Statistical Analysis Reveals Co-Expression Patterns of Many Pairs of Genes in Yeast Are Jointly Regulated by Interacting Loci

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Abstract
Expression quantitative trait loci (eQTL) studies have generated large amounts of data in different organisms. The analyses of these data have led to many novel findings and biological insights on expression regulations. However, the role of epistasis in the joint regulation of multiple genes has not been explored. This is largely due to the computational complexity involved when multiple traits are simultaneously considered against multiple markers if an exhaustive search strategy is adopted. In this article, we propose a computationally feasible approach to identify pairs of chromosomal regions that interact to regulate co-expression patterns of pairs of genes. Our approach is built on a bivariate model whose covariance matrix depends on the joint genotypes at the candidate loci. We also propose a filtering process to reduce the computational burden. When we applied our method to a yeast eQTL dataset profiled under both the glucose and ethanol conditions, we identified a total of 225 and 224 modules, with each module consisting of two genes and two eQTLs where the two eQTLs epistatically regulate the co-expression patterns of the two genes. We found that many of these modules have biological interpretations. Under the glucose condition, ribosome biogenesis was co-regulated with the signaling and carbohydrate catabolic processes, whereas silencing and aging related genes were co-regulated under the ethanol condition with the eQTLs containing genes involved in oxidative stress response process.

Introduction
eQTL studies aim to uncover the genetic architecture underlying expression regulation. In the past decade, they have been conducted in many organisms, including yeast, drosophila, mouse, human and many others [1–5]. A common approach in eQTL data analysis is to consider association between each expression trait and each genetic marker through regression analysis, and attention is usually focused on those trait-marker pairs whose associations are significant after multiple comparison adjustments. Despite great success with this approach, some regulatory signals may not be detected due to the complex nature of regulatory networks. For example, genetic buffering relationships often exist in the phosphorylation regulatory network in yeast [6], where pairs of regulators have overlap in function. Similar phenomena has also been observed in the transcriptional regulatory network in yeast [7]. Single marker analysis may not capture such regulatory patterns, where the genetic effects act through interactions between markers, necessitating the need to incorporate interactions in the analysis. However, extending beyond single marker analysis presents many challenges including the computational demand and the lack of statistical power, because a much larger number of models need to be considered and the need to control the overall false positive results. Storey et al. [8] developed a step-wise regression method to detect epistasis on the genome-wide scale. This method is computationally feasible but may miss epistatic effects involving markers having weak marginal effects. Wei et al. proposed a Bayesian partition model which may detect more loci having epistatic effects but weak marginal signals [9]. However, this Bayesian approach did not compare favorably with an exhaustive search scheme to detect features with weak marginal signals but strong epistatic effects in practice [10]. To reduce the model search space and increase statistical power, Lee et al. adopted genetic interaction networks identified by large-scale synthetic genetic array (SGA) analysis as prior for detecting epistasis in yeast [11]. Since they only consider interacting SNPs that have already been identified, its application is limited to those organisms where comprehensive prior knowledge is available, which is rare in practice.

Although most eQTL studies considered the expression levels of individual genes as response, a conceptually different approach was proposed by Li et al. [12] to consider “liquid association” (LA) between a pair of genes. LA aims to identify differential co-expressions, versus differential expressions, across different samples/conditions and the identified LA may offer insights that may not be captured by analysis based on single genes. Li and
Author Summary

eQTL studies collect both gene expression and genotype data, and they are highly informative as to how genes regulate expressions. Although much progress has been made in the analysis of such data, most studies have considered one marker at a time. As a result, those markers with weak marginal yet strong interactive effects may not be inferred from these single-marker-based analyses. In this article, using joint expression patterns between two genes (versus one gene) as the primary phenotype, we propose a novel statistical method to conduct an exhaustive search for joint marker analysis. When our method is applied to a well-studied dataset, we were able to identify many novel features that were overlooked by existing methods. Our general strategy has general applicability to other scientific problems.

