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


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## Keywords

Protein co-evolution, Evolutionary rates, Phylogenetic correlations, Protein interactions

## 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.

## 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 related[1]. Evolutionary rate covariation (ERC) is a measure of that correlation in relative evolutionary rates[2] (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.

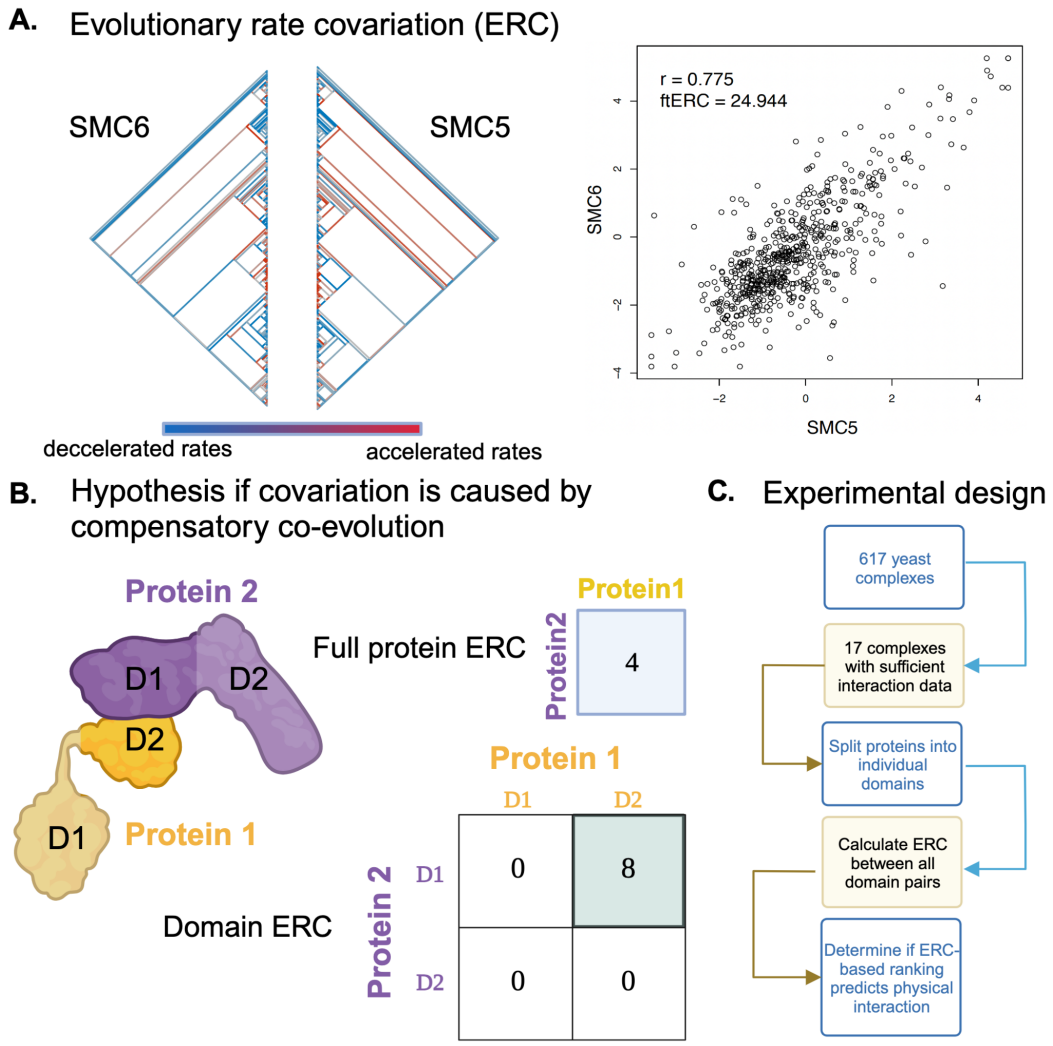


*Figure 1:*

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

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 meiosis[6] 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 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 residues[9], the binding neighborhood[18], and protein domains[17]. 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 roundworms[19]. This dataset started with 12,552 orthologous genes, which we parsed into annotated pathways from KEGG[20] and YeastPathway[21] and protein complexes from the EMBL-EBI yeast complex portal[22].

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

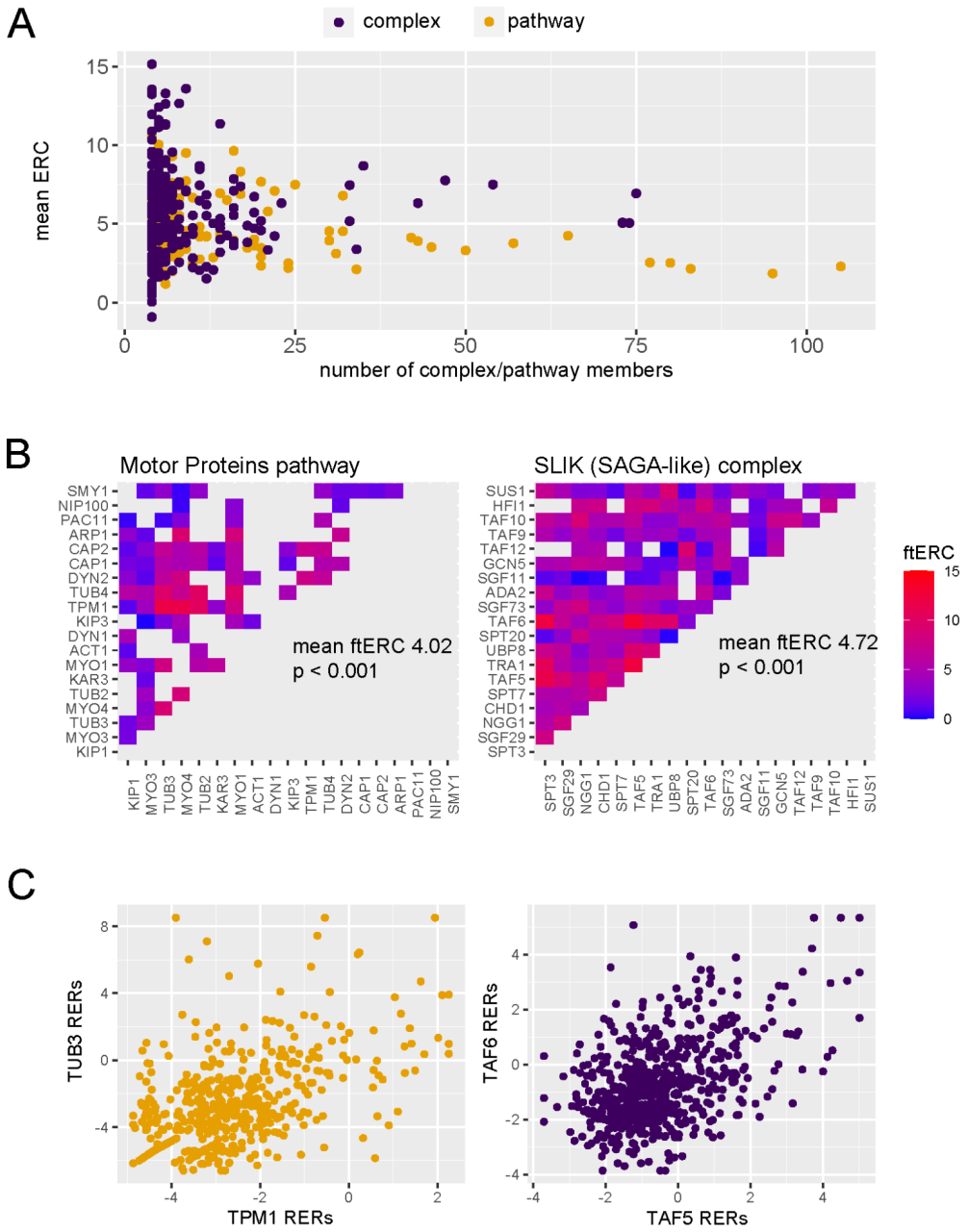


*Figure 2:*

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).

