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Influence of metabolic network structure and function on enzyme evolution
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Abstract

Background: Most studies of molecular evolution are focused on individual genes and proteins. However, understanding the design principles and evolutionary properties of molecular networks requires a system-wide perspective. In the present work we connect molecular evolution on the gene level with system properties of a cellular metabolic network. In contrast to protein interaction networks, where several previous studies investigated the molecular evolution of proteins, metabolic networks have a relatively well-defined global function. The ability to consider fluxes in a metabolic network allows us to relate the functional role of each enzyme in a network to its rate of evolution.

Results: Our results, based on the yeast metabolic network, demonstrate that important evolutionary processes, such as the fixation of single nucleotide mutations, gene duplications, and gene deletions, are influenced by the structure and function of the network. Specifically, central and highly connected enzymes evolve more slowly than less connected enzymes. Also, enzymes carrying high metabolic fluxes under natural biological conditions experience higher evolutionary constraints. Genes encoding enzymes with high connectivity and high metabolic flux have higher chances to retain duplicates in evolution. In contrast to protein interaction networks, highly connected enzymes are no more likely to be essential compared to less connected enzymes.

Conclusion: The presented analysis of evolutionary constraints, gene duplication, and essentiality demonstrates that the structure and function of a metabolic network shapes the evolution of its enzymes. Our results underscore the need for systems-based approaches in studies of molecular evolution.

Background
Molecular networks and the genes encoding their building blocks represent two different levels of biological organization that interact in evolution. On the one hand, genetic changes such as point mutations, gene deletions, and gene duplications influence the structure and evolution of these networks. Conversely, network function may constrain the kinds of mutations that can be tolerated, and thus how genes evolve. Existing work on the structure and evolution of molecular networks has mainly focused on protein interaction...
networks [1-6]. Such networks are very heterogeneous: they contain large macromolecular complexes, regulatory interactions, signaling interactions, and interactions of proteins that provide structural support for a cell. As a result, it is difficult to ascertain how network structure reflects network function. A large fraction of false positives and false negatives in protein interaction networks [7,8] further complicates the structure to function analysis. In contrast, cellular metabolic networks are relatively well-characterized in several model organisms such as Saccharomyces cerevisiae [9,10] and Escherichia coli [11]. Their function - biosynthesis and energy production - is also well understood, as is the relationship of network structure to network function.

In the present study, we ask how the topology of a metabolic network and the metabolic fluxes (a metabolic flux is the rate at which a chemical reaction converts reactants into products) through reactions in the network influence the evolution of metabolic network genes through point mutations and gene duplication. Our results suggest that both network structure and function need to be understood to fully appreciate how metabolic networks constrain the evolution of their parts. The present study has become possible with the recent publication of a comprehensive compendium of metabolic reactions in the yeast Saccharomyces cerevisiae [10]. This compendium comprises 1,175 metabolic reactions and 584 metabolites, and involves about 16% of all yeast genes.

Using the stoichiometric equations that describe chemical reactions, we calculate the connectivity of an enzyme as the number of other metabolic enzymes that produce or consume the enzyme’s products or reactants (see Materials and methods and Additional data file 1). In other words, a metabolic enzyme A and a metabolic enzyme B are connected if they share the same metabolite as either a product or reactant. Highly connected enzymes in this representation are enzymes that share metabolites with many other enzymes. Including the most highly connected metabolites and cofactors such as ATP or hydrogen in a network representation would render the network structure dominated by these few nodes, and would obscure functional relationships between enzymes. We thus excluded the top 14 most highly connected metabolites: ATP, H, ADP, pyrophosphate, orthophosphate, CO₂, NAD, glutamate, NADP, NADH, NADPH, AMP, NH₄⁺, and CoA [12]. The results we report below are qualitatively insensitive to the exact number of removed metabolites.

Results

Highly connected enzymes evolve slowly

We will first discuss how network structure - specifically, an enzyme’s position in the network - influences enzyme evolution. Generally, enzymes in central parts of metabolism such as the tricarboxylic acid cycle will have more neighbors than enzymes in peripheral metabolic pathways (Figure 1). The correlation shown in Figure 1 arises from the fact that more connected enzymes have a direct access to many network nodes and consequently have shorter path lengths to other enzymes in the network. The evolutionary constraints on a metabolic enzyme can be estimated through the normalized ratio of non-synonymous to synonymous substitutions per nucleotide site (Ks/Ka) that occurred in the gene coding for the enzyme [13]. A small Ks/Ka ratio suggests higher evolutionary constraints on the enzyme, that is, a smaller fraction of accepted amino acid substitutions. In our analysis, we used the average ratio Ks/Ka of unambiguous orthologs in four sequenced Saccharomyces species: S. cerevisiae, S. paradoxus, S. bayanus, and S. mikatae [14]. The average Ks/Ka values used in the main analysis were taken from the study by Kellis et al. [14]. We also recalculated the average ratios using the maximum-likelihood method of Yang and Nielsen [15] and obtained qualitatively similar results.