Statistical Analysis Reveals Epistasis-2D Modules

colleagues later introduced this 2D-trait concept into eQTL study [13]. The goal of such 2D-trait based eQTL analysis is to identify genetic markers that can affect the co-expression patterns between two genes. Since co-expression patterns reflect co-regulation status, such 2D-trait analysis can assess whether the co-regulatory relationship between two genes is associated with certain genetic markers, which is complementary to analyzing the expression patterns of individual genes. For example, in signal transduction pathways, transcriptional factors (TFs) are often regulated by post-transcriptional regulation such as phosphorylation and dephosphorylation. Such regulations are difficult to detect because there may be little change at the expression levels for these genes. However, post-transcriptional regulation does affect TFs’ activities, which further affect the expression levels of their target genes. In this case, if a genetic marker affects post-transcriptional regulation, its effect may be captured by the change of co-expression patterns of the targets of TFs, so a LA analysis may lead to the identification of such markers, where it may be difficult to detect these signals using single gene expressions as the response. Recently, Ho et al. [14] proposed a conditional bi-variate normal model to analyze LA that simultaneously captures means, variances, and correlation, and common influence of a pair of genes. Under a similar framework, Chen et al. [15] proposed a penalized likelihood approach to effectively detecting causal genetic loci using iterative reweighted least squares, and Daye et al. [16] further considered the heteroscedastic problem. Although these methods have broadened the scope of eQTL analysis, none have considered the possibility that markers may have no or weak marginal effects but strongly interact to affect the correlations patterns among gene expressions, which may happen if there is genetic buffering between the markers and this is the focus of our current manuscript.

One major challenge to consider interactions effects on 2D-trait is the large number of models to be examined. For example, with 6000 genes in yeast, a total of 18 million 2D-traits can be formed. If we collect 4000 markers from each yeast strain, considering each pair of markers for their interaction effects will involve 8 million pairs of markers. Therefore, an exhaustive search of all 2D traits versus all marker pairs will evaluate 8 million pairs of markers. Therefore, an exhaustive search considering each pair of markers for their interaction effects will be prohibitive number with the current computing power based on the existing methods mentioned above. In this manuscript, we propose a computationally efficient algorithm to identify these Epistasis-2D associations based on conditional bivariate models and likelihood ratio test. In our procedure, we proposed to use a statistic called PA (Potential of Association) to filter out trait and marker sets that are unlikely to be significant before performing the more rigorous likelihood ratio tests. When we applied our method to a yeast eQTL dataset, we were able to identify many “Epistasis-2D” associations that could not inferred from single marker based analysis, where 2D refers to our focus on gene co-expression patterns and epistasis refers to our focus on detecting how loci interact to affect 2D-trait.

Results

Detecting Epistasis-2D associations

Overview of our strategy. Figure 1 describes our strategy to detect Epistasis-2D associations. In this manuscript, we define a module as the collection of a pair of loci and a pair of genes, and our objective is to find Epistasis-2D modules where the two loci interact to affect the co-expression patterns between the two genes in the module. To facilitate statistical analysis, the joint conditional distribution of the two genes for a given pair of genotypes at the two loci is modeled as a bivariate normal distribution, where we are primarily interested in whether the correlation between two genes is dependent on the joint genotypes between two markers. Under the null hypothesis, all the conditional correlations are the same, whereas they differ under the alternative hypothesis. We used the likelihood ratio test to test the null hypothesis. Because it is computationally prohibitive to consider all possible modules using the likelihood approach, we employed a statistic called PA (Potential of Association) to filter out modules unlikely having an association signal. Due to our focus on 2D-traits, we are not interested in those modules containing linkages that can be identified using 1D-traits in this manuscript. Neither are we interested in those modules having only marginal signals.

Conditional bivariate models. Traditionally, for a given phenotype, the genetic effects of two loci are often modeled as

\[ \text{phenotypy} = x_0 + x_1A + x_2B + x_3A \times B, \]

where \( A \) and \( B \) are the coded genotypes at the two loci, \( x_1 \) and \( x_2 \) represent the effects of marker 1 and marker 2, and \( x_3 \) corresponds to the interaction effect between markers 1 and 2. Under this model, the presence of epistasis is captured by a non-zero \( x_3 \) term. Now for a pair of phenotypes, their bivariate phenotypes \((X,Y)\) can be modeled by a bivariate normal distribution [15],

\[ (X, Y) \sim N((\mu_1, \mu_2), \Sigma(\beta, A, B)), \]

where \( \mu_1 \) and \( \mu_2 \) are the mean values of \( X \) and \( Y \), which are set as constant in our model.

\[ \Sigma(\beta, A, B) = \left( \begin{array}{cc} \sigma_1^2 & \sigma_1 \sigma_2 \rho \beta \beta^T \sigma_1 \sigma_2 \beta \beta^T \\ \sigma_1 \sigma_2 \rho \beta \beta^T \sigma_1 \sigma_2 \beta \beta^T & \sigma_2^2 \end{array} \right) \]

is the covariance matrix and \( \sigma_1^2 \) and \( \sigma_2^2 \) are the variances for the two traits, respectively. As discussed above, our main interest in this paper is the co-expression between two genes, i.e. the correlation term in the matrix \( \rho \) which we model in the following form:

\[ \rho(\beta, A, B) = \rho_0 + \beta_1 A + \beta_2 B + \beta_3 A \times B, \]

where \( A \) and \( B \) were defined before and \( \beta_1, \beta_2, \) and \( \beta_3 \) have similar interpretations as \( x_1, x_2, \) and \( x_3 \) but quantify the marker effects on the correlation.