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.

| | AME1_1 | AME1_2 | AME1_3 | AME1_4 | OKP1_1 | OKP1_2 | OKP1_3 | CTF19_1 |
|---|---|---|---|---|---|---|---|---|
| MCM21_1 | 2.71 | 2.74 | 2.80 | 2.00 | 1.71 | 1.59 | 1.97 | 0.75 |
| MCM21_2 | 2.01 | 1.83 | 2.19 | 1.88 | 2.04 | 3.06 | 1.91 | 2.42 |
| AME1_1 | | | | | 3.82 | 1.20 | 3.06 | 0.07 |
| AME1_2 | | | | | 4.18 | 4.85 | 4.10 | 3.06 |
| AME1_3 | | | | | 5.02 | 5.56 | 5.05 | 2.38 |
| AME1_4 | | | | | 4.73 | 5.10 | 4.80 | 4.40 |
| OKP1_1 | | | | | | | | 2.26 |
| OKP1_2 | | | | | | | | 2.46 |
| OKP1_3 | | | | | | | | 1.58 |

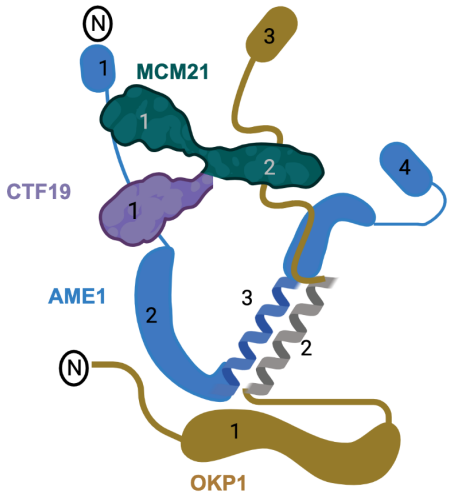


*Figure 3:*

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.

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 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.

*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) |

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.

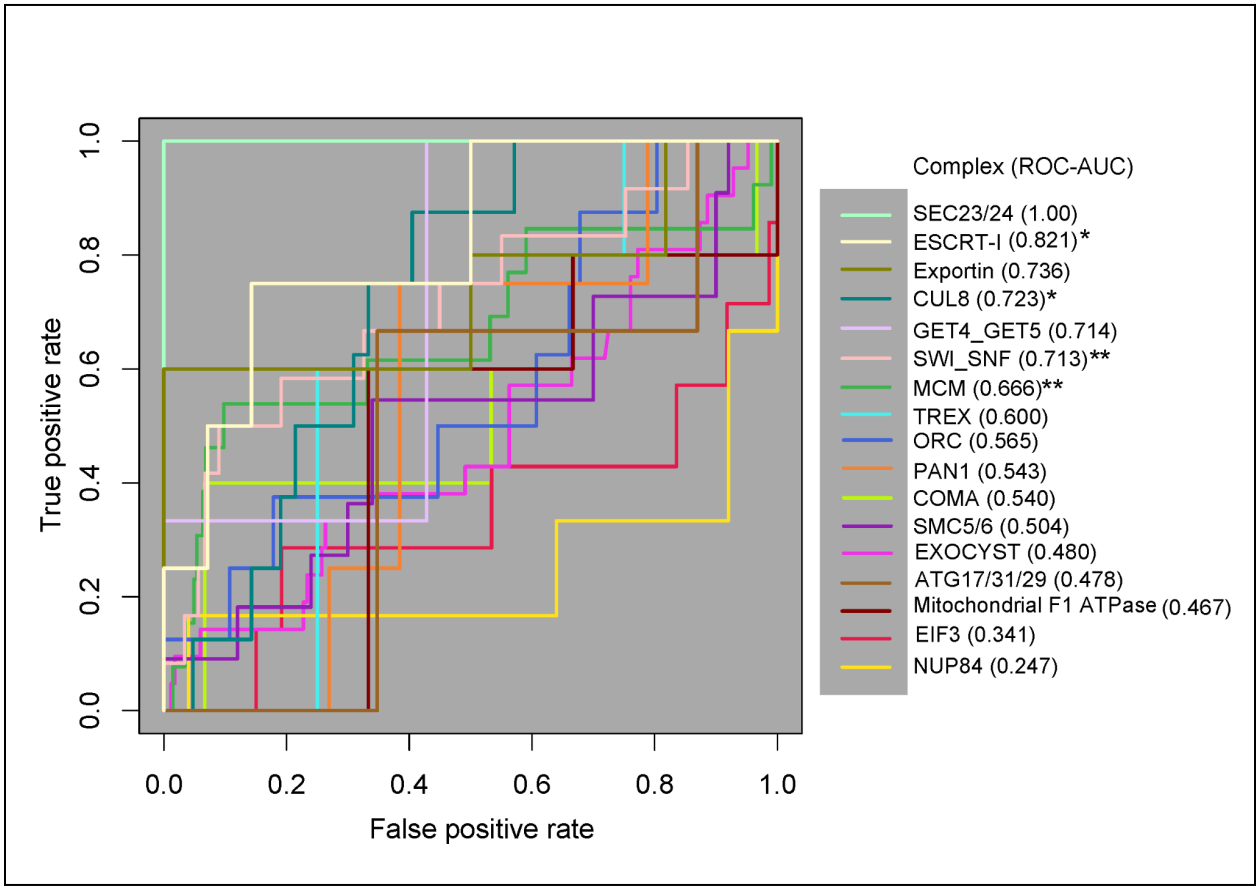


*Figure 4:*

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, $p < 0.05$*, $p<0.01$**

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 pathways[23] or core membership versus peripheral membership within the complex itself[24]. 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.

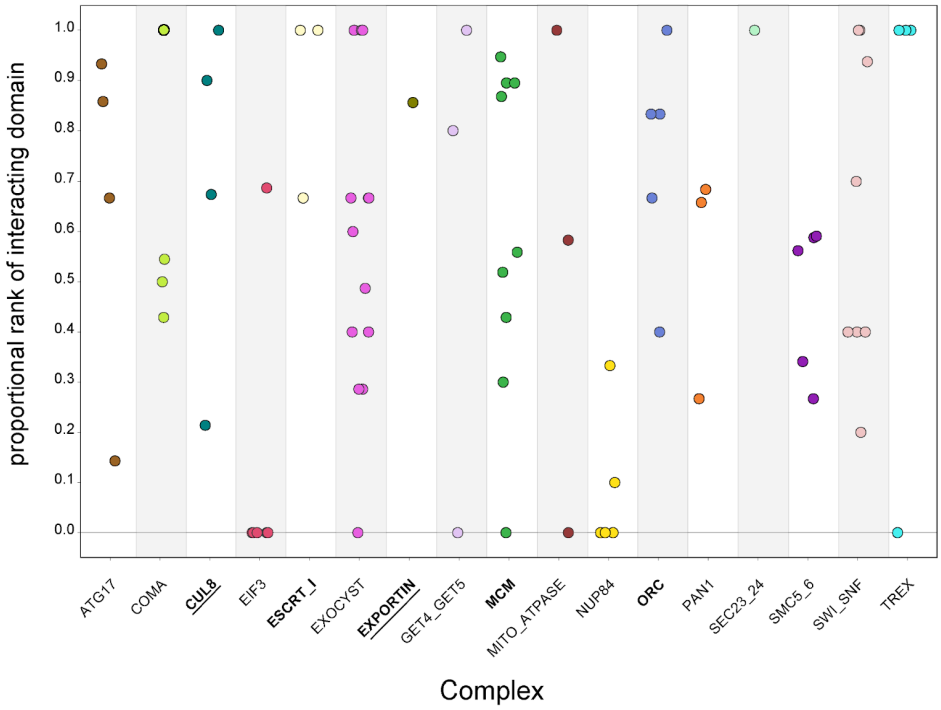


*Figure 5:*

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 rank of the 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**)