Figure 2 demonstrates a statistically significant negative correlation between the metabolic connectivity of an enzyme and the ratio Ks/Ka (Spearman’s rank correlation r = -0.20, P = 1.1 × 10⁻⁴; Pearson’s correlation r = -0.18, P = 7 × 10⁻⁴). The inset in Figure 2 shows that this negative association holds over a broad range of connectivities, and that it is not caused by a small number of highly connected proteins. Additional data file 2 demonstrates a weaker negative correlation between non-synonymous (amino acid changing) substitutions Ks and gene connectivity (Spearman’s rank correlation r = -0.13, P = 1.6 × 10⁻²). The reason is that using only Ks, instead of the preferable Ks/Ka, as a measure of evolutionary constraints does not compensate for gene-specific differences in synonymous substitution rates and thus introduces additional noise.

**Figure 1**
The correlation between enzyme connectivity and centrality in the yeast metabolic network. Spearman’s rank correlation r = -0.74, P < 0.0001; Pearson’s correlation r = -0.67, P < 0.0001. The centrality of an enzyme is equal to the mean length of network distances from the enzyme to all other enzymes in the networks (pairs of enzymes not connected by any path in the network were excluded from the calculation).
in the data. Additional data file 3 shows that synonymous (silent) substitutions Ks and enzyme connectivity are not significantly correlated (Spearman’s rank correlation r = 0.056, P = 0.30). This is to be expected, as synonymous substitutions do not cause amino acid changes and are thus selectively neutral for the purpose of our analysis.

Why do highly connected enzymes show greater evolutionary constraint (smaller Ka/Ks)? One possibility is that this correlation is primarily mediated by the corresponding gene expression level [3]. Indeed, confirming previous observations [3], we found a significant negative correlation between the ratio Ka/Ks and mRNA expression levels (Spearman’s rank correlation r = -0.33, P = 5.5 × 10^{-10}; Pearson’s correlation r = -0.30, P = 3.6 × 10^{-8}). Information on mRNA expression of metabolic genes was obtained from the study by Holstege et al. [16] in which the number of mRNA molecules per cell was estimated based on microarray data. We also found a relatively weak correlation between connectivity and expression levels (Spearman’s rank correlation r = 0.11, P = 4.6 × 10^{-2}). Nevertheless, a partial correlation analysis - controlling for mRNA expression levels - between gene connectivity and evolutionary constraint Ka/Ks shows that enzymes in highly connected parts of the network evolve slowly.
whether flux through enzymatic reactions is associated with different carbon sources (Table 1). Specifically, we asked yeast metabolic network [10], maximizing growth on several to relate metabolic flux and the rate of enzyme evolution, we used the isoenzyme with the smallest ratio Ka/Ks. The growth conditions we used vary in catalyzing the same reaction), we used the reaction with the different chemical reactions, we used the reaction with the largest flux; if an enzyme had several isoenzymes (enzymes enzyme-coding genes. In this analysis, for enzymes catalyzing the connectivity constraints described above (Figure 2).

Enzymes that carry large metabolic fluxes evolve slowly

How well a metabolic network supports cell growth can be computationally quantified through the apparatus of metabolic flux analysis [17]. In flux balance analysis, the constraints imposed by stoichiometry and reversibility of chemical reactions are used to restrict the space of feasible metabolic fluxes. The constrained system can be subjected to an optimization procedure to obtain a flux distribution that maximizes some desirable metabolic property. Because cellular growth-rate is an important component of the fitness in a single-cell organism, biomass production is often used as the property being optimized. The predictions of flux balance analysis are often in good agreement with experimental results for E. coli [18,19] and S. cerevisiae [20].