We note that there is an extensive literature on the difference between statistical interactions and biological interactions because
the presence or absence of statistical interactions depends on the specific statistical models used and the scale of the response variable. When each marker has two genotypes (coded by 0 and 1), models (1) and (3) fully parameterize the relationship between phenotype and the four possible genotypes, namely (1,1), (1,0), (0,1) and (0,0) [8]. In this case, we can reformulate model (3) as

$$p(\beta_{ij},A,B) = \sum_{i \in \{0,1\}, j \in \{0,1\}} \beta_{ij} I(A=i)I(B=j),$$

where $I$ is the indicator function, i.e. $I(A=i) = 1$ if $A=i$ and 0 otherwise.

Although we could use this most general model to identify interesting modules, a model with fewer parameters may be preferred to achieve a balance between the goodness of fit and parsimony of the model. For example, Figure 2A–2B illustrate two examples where fewer than four parameters are needed to model the co-expression pattern. In Figure 2A, the correlation between the two genes for samples having genotype (1,1) ($\beta_{11}$) is different from the samples with other genotypes. Hence two instead of four parameters are needed to model this module. Similarly, three parameters are needed for the example shown in Figure 2B where samples having genotypes (0,1) or (0,0) have uncorrelated phenotypes. For the best fitted parameter settings with two or
three parameters, the p-values of these two example are $1.5 \times 10^{-13}$ and $7.5 \times 10^{-13}$, whereas those for the full model are $4.6 \times 10^{-12}$ and $2.9 \times 10^{-12}$. In this article, we test all possible parameter settings for each module and select the model with the most significant p-value. This approach may yield simpler interpretations of the modeling results when fewer parameters are used. In addition, more modules were identified at the same false discovery rate control using our approach compared to the approach based on the full model (Materials and Methods, Text S1).

**Filtering step to reduce computation burden.** There is a computational barrier to directly apply our model to large scale data because estimating parameters in the conditional bivariate models needs numerical solution through iteration [15]. To reduce computation burden, we propose a filtering step which removes modules with low possibility to be significant from further considerations using a statistic called “PA” (Potential of Association). This statistic estimates the lower bound of the p-value for each module. Because PA can be directly calculated from the
observed values (expression values and genotypes) without numerical iterations, it can be used to remove most modules before applying the conditional bivariate model. Our real data analysis showed an average reduction of 16 fold computational time with this filtering step (Materials and Methods, Text S1).

Application to yeast eQTL data

We applied our method to a dataset containing gene expressions and genotypes for 109 segregants from a cross between laboratory (BY, noted as 1) and wild (RM, noted as 0) strains of *Saccharomyces cerevisiae* [3]. The expression levels were measured under two different conditions: glucose and ethanol. We applied our method to the expression data collected under these conditions, and identified 225 and 224 pairs of genes (2D-traits), respectively, whose correlation patterns were under the epistatic control of pairs of markers at an estimated false discovery rate (FDR) < 0.2 (Materials and Methods, Text S1, Table S3). As far as we are aware, none of these detected marker interactions have been reported to affect expression traits, and our results revealed a new group of regulation patterns that have been overlooked in the literature. Among the 225 and 224 gene pairs, there is an enrichment of pairs having the same functional annotations (31 out of 225 with a p-value of 0.05 and 58 out of 224 with a p-value of $2 \times 10^{-11}$) according to GO slim. Despite this statistically significant enrichment, most pairs have different functional annotations suggesting either unknown functions for these genes or interactions between different biology processes.

We observed that the functional distributions of the Epistasis-2D associations depend on the environment condition under which the eQTL data were collected. This is consistent with the literature on the importance of the environment on gene expression regulations [17]. Also consistent with previous finding that the trans-acting linkages differ under different environmental conditions [18], our results suggest that trans-acting loci are related to the environment related stress response pathways. The modules identified by our method may be followed up with experimental studies for validation and learning to gain further insights on their biological relevance.

