we capture with it, we have used your suggested title.

There are an outsized number of complexes that had ROC-AUCs greater than 0.5 which is why we performed the permutation tests to determine how significant each of the individual ROC-AUCs were given the differing number of protein/domain pairs in each complex. Between the statistical methods used only 3 of the 17 complexes ranked physical interactions significantly higher than non-physically interacting domains in every analysis. Even among the 3 that were statistically significant some of the physically interacting domains still fell among the bottom portion of the ERC scores for that complex (Figure 5: MCM and CUL8 complexes) This is why we concluded that physical interactions are not the sole driving force of the signal captured by ERC.

-Abstract: related to my preceding comment, the word "negligible" in the abstract is misleading. If physical interactions were truly entirely negligible, the comparisons of physically interacting vs non-physically interacting domains would yield 0.5. Instead, these comparisons always yielded results greater than 0.5. Consider rewording.

Thank you for the suggestion this phrasing has been changed to "Therefore, we conclude that coevolution due to physical interaction is weak, but present in the signal captured by ERC"

We agree that "negligible" may be too strong of a word, however, the comparisons do not always yield results greater than 0.5.

5 of the 17 complexes do not reach the 0.5 threshold for the initial ROC analysis and even among those that do, only 4 had significantly high ROC-AUCs. You are correct that the signal is not completely negligible which is why we continued by determining if the physical interaction was driving high ERC only within proteins (Figure 5)

-Figure 3: I think there may be an error in the domain labeling in Figure 3. The comparison between OKP1_2 and AME1_3 is the highest ERC value in the matrix. From the complex structure, it appears that OKP1_2 and AME1_3 are two helix domains that appear to physically interact. However, in the ERC matrix, they are not shaded to indicate they are a physical interaction pair. Please double-check that the interacting domains are properly annotated, since mis-annotation would have a large impact on the interpretation of this figure with respect to the overall question the paper addresses.

Thank you for catching this - fixed.

Minor comments:

*-Methods: "The full ERC pipeline can be found at (Github)." Provide github URL here?
Thanks for the catch, fixed*

-Discussion: "Evidence for physical coevolution however was tempered by a global permutation test, which did not reach significance, indicating that this inference is sensitive to approach and further underlines the relatively weak contribution of physical coevolution." The word "relatively" may not be a good choice of words. In comparison to what? As is, the phrasing could be interpreted as implying "in comparison to non-physical interactions". This would not be accurate, because the results show that in general, physical interactions are a stronger contributor to ERC (consistent trend but varied significance, depending on methodology) than non-physical interactions.

Thank you for your help with clarification. The word relatively was removed.

However, we do not agree that in general physical interactions are a stronger contributor to ERC than non-physical interactions (such as gene expression, codon adaptation, etc.). In all of our statistical tests a maximum of 5 of the 17 complexes ranked physical interactions significantly higher than non-physical interactions. While the ROC-AUC is greater than 0.5 for 12 of the 17 complexes only 4 of those were significant.

-I have not seen Fisher-transformed correlation coefficients used in the context of ERC. I understand that it's helpful in normalizing the results so that they are comparable between ERC comparisons with differing numbers of overlapping branches (i.e. points on a linear correlation plot). A reference of where the authors got this idea or a little more verbiage to describe the rationale would be helpful. On a related note, I would expect that using linear correlation p-value instead of R-squared would account for differences in overlapping branches, eliminating the need to apply fisher-transformation. It would be helpful for the authors to outline their rationale for using a correlation coefficient rather than a P-value.

We agree that this method could be made clearer. We made a methodological choice to use Fisher transformation over linear correlation p-value. Both methods should achieve the same end result by taking the number of branches into consideration. We have added additional explanation to the results section "Both protein pathways and complexes have elevated ERC":

"ERC was calculated for all pairs of the 12,552 genes. For each pair the correlation is Fisher transformed to normalize for the number of shared branches that contribute to the correlation. This normalization is necessary to reduce false positives that have high correlation solely due to a small number of data points. This normalization also allows for direct comparison of ERC between gene pairs that have differing numbers of branches contributing to the score."

We also added additional explanation in the methods section including the formula used to calculate the Fisher transformation

-Did the authors use Pearson or Spearman correlation coefficient?

Pearson. We clarified this in the methods section, "Calculating evolutionary rate covariation": "Evolutionary rate covariation is calculated by correlating relative evolutionary rates (RERs) between two gene trees using a Pearson correlation."

-Did the authors explore ERC between domains within a single protein? Do domains within a protein exhibit ERC? I would expect that they do. If they do, this could likely be attributed to linkage/genetic hitchhiking, representing a new angle/factor beyond

physical interaction that could lead to ERC. This is just an idea for a future analysis, not necessarily a request within the scope of the present paper.

We did calculate the ERC between domains of a single protein but did not include them in the analysis since they didn't address the specific question we posed. As expected they are highly correlated, and past unpublished studies in the lab do find a very weak, but detectable genome-wide, signature of rate covariation between neighboring colinear genes on a chromosome. That signal was however so weak as to be eclipsed by true functional relationships, when present.

Reviewer #3 (Recommendations For The Authors):

Please read the literature and revise accordingly.

We understand the confusion surrounding previous literature on the relationship between expression levels and evolutionary rates when comparing between different proteins. Those studies clearly showed how expression level is highly predictive of a given protein's average evolutionary rate. However, we are studying the change in evolutionary rate over branches for single proteins. This is inherently different because we're following rate fluctuations in the same protein over time. To our knowledge it has not yet been shown that expression level commonly varies enough over time to produce large rate variations over time in the same protein, and if it is responsible for the correlations of rate we observe between co-functional proteins. It is however reasonable to expect that what governs between-protein differences in rate could also contribute to between-branch differences (over time for a single protein). In fact, our earlier study approached this (Clark et al. Genome Research 2012). We expect expression level could influence rate over time and lump its effect together with general non-physical forces, such as selection pressures. We recognize we could do better in defining more of the non-physical forces and the past literature. We added the following section to the introduction and many other clarifying statements throughout the manuscript:

“For the purposes of this study, the forces that contribute to correlated evolutionary rates are grouped into two bins, physical and non-physical. The physical force is coevolution occurring at physical interaction interfaces. Non-physical forces include gene co-expression, codon adaptation, selective pressures, and gene essentiality. There is a well accepted negative relationship between gene expression and rate of protein evolution where genes that are highly expressed generally have slower rates of evolution^{14,15}. However, Cope et al.¹⁶ found that there is a weak relationship between both gene expression and the number of interactions a protein has with the coevolution of expression level. Conversely, they found a strong relationship between proteins that physically interact and the coevolution of gene expression. These findings illuminate the difference between the strong relationship of gene expression level on the average evolutionary rate of a protein and the weak contribution of gene expression level to correlated evolutionary rates of proteins across branches. The finding that physically interacting proteins have strong expression level coevolution brings to question how much coevolution of physically interacting proteins contributes to overall covariation in protein evolutionary rates.”