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 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:

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

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 portal[22] and curated yeast pathways from KEGG[20] and YeastPathway[21]. 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 interpro scan[25]. New gene trees were generated for each domain using phangorn[26], 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 package[27]. 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:

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

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**

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## References

1. Clark, N. L., Alani, E. & Aquadro, C. F. Evolutionary rate covariation reveals shared functionality and coexpression of genes. *Genome Res.* **22**, 714–720 (2012).

2. Kowalczyk, A. *et al.* RERconverge: an R package for associating evolutionary rates with convergent traits. *Bioinformatics* **35**, 4815–4817 (2019).
3. Findlay, G. D. *et al.* Evolutionary Rate Covariation Identifies New Members of a Protein Network Required for Drosophila melanogaster Female Post-Mating Responses. *PLOS Genet.* **10**, e1004108 (2014).
4. Steenwyk, J. L. *et al.* PhyKIT: a broadly applicable UNIX shell toolkit for processing and analyzing phylogenomic data. *Bioinformatics* **37**, 2325–2331 (2021).
5. Steenwyk, J. L. *et al.* An orthologous gene coevolution network provides insight into eukaryotic cellular and genomic structure and function. *Sci. Adv.* **8**, eabn0105 (2022).
6. Clark, N. L., Alani, E. & Aquadro, C. F. Evolutionary Rate Covariation in Meiotic Proteins Results from Fluctuating Evolutionary Pressure in Yeasts and Mammals. *Genetics* **193**, 529–538 (2013).
7. Gershoni, M. *et al.* Coevolution Predicts Direct Interactions between mtDNA-Encoded and nDNA-Encoded Subunits of Oxidative Phosphorylation Complex I. *J. Mol. Biol.* **404**, 158–171 (2010).
8. Fraser, H. B., Hirsh, A. E., Wall, D. P. & Eisen, M. B. Coevolution of gene expression among interacting proteins. *Proc. Natl. Acad. Sci.* **101**, 9033–9038 (2004).
9. Hakes, L., Lovell, S. C., Oliver, S. G. & Robertson, D. L. Specificity in protein interactions and its relationship with sequence diversity and coevolution. *Proc. Natl. Acad. Sci.* **104**, 7999–8004 (2007).
10. Juan, D., Pazos, F. & Valencia, A. High-confidence prediction of global interactomes based on genome-wide coevolutionary networks. *Proc. Natl. Acad. Sci.* **105**, 934–939 (2008).
11. Ramani, A. K. & Marcotte, E. M. Exploiting the Co-evolution of Interacting Proteins to Discover Interaction Specificity. *J. Mol. Biol.* **327**, 273–284 (2003).
12. Salmanian, S., Pezeshk, H. & Sadeghi, M. Inter-protein residue covariation information unravels physically interacting protein dimers. *BMC Bioinformatics* **21**, 584 (2020).

13. Goh, C. S., Bogan, A. A., Joachimiak, M., Walther, D. & Cohen, F. E. Co-evolution of proteins with their interaction partners. *J. Mol. Biol.* **299**, 283–293 (2000).

14. Drummond, D. A., Raval, A. & Wilke, C. O. A Single Determinant Dominates the Rate of Yeast Protein Evolution. *Mol. Biol. Evol.* **23**, 327–337 (2006).

15. Drummond, D. A., Bloom, J. D., Adami, C., Wilke, C. O. & Arnold, F. H. Why highly expressed proteins evolve slowly. *Proc. Natl. Acad. Sci.* **102**, 14338–14343 (2005).

16. Cope, A. L., O'Meara, B. C. & Gilchrist, M. A. Gene expression of functionally-related genes coevolves across fungal species: detecting coevolution of gene expression using phylogenetic comparative methods. *BMC Genomics* **21**, 370 (2020).

17. Jothi, R., Cherukuri, P. F., Tasneem, A. & Przytycka, T. M. Co-evolutionary Analysis of Domains in Interacting Proteins Reveals Insights into Domain–Domain Interactions Mediating Protein–Protein Interactions. *J. Mol. Biol.* **362**, 861–875 (2006).

18. Kann, M. G., Shoemaker, B. A., Panchenko, A. R. & Przytycka, T. M. Correlated evolution of interacting proteins: looking behind the mirrortree. *J. Mol. Biol.* **385**, 91–98 (2009).

19. Shen, X.-X. *et al.* Tempo and Mode of Genome Evolution in the Budding Yeast Subphylum. *Cell* **175**, 1533-1545.e20 (2018).

20. Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M. & Ishiguro-Watanabe, M. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res.* **51**, D587–D592 (2023).

21. Cherry, J. M. *et al.* Saccharomyces Genome Database: the genomics resource of budding yeast. *Nucleic Acids Res.* **40**, D700–D705 (2012).

22. Meldal, B. H. M. *et al.* Complex Portal 2018: extended content and enhanced visualization tools for macromolecular complexes. *Nucleic Acids Res.* **47**, D550–D558 (2019).

23. Mani, M. *et al.* MoonProt: a database for proteins that are known to moonlight. *Nucleic*

*Acids Res.* **43**, D277–D282 (2015).

24. Chakraborty, S., Kahali, B. & Ghosh, T. C. Protein complex forming ability is favored over the features of interacting partners in determining the evolutionary rates of proteins in the yeast protein-protein interaction networks. *BMC Syst. Biol.* **4**, 155 (2010).

25. Jones, P. *et al.* InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30**, 1236–1240 (2014).

26. Schliep, K. P. phangorn: phylogenetic analysis in R. *Bioinformatics* **27**, 592–593 (2011).

27. Grau, J., Grosse, I. & Keilwagen, J. PRROC: computing and visualizing precision-recall and receiver operating characteristic curves in R. *Bioinformatics* **31**, 2595–2597 (2015).

28. Politis, A. *et al.* Topological Models of Heteromeric Protein Assemblies from Mass Spectrometry: Application to the Yeast eIF3:eIF5 Complex. *Chem. Biol.* **22**, 117–128 (2015).

29. Frigola, J. *et al.* Cdt1 stabilizes an open MCM ring for helicase loading. *Nat. Commun.* **8**, 15720 (2017).

30. Shi, Y. *et al.* Structural Characterization by Cross-linking Reveals the Detailed Architecture of a Coatomer-related Heptameric Module from the Nuclear Pore Complex. *Mol. Cell. Proteomics* **13**, 2927–2943 (2014).

31. Feng, X. *et al.* The structure of ORC–Cdc6 on an origin DNA reveals the mechanism of ORC activation by the replication initiator Cdc6. *Nat. Commun.* **12**, 3883 (2021).

32. Complex Portal - CPX-426. https://www.ebi.ac.uk/complexportal/complex/CPX-426.

33. Yu, Y. *et al.* Integrative analysis reveals unique structural and functional features of the Smc5/6 complex. *Proc. Natl. Acad. Sci.* **118**, e2026844118 (2021).