**Examples of Epistasis-2D modules.** Among the Epistasis-2D modules (Table S3) identified by our method, many are biologically meaningful. For example, Figure 2A shows a module detected under the glucose condition where the two genes (*GOT1* and *ERV14*) are functional in ER to Golgi vesicle-mediated transport, whereas the two markers interacting with each other affect the co-expression patterns between these two genes are located at chromosome VII:833786-858604 and chromosome I:187640-193251. These two chromosomal intervals contain 14 and 2 genes, with each having a candidate that also functions in ER to Golgi vesicle-mediated transport: *HIP1* and *MST28* ($p = 7 \times 10^{-3}$, Materials and Methods). Both *HIP1* and *MST28* are integral membrane proteins that are involved in COPII transport vesicle formation [19,20]. Literature suggests synthetic lethality of *HIP1* with the heterodimer of the COPII vesicle coat Sec23-Sec24 and physical interaction between *MST28* and Sec23-Sec24 [19,20]. These experimental results suggest potential interactions between *HIP1* and *MST28*. In addition, *GOT1* was identified as a suppressor functioning in the same pathway as *HIP1* that regulates biogenesis of COPII vesicle [21]. This observation suggests a regulatory relationship between *HIP1* and *GOT1*. Finally, *ERV14* is involved in vesicle formation [22] and interacts genetically with both *GOT1* [23,24] and Sec23 [25]. In summary, our results and the literature suggest that *HIP1* and *MST28* may interact to regulate the co-expression of *GOT1* and *ERV14*, and the heterodimer Sec23-Sec24 may mediate their effects (Figure 2C).

Another module is shown in Figure 2B with two genes, *PTP2* and *GABI*, whose co-expression patterns are epistatically regulated by two loci on chromosomes XII and III. *PTP2* is a phosphatase that dephosphorylates Hog1 in high osmolarity sensing ([HOG]) mitogen-activated protein kinase ([MAPK]) pathway. *GABI* is a GPI transamidase subunit and may play a role in the recognition of the attachment signal. The two chromosomal intervals (chromosome XII:370434-388953, chromosome III:240351-264124) contain 8 and 15 genes, and each contains a candidate gene that functions in the HOG MAPK pathway, *HOG1* and *SRK22* ($p = 5 \times 10^{-3}$, Materials and Methods). *Srk22* is a MAP kinase kinase kinase ([MAPKKK]) and *Hog1* is a MAP kinase ([MAPK]). *PTP2* is known to be induced by Hog1-dependent transcripational factor Rim1 [26]. *GABI* is transcriptionally regulated by Yap1 [27,28], which is also the substrate of Hog1 [29]. Hence, the genetic interaction between *HOG1* and *Srk22* and their regulation on *PTP2* and *GABI* is supported by existing literature on the HOG pathway (Figure 2D).

Clustering in the epistasis map reveals functional genetic modules. The locus pairs identified that epistatically interact to regulate the co-expression patterns of gene pairs may be inferred to have genetic interactions and such interactions can be used to develop a global genetic interaction map. We applied the hierarchical clustering to this interaction map and found densely interacting locus clusters (Figure 3A, Materials and Methods). Under the glucose condition, there was one cluster containing eight genetic intervals (Figure 3B). These pairs share similar target gene pairs including six genes (*COX4, QCR9, ATP14, TIM11, STF1* and *DBP8*), all except *DBP8* are encoding proteins functional in oxidative phosphorylation, whereas *DBP8* is a ribosomal gene. The expressions of oxidative phosphorylation genes are repressed by glucose, whereas the expression of ribosomal genes are induced by high glucose signal. Therefore, it is plausible that the expression of *DBP8* is correlated with oxidative phosphorylation genes for samples with certain genotypes under glucose condition. The eight intervals are enriched with oxidative phosphorylation candidates ($p = 3.5 \times 10^{-6}$, Materials and Methods). More specifically, six intervals contain one candidate annotated to function in oxidative phosphorylation, including *COX1, QCR6, QCR8, Cyt1, Cor1* and *QCR6*. The other two intervals contain *MRP14* and *PET100* which participate in the oxidative phosphorylation process although they are not noted in GO. Among the proteins encoded by these candidates, Yip043w is responsible for the oxidation of succinate and production of ubiquinone, which is the substrate for cytochrome c reductase complex containing *Cor1*, *Qcr6*, *Qcr8*, *Cyt1* and the target *Qcr9*. In addition, *Mrp14* is associated with the Cbp3-Cbp6 complex to promote cytochrome c reductase complex synthesis and assembly [30]. The cytochrome c reductase complex oxidizes ubiquinone while reducing cytochrome c, which in turn serves as the substrate for cytochrome c oxidase complex including the candidates *Cor6* and the target *Cox4*. Pet100 is a chaperone that specifically facilitates the assembly of cytochrome c oxidase. During these processes protons are transferred out of the mitochondrial membrane, and back into the mitochondrial matrix. The energy derived from the movement of these protons is used in ATP synthesis, and the targets *Apf14*, *Tim11*, *Stf1* are functional in the F1F0-ATP synthesis (Figure 3C). Hence, these detected epistatic relationships are well supported by their close connections in the oxidative phosphorylation pathway.