To relate metabolic flux and the rate of enzyme evolution, we used flux balance analysis to calculate metabolic fluxes in the yeast metabolic network [10], maximizing growth on several different carbon sources (Table 1). Specifically, we asked whether flux through enzymatic reactions is associated with the evolutionary constraint Kₙ/Kₑ on the corresponding enzyme-coding genes. In this analysis, for enzymes catalyzing different chemical reactions, we used the reaction with the largest flux; if an enzyme had several isoenzymes (enzymes catalyzing the same reaction), we used the isoenzyme with the smallest ratio Kₙ/Kₑ. The growth conditions we used vary in the available carbon sources and in different uptake rates for oxygen (Table 1). The calculated distribution of flux values in the metabolic network is highly non-uniform [11] with several fluxes - usually representing glycolytic enzymes - more than two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4). To eliminate the disproportionate effect of these large fluxes, we removed the fluxes that are two orders of magnitude larger than the rest (Additional data file 4).

Gene duplication correlation with connectivity and flux

Gene duplications have effects opposite from those of most amino acid changes: they may increase rather than reduce flux through an enzymatic reaction. We established that highly connected enzymes and enzymes with high associated flux are especially sensitive to amino acid changes (Figures 2 and 3). Are their enzyme-coding genes, conversely, also more likely to undergo duplication? Figure 4 shows that this is indeed the case for enzyme connectivity. The figure demonstrates an association between an enzyme-coding gene’s number of duplicates and enzyme connectivity (only enzymes with sequence identity higher than 40% were considered as duplicates). Mean connectivity for genes with no duplicates is 15.0, and for genes with duplicates it is 19.2 (non-parametric Wilcoxon test, \( P = 1.4 \times 10^{-4} \)). This result suggests that duplicates of enzymes producing or consuming widely used metabolites are more likely to be retained in evolution. Figure 5 and Additional data file 5 demonstrate that a similar association exists between non-zero enzymatic flux through a reaction and the number of duplicates of the respective enzyme’s coding gene. Specifically, the higher the flux through a reaction, the more duplicates an enzyme-coding gene has. Qualitative association between enzymatic flux and gene duplication was also recently shown by Papp et al. [22].
Connectivity, essentiality, and metabolic robustness

Evolutionary constraints on enzymes are indirect indicators of metabolic robustness to amino acid changes, changes that a metabolic network tolerated for well over millions of years of evolution. Another type of biological robustness is that against complete gene deletions. Robustness against gene deletions can be derived from laboratory studies in which the effects of gene deletions on growth rate and other indicators of fitness are studied [23,24]. These studies determine essential genes, that is, genes whose elimination in one or more laboratory environments is effectively lethal. Our use of available essentiality data is motivated by the observation that highly connected proteins in protein interaction networks may be more likely to be essential to a cell [1]. We carried out analyses of fitness are studied [23,24]. These studies determine essential genes, that is, genes whose elimination in one or more laboratory environments is effectively lethal. Our use of available essentiality data is motivated by the observation that highly connected proteins in protein interaction networks may be more likely to be essential to a cell [1]. We carried out analyses...
using data on essential genes derived from a large scale gene deletion study by Giaever et al. [23], and used the *Saccharomyces* genome database (SGD) [25] to collect the essentiality data.

Our analyses of essential enzyme-coding genes show the clearest deviations from behavior observed in protein interaction networks. In the metabolic network from which the most highly connected metabolites (such as ATP or hydrogen) have been excluded, the mean connectivity of essential enzymes is significantly smaller than the mean connectivity of non-essential enzymes (Figure 6). For the yeast network, mean connectivity of essential enzymes is 13.2, and for non-essential enzymes (Figure 6). For the yeast network, mean connectivity of essential enzymes is significantly smaller than the mean connectivity of non-essential enzymes (non-parametric Wilcoxon test, \( P = 4.0 \times 10^{-4} \)). No statistically significant difference in connectivity of essential and non-essential enzymes is observed if all metabolites are used to establish network connections. Consequently, highly connected metabolic enzymes are no more likely to be essential than low connected enzymes. Similarly, as Mahadevan et al. [26] demonstrated recently, removal of highly connected metabolites is no more essential than removal of low connected metabolites. As we show above, highly connected enzymes and enzymes carrying high fluxes are more likely to have duplicates (often with the same or similar biochemical function). This suggests that highly connected enzymes are no more likely to be essential because they often have duplicates that can compensate for loss-of-function mutations [27]. Indeed, we find that the average number of duplicates for essential metabolic enzymes is 0.19 while the average number of duplicates for non-essential enzymes is 0.8 (non-parametric Wilcoxon test, \( P = 8 \times 10^{-49} \)). In addition to gene duplications, flux rerouting may provide another mechanism to make highly connected genes less essential. In highly connected parts of a metabolic network, metabolic fluxes may be rerouted through alternative pathways after a loss-of-function mutation [19,28,29]. This does not hold for linear metabolic pathways at a metabolic network's periphery, where a loss-of-function mutation may be fatal because no rerouting is possible.