34. Xie, Y. *et al.* Cryo-EM structure of the yeast TREX complex and coordination with the SR-like protein Gbp2. *eLife* **10**, e65699 (2021).

35. Ganesan, S. J. *et al.* Integrative structure and function of the yeast exocyst complex. *Protein Sci.* **29**, 1486–1501 (2020).

36. Fischböck-Halwachs, J. *et al.* The COMA complex interacts with Cse4 and positions

Sli15/Ipl1 at the budding yeast inner kinetochore. *eLife* **8**, e42879 (2019).

37. Han, Y., Reyes, A. A., Malik, S. & He, Y. Cryo-EM structure of SWI/SNF complex bound to a nucleosome. *Nature* **579**, 452–455 (2020).

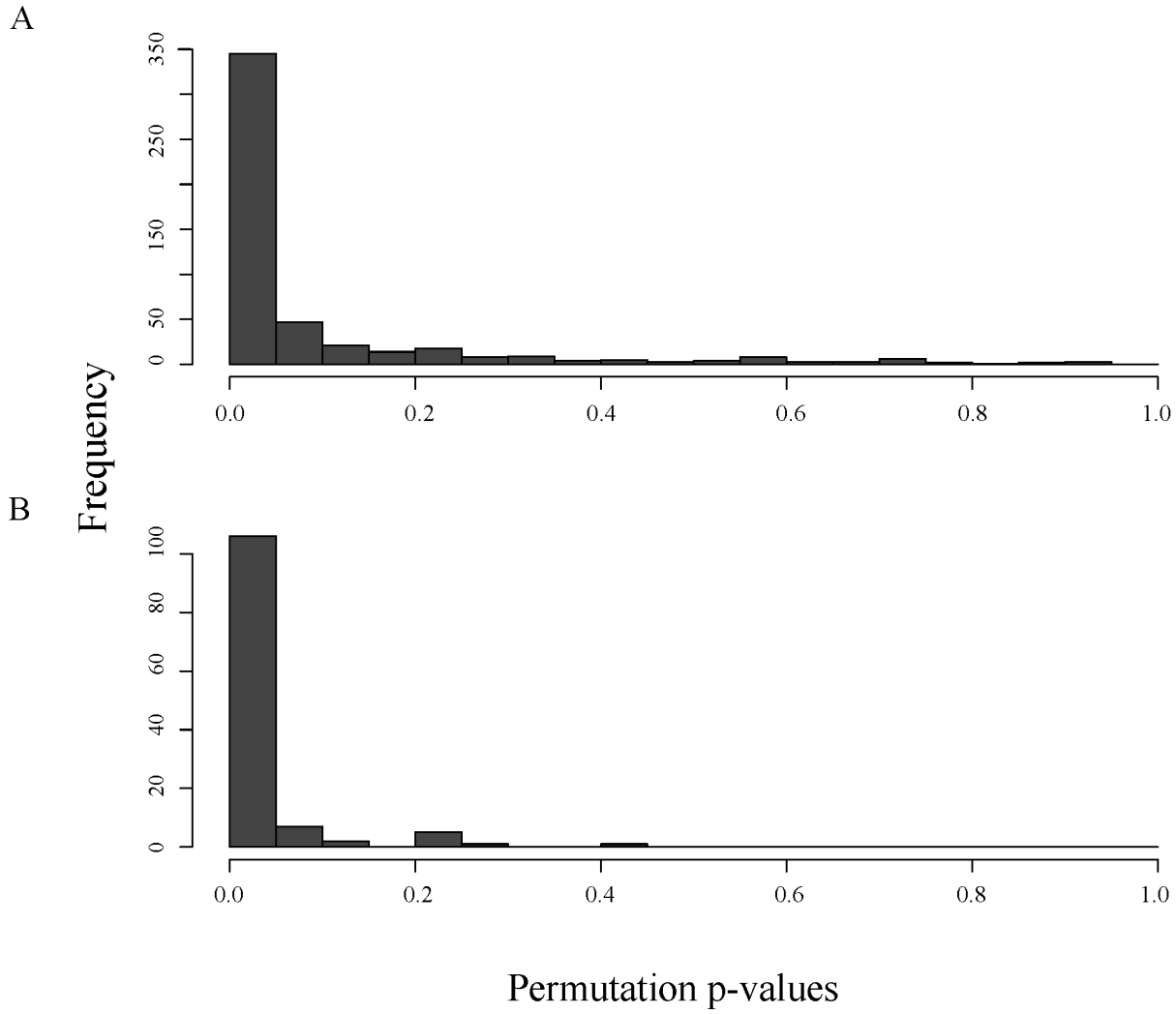
38. Schubert, H. L. *et al.* Structure of an actin-related subcomplex of the SWI/SNF chromatin remodeler. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3345–3350 (2013).

39. Mimura, S. *et al.* Cul8/Rtt101 forms a variety of protein complexes that regulate DNA damage response and transcriptional silencing. *J. Biol. Chem.* **285**, 9858–9867 (2010).

40. Chang, Y.-W. *et al.* Crystal Structure of Get4-Get5 Complex and Its Interactions with Sgt2, Get3, and Ydj1♦. *J. Biol. Chem.* **285**, 9962–9970 (2010).

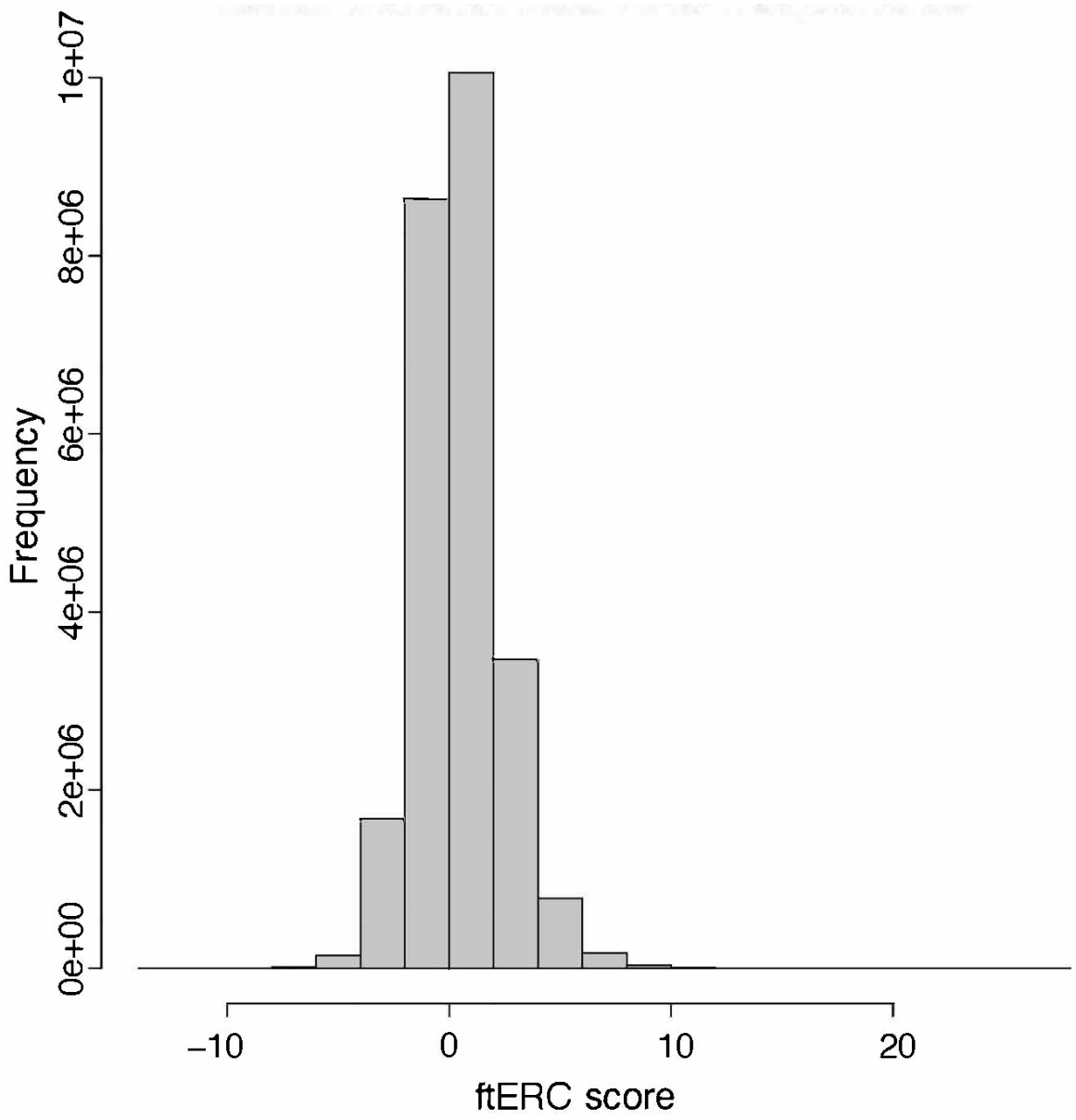
41. Ragusa, M. J., Stanley, R. E. & Hurley, J. H. Architecture of the Atg17 complex as a scaffold for autophagosome biogenesis. *Cell* **151**, 1501–1512 (2012).