**Function analysis reflects how environment modulates regulatory modules.** To understand how environmental conditions modulate the effects of genetic variants on phenotypic traits, we investigated whether the gene pairs in the inferred Epistasis-2D modules are enriched for certain biological processes. For the 225 and 224 2D-traits identified under the two conditions,
Figure 4 summarizes the pairs of functions enriched for co-regulated gene pairs (Materials and Methods). It can be seen that the patterns are quite different between the two conditions. Under the glucose condition, ribosome biogenesis tends to be co-regulated with carbohydrate metabolic process ($p \approx 8.9 \times 10^{-6}$) and signaling ($p \approx 3.2 \times 10^{-3}$). Genes within the cellular respiration process also tend to be co-regulated ($p \approx 9.5 \times 10^{-5}$) (Figure 4A). Under the ethanol condition, genes within the RNA metabolic process ($p \approx 1.4 \times 10^{-5}$) and translation ($p \approx 2.0 \times 10^{-4}$) tend to be co-regulated (Figure 4B).

**Glucose response pathway modulates ribosome-related modules.** To understand the differences observed between the two conditions, we investigated the regulatory loci to bridge the gap between environment and co-regulated processes. Ribosome biogenesis is associated with the glucose condition, with ribosome biogenesis genes induced in response to high, but not low, glucose signals [31]. This is consistent with our observation that ribosome biogenesis related regulation was only identified under the glucose condition. In addition, yeast responds to glucose via several glucose-sensing and signaling pathways, two of which are reflected in our results: the main glucose repression pathway through the complex SNF1 complex to inhibit the Mig1 repressor-containing complex, and the Gpr1/Gpa2 glucose-sensing pathway which activates cAMP synthesis [32].

Figure 3. Clustering in the epistasis map reveals a functional genetic module in oxidative phosphorylation pathway. (A) The clustering heatmap of the detected epistasis under the glucose condition. (B) One cluster contains eight highly interacting loci. Their co-targets in the module are mostly functional in the oxidative phosphorylation pathway. There are candidates also functional in the same pathway at these eight loci. (C) A diagram showing the regulatory pathway from the literature, where the green circle represents the candidates and the grey circle represents their targets in the modules. doi:10.1371/journal.pgen.1003414.g003
To gain insights of this environment modulated regulatory relationship, we first studied the linkage between ribosome biogenesis and signaling process. In our results, two signaling genes (GPG1 and TFS1) were co-regulated with ribosomal genes under the glucose condition. They both encode proteins that function in the glucose signaling pathway. Gpg1 interacts with the glucose sensor Grp1 and Gpa2 [33], and Tfs1 could activate the cAMP/PKA pathway. The regulatory loci of these modules are enriched with glucose metabolic genes ($p = 3.8 \times 10^{-3}$, Materials and Methods). GPG1 is co-regulated with three ribosomal genes (REX4, RNT1, UTP9), and associated with two genetic intervals: chromosome XVI:387239-420441 and chromosome XIV:558284-595885. These two chromosomal intervals contain 14 and 16 genes and each contains a candidate that functions in the glucose response process: GCR1 and SXX8. Gcr1 forms a complex with Rap1 and Gcr2 to transcriptionally activate glycolytic genes [34,35], and Rap1 was detected to be the transcription factor of GPG1 and REX4 [27,28]. SXX8 encodes the RNA polymerase II holoenzyme and is involved in glucose repression [36], it is repressed by SNF1 complex and also physically interacts with Snf1 (Figure 5). High-throughput study has detected genetic interaction between Gcr2 and Ssn8 [37], which may be related to the epistatic interaction between Gcr1 and Ssn8. How this interaction could influence the regulation of the genes in the modules maybe an interesting direction for future studies. The co-regulated ribosomal genes pairs TFS1 and NOC3 are associated with chromosomal intervals: XII:514835-516700 and V:430931-458085. The former interval is located near TFS1 and the latter contains 11 genes, among which one candidate GLC7 is a well known regulator in glucose response. Glc7 is the phosphatase that inhibits Snf1 complex (Figure 5). Snf1 complex inhibits the Mig1 complex, which is the transcriptional regulator of TFS1 [27,28]. Further more, we can also find glucose response genes among the co-regulatory loci of ribosome biogenesis and carbohydrate metabolic process. For example, one module contains two genes GDB1 and NOC3. GDB1 encodes a glycogen debranching enzyme and NOC3 is involved in ribosome biogenesis. Their regulatory loci are located at two chromosomal intervals: IV:1149761-1185630 and XIII:286122-289193, where enriched with glucose transport genes ($p = 1.2 \times 10^{-6}$, Materials and Methods). The former contains 15 genes and three candidates (HXT3, HXT6, HXT7) encode glucose transporters in glucose response pathway. The latter interval contains two genes and one candidate HXT2 also encodes a transporter in glucose response pathway (Figure 5).