**Discussion**

In sum, we demonstrate that both highly connected enzymes and enzymes that carry high metabolic fluxes in the yeast metabolic network have tolerated fewer amino acid substitutions in their evolutionary history. Why are enzymes carrying larger fluxes more constrained? The likely answer comes from the observation that most mutations affecting enzymatic activity may reduce rather than increase flux. Enzymes carrying high fluxes tend to have reaction products that enter a large number of metabolic pathways. Consequently, a mutational reduction in the activity of such enzymes should be more detrimental than a reduction in the activity of enzymes with lower flux.

We also show that the genes encoding enzymes with high flux have more duplicates. Importantly, we do not argue that duplications arise more frequently for genes whose products carry high flux, but that such duplications are more likely to be preserved in evolution, because of the advantage - higher flux - they provide. While a gene's duplicates can initially be preserved through an advantageous increase in metabolic flux, after divergence they may provide other functional benefits [30]. Divergence of metabolic genes in their expression and regulation is well-established for gene in intensely studied parts of metabolism, such as tricarboxylic acid cycle enzymes [31].

We found that the association between predicted enzymatic flux and evolutionary rate is most pronounced for carbon sources that dominate the natural environment of yeast. This suggests that one can use the association between flux and evolutionary constraint to search for conditions that dominated the evolution of metabolic networks. Similar analyses, which use genomic data to infer the environment that has shaped an organism’s evolution, have been used before to show that carbon limitation may have influenced the evolution of the *E. coli* metabolic network more strongly than nitrogen limitation [19], and to show that yeast evolution favored fermentation over respiration [32].

A previous study by Hahn et al. [6] reported that, based on amino acid divergence, in the *E. coli* metabolic network there exists no statistically significant association between enzyme connectivity and evolutionary constraint. We emphasize that any contradiction between this earlier work and our results is only apparent. First, the earlier study was based on a much smaller set of enzymes (\( n = 108 \) as opposed to \( n = 350 \) here), and thus had less statistical power. Nevertheless, two differ-
ent statistical measures in the previous study showed, like we do here, a negative association between connectivity and evolutionary constraint, albeit not at $P < 0.05$. Second, because of the lack of sufficient sequence information for a closely related sister species of E. coli, the previous study used only amino acid divergence $K_a$ and not the preferable $K_a/K_s$ to indicate evolutionary constraint. In fact, the correlation between connectivity and $K_a$ is very similar between the present study and the previous work (Spearman’s rank correlation $r = -0.13$, $P = 1.2 \times 10^{-2}$ here versus Spearman’s rank correlation $r = -0.15$, $P = 7 \times 10^{-2}$ in the study by Hahn et al.).

It should not be surprising that the observed associations are weak in magnitude. The reason for the low magnitude is that many other factors influence the evolution of enzyme-coding genes. Two of these factors are gene expression levels (discussed in the paper) and constraints stemming from the tertiary and quaternary structure of enzymes, which may differ among enzymes (little is known about such constraints). The key point is that besides all these other factors, metabolic network function and structure also has a clear influence on protein evolution.

How do our results on the yeast metabolic network relate to earlier work on protein interaction networks? There, a similar relationship between protein connectivity and evolutionary constraint has been suggested [4,5]; however, this association exists for different reasons. Highly connected proteins in protein interaction networks may evolve slowly because a larger fraction of a highly connected protein’s sequence is involved in protein interactions and may thus be evolutionarily constrained [4]. In contrast, high protein connectivity in the metabolic network is established not through protein-protein interactions, but through consumption or production of widely used metabolites. In metabolic networks, mutations in enzyme-coding genes - changing reaction rates and concentrations - may have especially deleterious consequences for widely used metabolites. Consequently, highly connected metabolic enzymes may evolve slow due to functional as opposed to structural constraints. Our ability to consider fluxes through enzymes in a metabolic network allows us to relate the functional role of each enzyme in a network to its rate of evolution. Such a functional analysis of a genome-scale network has no counterpart in any other genome-scale network studied thus far.

In conclusion, our analysis of evolutionary constraints, gene duplication, and essentiality demonstrates that the structure and function of a metabolic network shapes the evolution of its enzymes. In the long run, system analyses of biological networks will allow us to increasingly place the evolution of genes in the larger context in which they operate, as building blocks of cellular networks.