42. PMC, E. Molecular Architecture and Functional Model of the Complete Yeast ESCRT-I Heterotetramer. *Cell* **129**, 485 (2007).



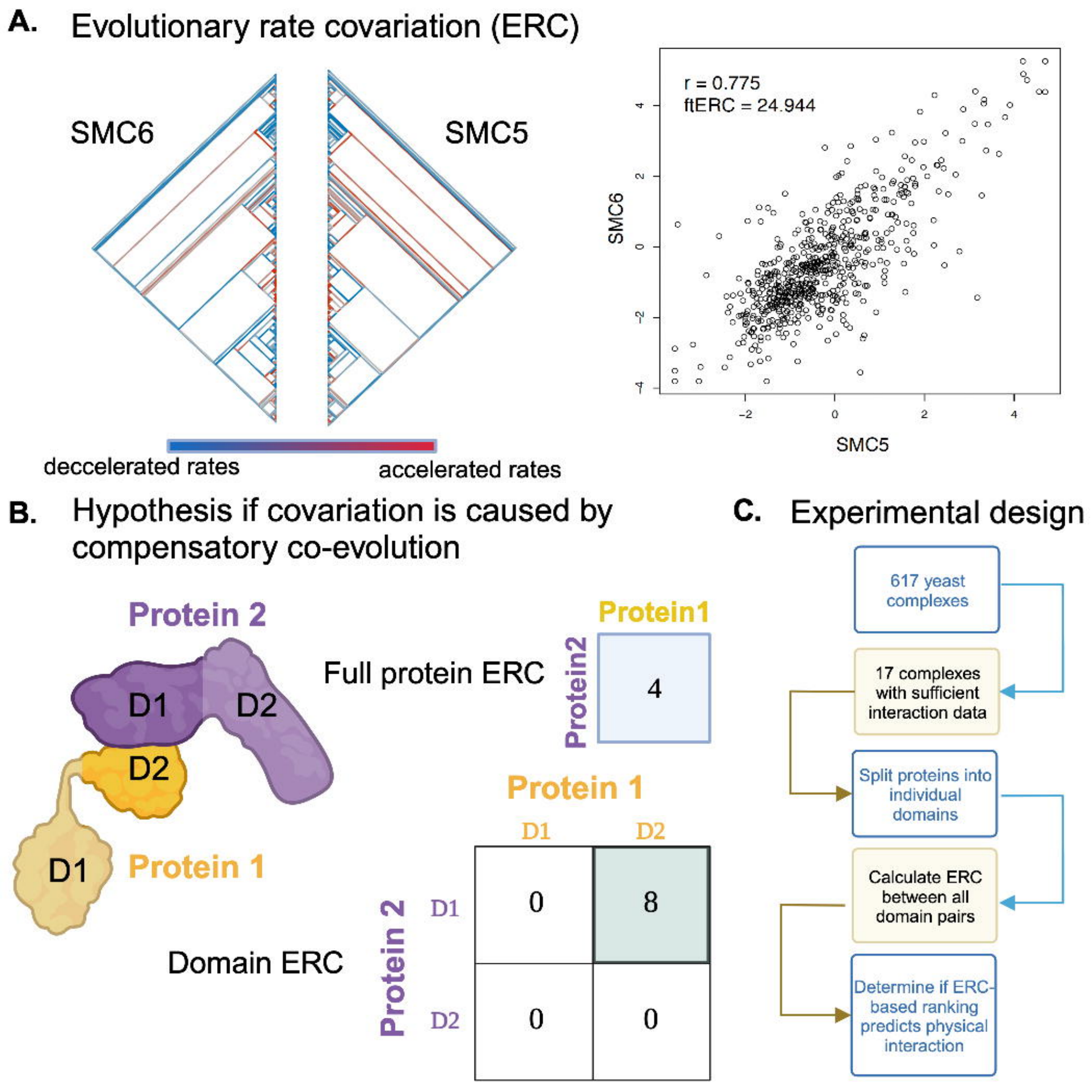

*Supplementary figure 1:*

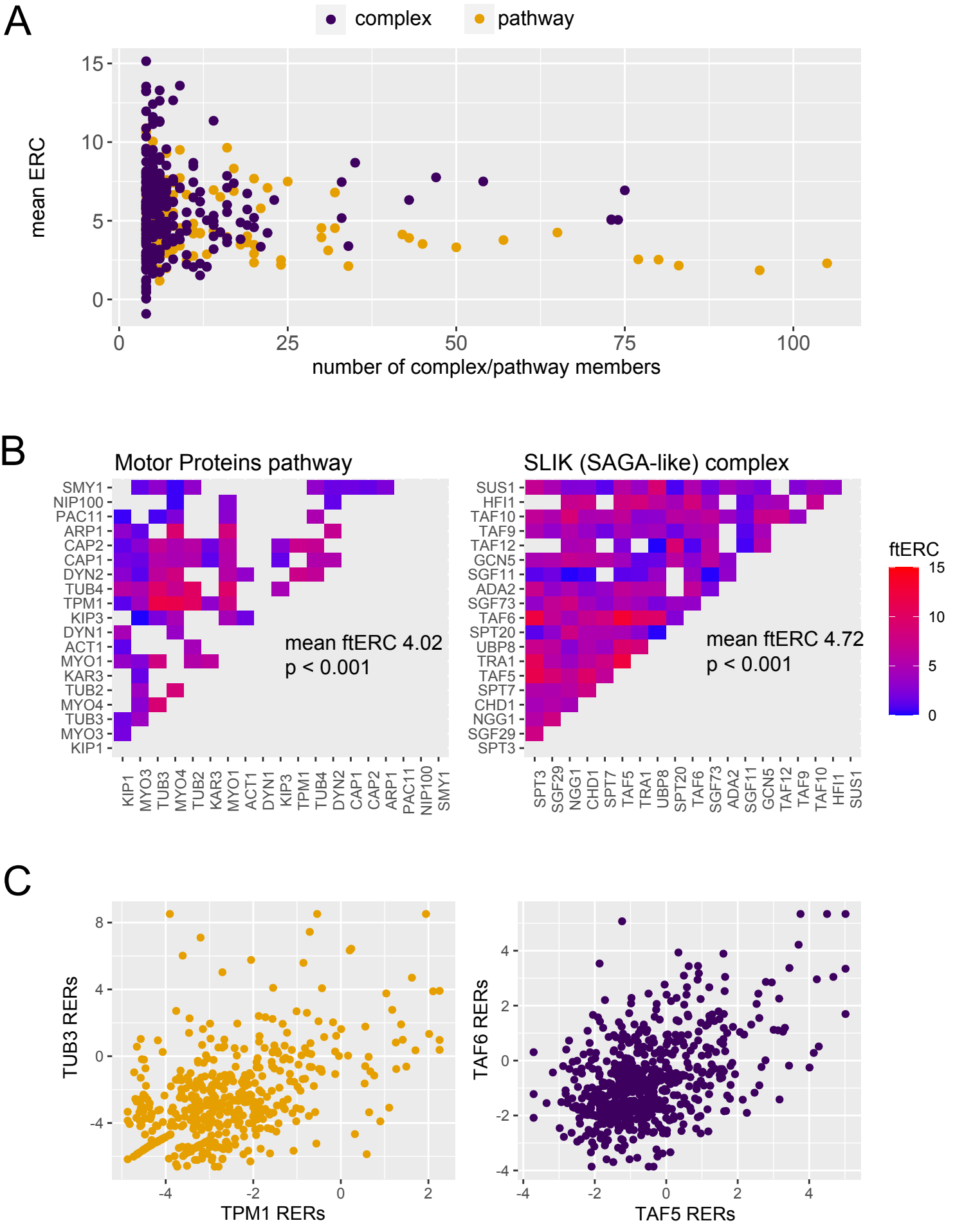
Permutation p-value distribution of 617 protein complexes (top) and 