**Oxidative stress in ethanol modulates aging-related modules.** Under the ethanol condition, 25 pairs of RNA metabolic genes were co-regulated among the 224 identified 2D-trait containing 43 unique genes. We note that 11 genes function in the silencing process, which are known to be related to aging [38,39]. In addition, two other genes RAS2 and MSN2 are known to function in the aging process [40]. In total, 16 of these 25 (64%) pairs contain at least one aging related gene. These aging-related modules and proposed regulatory candidates are shown in Table S5. Among the 11 silencing genes, six form three co-regulated pairs: UBP10-HMLALPHA1, SPT23-RPD3, and EXC2-ZDS2. All the regulatory loci in the first two modules and many regulatory loci in the other aging-related modules contain oxidative stress response related regulators (Table S5), which is interesting because there is a conjecture that oxidative stress could induce aging in ethanol environment [38,39]. The metabolism of ethanol is assumed to induce aging through increased damage from reactive oxygen species (ROS) produced in oxidative stress response [38,39], but the exact mechanism is not clear. We discuss the details of these modules in the following to gain some insights on how oxidative stress response pathway regulate aging-related process.
HOG1 and GRE2 are involved in the oxidative stress response and regulated by gene GRE2 which contains two and eight genes, respectively. Only one chromosome XV:37207-44482 and chromosome XII:370434-43,44. The regulatory loci in this module are located at [42]. The SIR complex is also known to link silencing and aging, assembly of silencing complex at telomeres and mating-type loci through direct binding [53]. Although the latter regulation is not discovered under oxidative stress and it is not clear whether this regulation is related to the function of Spt23 in silencing, these known regulations provide a potential framework to interpret the regulation between Vms1 and Spt23. In addition, high-throughput experiments have captured the genetic interaction between Spt23 and Vms1 [7,54]. To understand the epistatic interaction between Vms1 and HOG pathway, we investigated the upstream regulators of Vms1. Under oxidative stress Vms1 is genetically interacted with Sod2 [51]. Sod2 is downstream of Sch9, and Sch9 is regulated by Tor1. Tor1 is the main kinase in TOR pathway that is well known to regulate aging under caloric restriction [38]. VMS1 was also found to be regulated by Tor1 [55]. From observing the co-expression pattern in the module (Figure 6B), we note that only when the genotype of Ste11 is 0 (wild RM), SPT23 and RPD3 could be co-regulated, moreover the sign of their expression correlation depends on the genotype of Vms1. It seems that Ste11 provides the signal upstream of Vms1. In addition, the regulation of VMS1 in this module also exists under oxidative stress. Hence, there may be some crosstalk between oxidative stress and caloric restriction response pathways. This is plausible because the two stresses could both be induced by ethanol environment and cause silencing and aging. Interestingly, Huang et al. [56] recently demonstrated that Sch9 could integrate nutrient signals from the TOR pathway and oxidative stress signals from sphingolipids to regulate aging. Although their proposed signal transduction from oxidative stress to Sch9 was not related to the HOG pathway, other studies have shown the close interactions between Sch9 with HOG-related genes like Hog1, Gre2, Msn2,4 and Rpd3 in other processes [57–59]. Combined with our results, it is possible that Sch9 could integrate oxidative stress signal from HOG pathway to regulate aging related processes under the ethanol condition.