### Materials and methods

#### Metabolic network

We used a comprehensive collection of the yeast *S. cerevisiae* metabolic reactions by Foster et al. [10] to calculate metabolic enzyme connectivities. In addition to enzymatic reactions assigned to 671 open reading frames (ORFs), the collection contains reactions unassigned to known ORFs, transport reactions, and reactions represented by large macromolecular complexes. These reactions were used to calculate other enzyme connectivities but were excluded from the main analysis. Large macromolecular complexes (containing several ORFs) were represented by single enzymatic nodes in the calculation of connectivities for other metabolic enzymes. In order to include only functional relationships in the calculation of the enzyme connectivities, we excluded the 14 highly connected metabolites and co-factors (as described in the main text). As a result of the exclusion, a small fraction (5%) of network enzymes became disconnected from the network (they have zero connectivity). These enzymes were not included in the analysis.

#### Flux balance analysis

Flux balance analysis (FBA) was used to obtain metabolic flux distribution as described previously [10,17,19]. The network by Forster et al. [10] was used in all flux balance calculations. The in silico network of yeast metabolism includes central carbon metabolism, transmembrane transport reactions, pathways responsible for the synthesis and degradation of amino acids, nucleic acids, vitamins, cofactors, and lipids. In total, the network consists of 733 metabolites and 1,775 metabolic reactions. In the flux-balance analysis, the constraints limiting nutrient uptake, reaction irreversibility, and steady-state conservation of metabolite concentrations are applied. The fluxes optimal for growth are then obtained by maximization of biomass production using linear optimization. Linear optimization was performed using the GNU Linear Programming Kit [33].

#### Molecular evolution

We identified duplicates in the *S. cerevisiae* genome using a previously described whole-genome analysis tool [34]. Briefly, the tool locates gene duplicates in a genome using BLASTP [35] and aligns them globally with the Needleman and Wunsch dynamic programming alignment algorithm [36]. Putative duplicate pairs with less than 40% amino acid similarity or less than 100 aligned amino acid residues were excluded; for the remaining pairs we calculated the number of substitutions per synonymous site ($K_s$) and the number of substitutions per non-synonymous site ($K_a$) using the maximum likelihood models of Muse and Gaut [37] and Goldman and Yang [38].

The average $K_a/K_s$, $K_a$, and $K_s$ values used in the analysis were obtained from the study by Kellis et al. [14]. In a complementary approach, we also recalculated the average ratios using
the maximum-likelihood method of Yang and Nielsen [15] and obtained qualitatively similar results.

**Additional data files**
The following additional data are available with the online version of this paper. Additional data file 1 is a figure showing examples of metabolic connectivity. (a) An example of the metabolic reaction network from sphingoglycolipid metabolism; metabolites are drawn as small circles (DHSP, sphinganine-1-phosphate; PETHM, ethanolamine phosphate; SPH, sphinganine; CD Peterson, CD Petethanolamine; ETHM, ethanolamine) and enzyme-encoding genes are shown in rectangles. (b) Metabolic connectivity of the dpl1 gene (solid edges), as defined by the reactions shown in (a). The dpl1 gene has a total of six metabolic connections: two established through ethanolamine phosphate (red edges); and four through sphinganine-1-phosphate (blue edges). Metabolic connections between other enzymes are show by dashed edges. Additional data file 2 demonstrates the relationship between enzyme connectivity and the average amino acid divergence $K_s$. Spearman's rank correlation $r = -0.13$, $P = 1.6 \times 10^{-2}$. Additional data file 3 shows the relationship between enzyme connectivity and the average silent divergence $K_s$. Spearman's rank correlation $r = -0.056$, $P = 0.30$. Additional data file 4 is a histogram of the calculated metabolic fluxes in the yeast network for aerobic growth on glucose (maximal uptake rate $V_{max}$). Additional data file 5 shows the correlation between enzyme connectivity and the average amino acid divergence $K_s$. Spearman's rank correlation $r = -0.13$, $P = 1.6 \times 10^{-2}$. Additional data file 3 demonstrates the relationship between enzyme connectivity and the average amino acid divergence $K_s$. Spearman's rank correlation $r = -0.056$, $P = 0.30$. Additional data file 4 is a histogram of the calculated metabolic fluxes in the yeast network for aerobic growth on glucose (maximal uptake rate $V_{max}$).

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