125 pathways (bottom) when compared to a null distribution of 1000 samples.



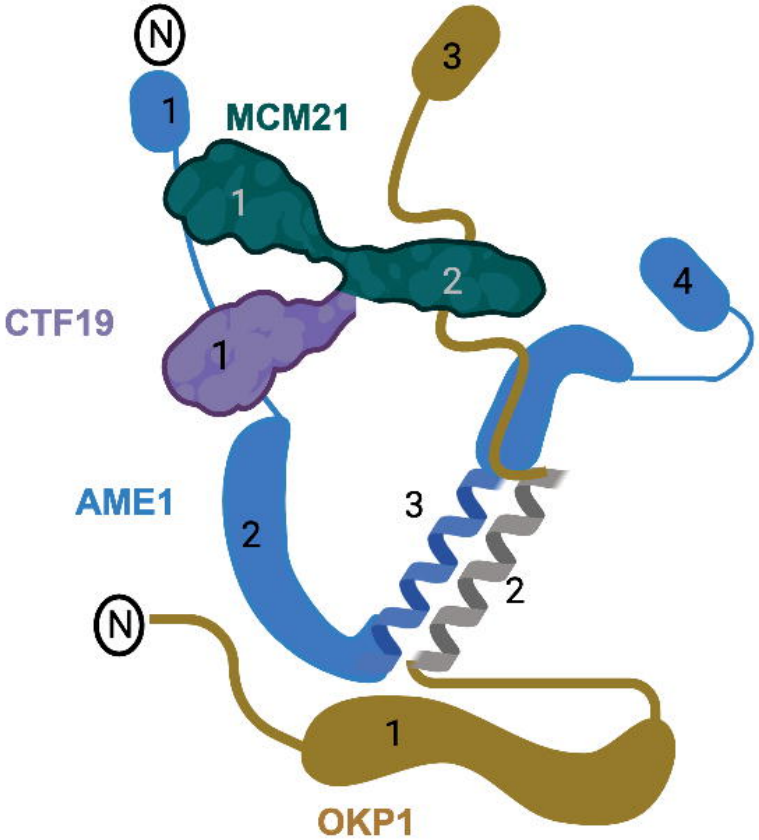

*Supplementary figure 2:*

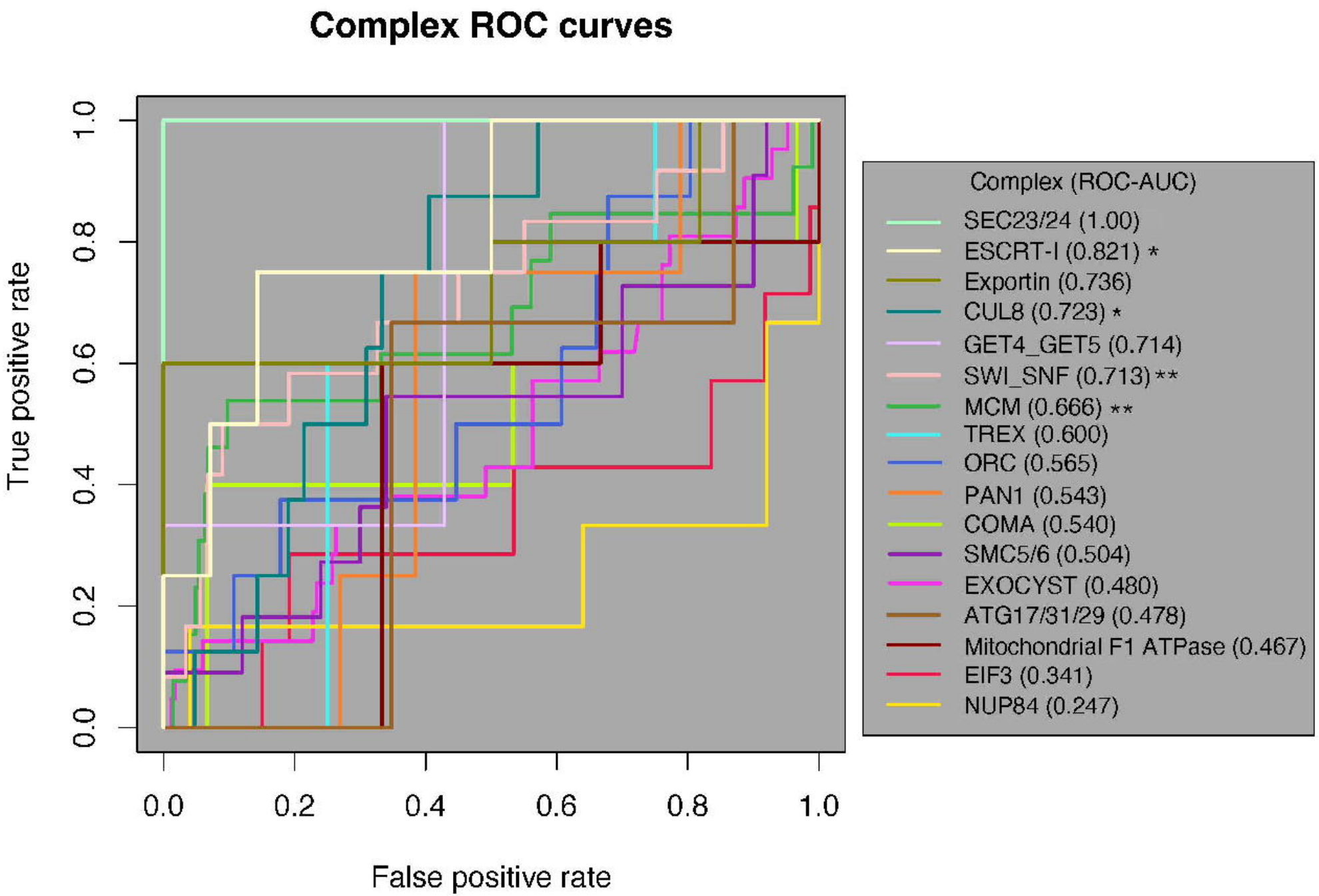
Histogram of the Fisher transformed ERC values for all 12,552 orthologous genes in the 343 yeast dataset. Median = 0.953.