Comparisons between our method and other methods. In this section, we compare the results from our method to those from two other methods: a forward search
strategy to identify Epistasis-2D modules (instead of the exhaustive search scheme adopted by us) and an analysis focusing on 1D traits. For the forward search method, we extend a forward search algorithm proposed by Storey et al. [8] to consider 2D-traits as follows:

- For each pair of traits, identify the locus primarily associated with the co-expression patterns where the correlation between the two traits is modeled as $r(\beta) = \beta_0 + \beta_1 L_1$, that is, we first identify single loci affecting the changes in correlations between two traits.

- After the identification of the first locus, we conduct the forward search to identify other loci that interact with the first locus to affect the correlations between the two traits using the conditional bivariate test described above for our method. We also test all possible interaction models for each candidate module with two genes and two loci and select the model with the most significant p-value.

Because our method is based on an exhaustive search scheme, we only focused on the modules identified by our method as the

Figure 6. Ethanol modulates aging-related modules through oxidative stress response pathway. (A) One module contains two silencing genes, UBP10 and HMLALPHA1. The regulatory loci contain two oxidative stress response genes, Gre2 and Hog1; (B) One module contains two silencing genes, SPT23 and RPD3. The regulatory loci contain two oxidative stress response genes, Ste11 and Vms1; (C) A diagram showing the module A related pathway, which indicates a potential regulatory relationship between oxidative stress response and Ubp10 induced silencing process; (D) A diagram showing the module B related pathway. The epistatic interaction between the two candidates Vms1 and Ste11 indicates the potential crosstalk between caloric restriction response and oxidative stress. Moreover, Ste11 may also regulate Pbs2-Hog1 signaling pathway under oxidative stress besides osmotic stress response.

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interacting loci through the analysis of eQTL data. Our approach is based on modeling the joint expression levels with a bivariate normal distribution whose covariance matrix is dependent on the joint genotypes at two candidate loci. Although different model search strategies have been proposed to jointly analyze multiple markers and their interactions based on genome wide data, e.g. marginal search, forward search and exhaustive search, the ability to conduct an exhaustive search allows us to identify interacting loci with weak marginal effects [63]. To facilitate an exhaustive search of all gene pairs versus locus pairs, we also proposed a filtering process to only focus on those modules that are likely to be statistically significant. This filtering process is one important component of our strategy to reduce the computational burden without reducing statistical power for discoveries. The application of our method to a yeast data set has identified many interacting loci with weak marginal signals which would not have been found without the exhaustive search strategy. Compared to the existing methods to detect epistasis, we considered the 2D-trait, especially their co-expression patterns, as the phenotype. As discussed in the introduction section, using such 2D-trait may help to detect post-transcriptional regulation from the change of expression correlations between downstream genes. As shown with the examples in the results section, we detected many regulatory loci containing candidate genes encoding kinases or phosphatases that regulate the co-expression correlation between the targets of their TF substrates. None of these modules could have been detected using 1D-trait. Since we only focus on modules which can not be detected using 1D-mapping in this paper, we may miss potential Epistasis-2D modules with genotype-dependent mean values (\( \mu \)) through 1D-Map filtering and assuming \( \mu_1 = \mu_2 = 0 \) in our model. About 13% modules were filtered using 1D-Map filtering, therefore around 13% Epistasis-2D modules could be missed by our method. Although we could introduce more parameters in our model to allow for genotype-dependent mean values, this may introduce noises that lead to reduced statistical power with limited sample size. More detailed discussion on the trade-off between statistical power and model adequacy is provided in the supplementary materials (Text S1, Figures S6 and S7).

We applied our strategy to a well studied yeast eQTL dataset and detected many epistasis modules, most of which have not been discovered to date and many may be interpreted with existing biological literature. We found that the co-regulated genes in the modules inferred under different environments were enriched for different biological processes. For example, under the glucose condition, ribosome biogenesis tends to be co-regulated with glucose response and glucose metabolic processes. The loci jointly regulating their expression patterns are enriched with genes in the glucose response pathway. Under the ethanol condition, silencing and aging related genes were found to be co-regulated. The loci jointly regulating these genes are enriched with genes in the oxidative response pathway, consistent with the hypothesis that the metabolism of ethanol would induce aging through increased damage from ROS produced in oxidative stress response. Through detailed discussion of several identified modules, we proposed potential regulatory mechanisms between oxidative stress signal and aging process.