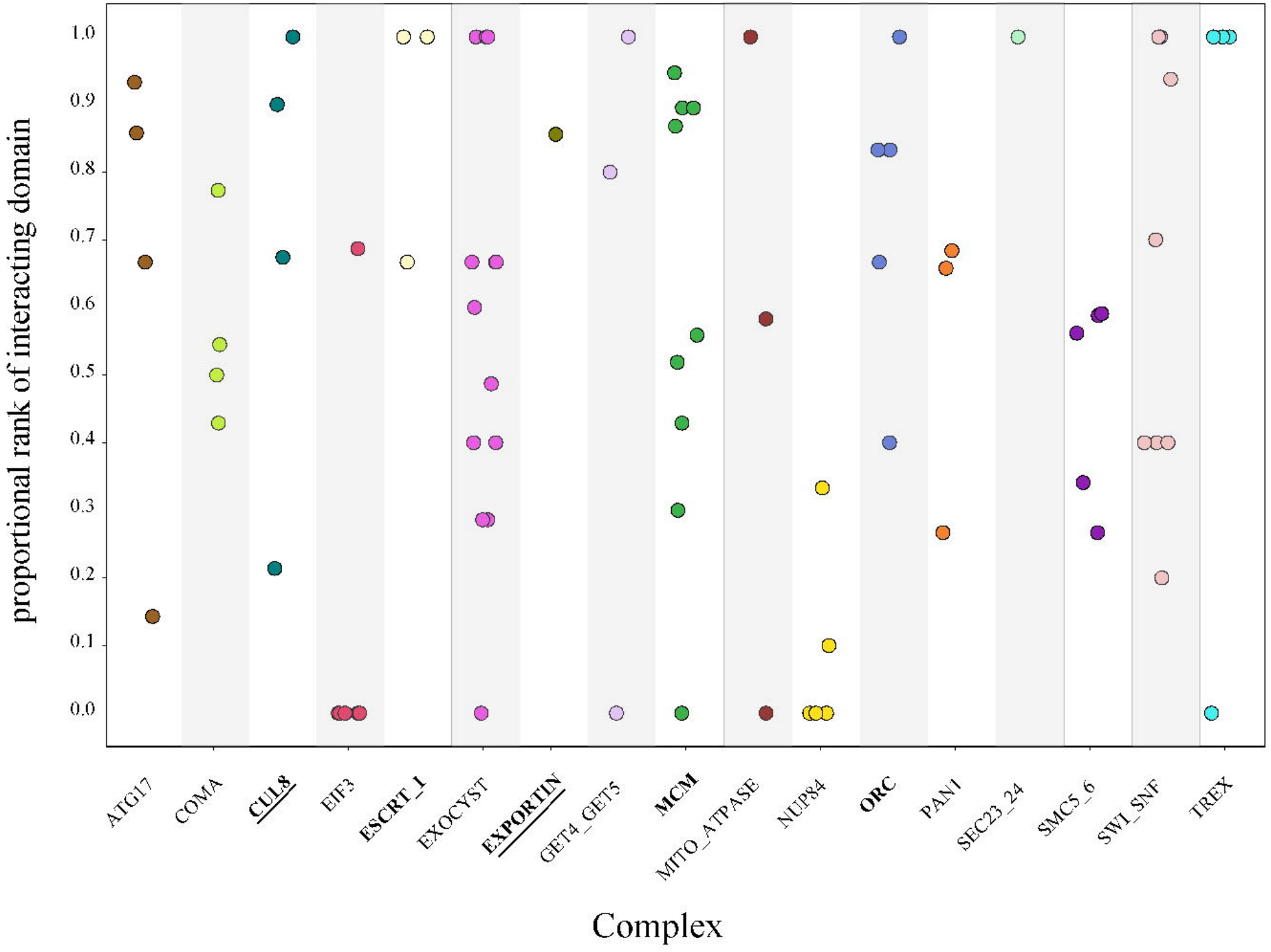

A. Evolutionary rate covariation (ERC)
SMC6
SMC5
deccelerated rates
accelerated rates
r = 0.775
ftERC = 24.944
SMC6
SMC5
B. Hypothesis if covariation is caused by compensatory co-evolution
Protein 2
D1
D2
D2
D1
Protein 1
Full protein ERC
Protein1
Protein2
4
Protein 1
D1
D2
Domain ERC
Protein 2
D1
D2
0
8
0
0
C. Experimental design
617 yeast complexes
17 complexes with sufficient interaction data
Split proteins into individual domains
Calculate ERC between all domain pairs
Determine if ERC-based ranking predicts physical interaction

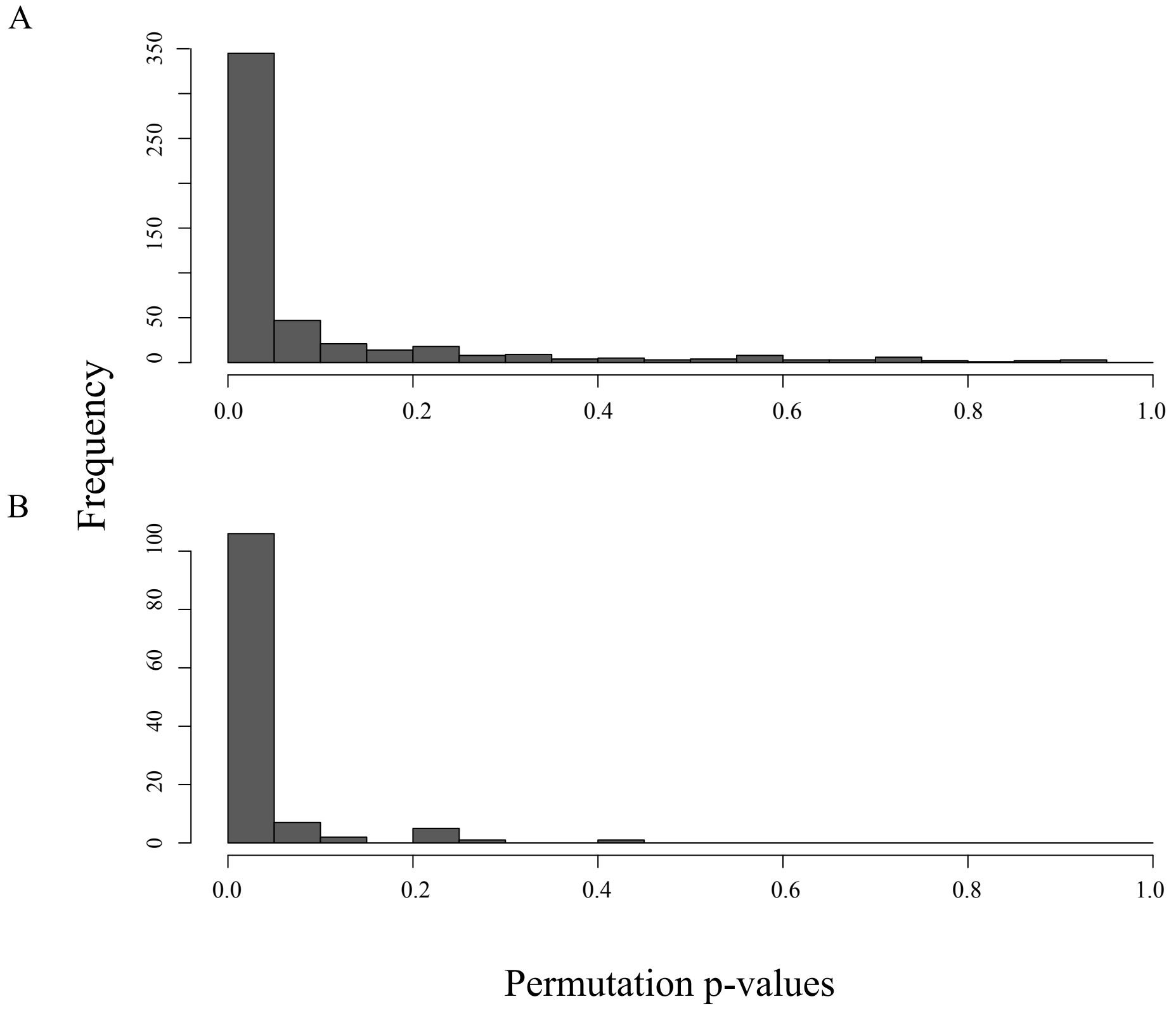

A
complex
pathway
mean ERC
number of complex/pathway members
B
Motor Proteins pathway
mean ftERC 4.02
p < 0.001
SLIK (SAGA-like) complex
mean ftERC 4.72
p < 0.001
ftERC
C
TUB3 RERs
TPM1 RERs
TAF6 RERs
TAF5 RERs

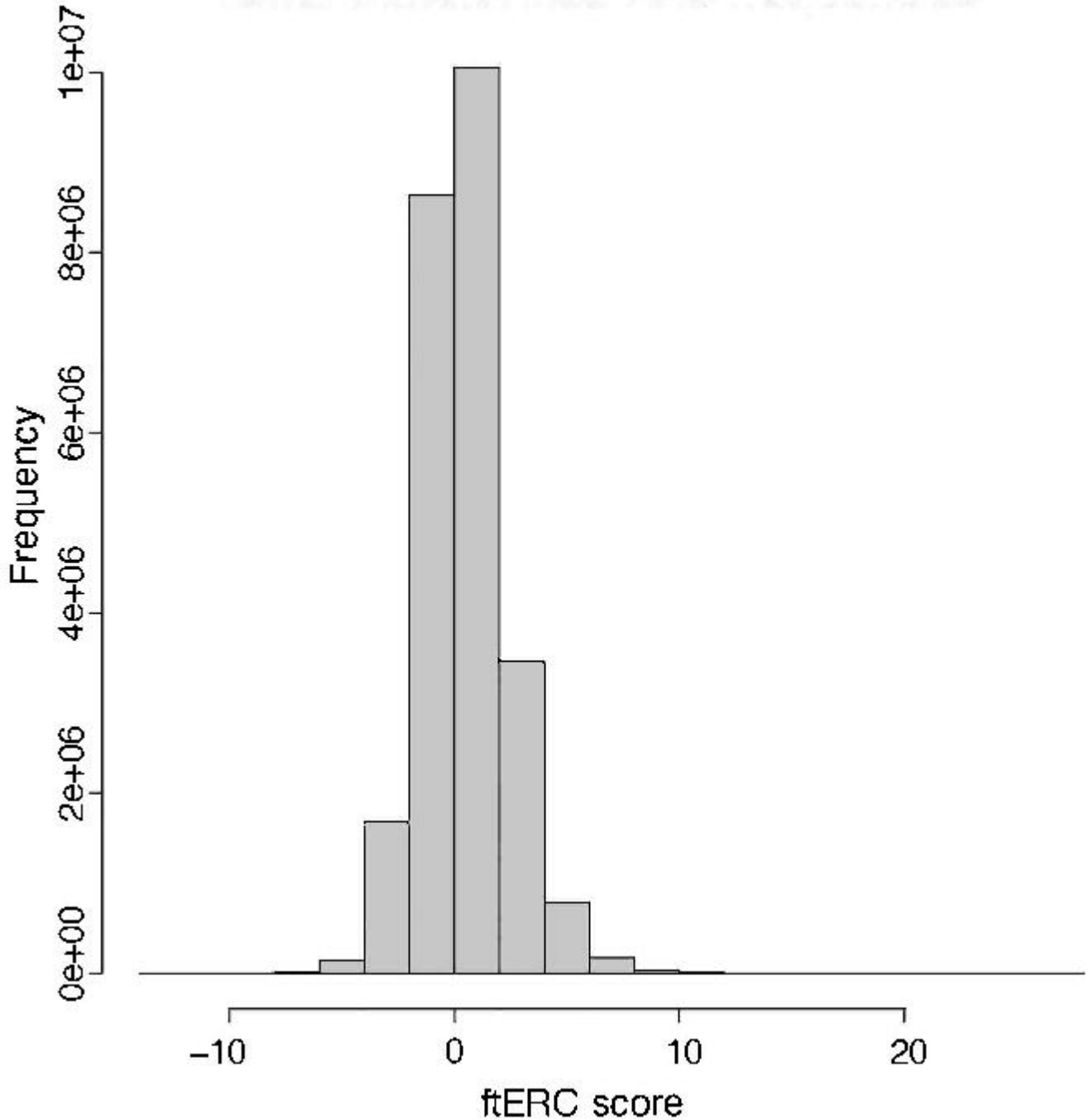
| | AME1_1 | AME1_2 | AME1_3 | AME1_4 | OKP1_1 | OKP1_2 | OKP1_3 | CTF19_1 |
|---|---|---|---|---|---|---|---|---|
| MCM21_1 | 2.71 | 2.74 | 2.80 | 2.00 | 1.71 | 1.59 | 1.97 | 0.75 |
| MCM21_2 | 2.01 | 1.83 | 2.19 | 1.88 | 2.04 | 3.06 | 1.91 | 2.42 |
| AME1_1 | | | | | 3.82 | 1.20 | 3.06 | 0.07 |
| AME1_2 | | | | | 4.18 | 4.85 | 4.10 | 3.06 |
| AME1_3 | | | | | 5.02 | 5.56 | 5.05 | 2.38 |
| AME1_4 | | | | | 4.73 | 5.10 | 4.80 | 4.40 |
| OKP1_1 | | | | | | | | 2.26 |
| OKP1_2 | | | | | | | | 2.46 |
| OKP1_3 | | | | | | | | 1.58 |




Complex ROC curves
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Complex (ROC-AUC)
SEC23/24 (1.00)
ESCRT-I (0.821) *
Exportin (0.736)
CUL8 (0.723) *
GET4_GET5 (0.714)
SWI_SNF (0.713) **
MCM (0.666) **
TREX (0.600)
ORC (0.565)
PAN1 (0.543)
COMA (0.540)
SMC5/6 (0.504)
EXOCYST (0.480)
ATG17/31/29 (0.478)
Mitochondrial F1 ATPase (0.467)
EIF3 (0.341)
NUP84 (0.247)


proportional rank of interacting domain
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ATG17
COMA
CUL8
EIF3
ESCRT_I
EXOCYST
EXPORTIN
GET4_GET5
MCM
MITO_ATPASE
NUP84
ORC
PAN1
SEC23_24
SMC5_6
SWI_SNF
TREX
Complex


A
B
Frequency
0
50
150
250
350
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
Permutation p-values


Frequency
0e+00
2e+06
4e+06
6e+06
8e+06
1e+07
−10
0
10
20
ftERC score