Interpretation is difficult in eQTL linkage studies because the detected eQTLs often have low resolution, e.g. large intervals, with many candidate genes. Traditional linkage analysis with single genes and one locus often offers limited information to identify a candidate gene around the locus to understand the linkage signal. Since Epistasis-2D modules detected in our study involve two genes and two loci, the biological association of the two genes offers additional information to prioritize candidate genes. For example, Figure 7 shows an example where the expression of the two genes, \textit{GPG1} and \textit{RNT1}, were both affected by hotspots. We can see that their co-expressions were regulated by two other markers located at chromosome XIV:558284-595885 and chromosome XVI:368296-408883. The candidate \textit{IRA2} involved in hotspot 2 mediates glucose response via the \textit{cAMP}-dependent pathway. We found candidates \textit{SSN8} and \textit{GCR1} in chromosome XIV:558284-595885 and chromosome XVI:368296-408883 which also function in glucose response as introduced above. This observation suggests that different forms of regulation may exist. \textit{Ira2} globally regulates gene expressions in the glucose response related pathways including \textit{GPG1} and \textit{RNT1} [8], whereas \textit{Ssn8} and \textit{Gcr1} specifically regulate the co-expression between \textit{GPG1} and \textit{RNT1}. Cis-acting eQTL may affect the gene expression through affecting transcription factor binding [61,62]. Additional association signals besides cis-acting loci suggest other regulatory mechanisms for these genes. Hence, analysis based on 1D-traits and 2D-traits complement each other in identifying regulatory signals and they may reflect different regulation mechanisms.


discussion

We have developed a novel statistical approach to identifying gene pairs whose co-expression patterns are jointly regulated by
genes in the inferred loci as shown in the examples in the results section. However, significant challenges remain to identify candidate genes in the inferred loci and interpret the results. First, there is genetic buffering in a robust regulatory network, and we may not be able to infer all the direct linkages from eQTL studies. The mediators not observed between indirectly linked loci and genes make it more difficult to interpret the regulatory linkage. Second, in the Epistasis-2D modules, the genetic loci may affect one of the two target genes or both of them, and either situation will cause the variation of the co-expression pattern. This also increases difficulty for interpreting the linkage results. Therefore it is often necessary to incorporate information from other resources to interpret the detected modules. For example, in the oxidative phosphorylation pathway modules we illustrated in the results section, the co-expression patterns between Dhp8 and other oxidative phosphorylation genes are co-regulated. Since the candidates we predicted are all involved in the oxidative phosphorylation pathway, it is quite possible that only the expression of oxidative phosphorylation genes in Dhp8-related modules, but not the expression of Dhp8, is actually affected. Similarly, we also investigated different types of databases to collect evidences and interactions for interpreting other discussed modules. It is important to integrate other data sources including protein interactions, transcription and proteomics data under a consistent framework to better interpret the results. This generic idea has been formalized in different ways for interpreting one-to-one linkages [64–67], and more work is needed to adapt these methods to interpret the modules identified by our method. Utilizing our results through integration of multiple data sources is an interesting future direction. Our strategy could also be applied to other eQTL data in mouse or human. For example, in the mouse eQTL data, there are around 2000 markers which is

Figure 7. 1D-trait and 2D-trait reflect different genetic regulations. \( \text{RNT1} \) is up-regulated in segregants bearing the \( \text{BY} \) allele at \( \text{IRA2} \) (\( \text{IRA2} = 1 \)); \( \text{GPG1} \) is up-regulated in segregants bearing the \( \text{RM} \) allele at \( \text{IRA2} \) (\( \text{IRA2} = 0 \)); the expressions of \( \text{RNT1} \) and \( \text{GPG1} \) are negative correlated except in segregants bearing the \( \text{BY} \) allele at two other loci containing candidates \( \text{GCR1} \) and \( \text{SSN8} \). doi:10.1371/journal.pgen.1003414.g007
Statistical Analysis Reveals Epistasis-2D Modules

Materials and Methods

Conditional bivariate model

We define a module in this manuscript as the collection of a pair of loci and a pair of genes, denoted as \( M = (L_1,L_2,G_1,G_2) \), where \( L_1 \) and \( L_2 \) represent two loci and \( G_1 \) and \( G_2 \) represent two genes. Our objective is to identify Epistasis-2D modules where \( L_1 \) and \( L_2 \) interact to affect the co-expression patterns of \( G_1 \) and \( G_2 \). To formally describe our model, we use \((A,B,X,Y)\) to denote the genotypes of \( L_1 \) and \( L_2 \) and the expressions of \( G_1 \) and \( G_2 \). We assume that

\[
\begin{split}
(X | Y) &\sim N \left( 
\begin{pmatrix} \mu_1 \\ \mu_2 \\ \mu_3 \\ \mu_4 \\ \mu_5 \\ \mu_6 \\ \mu_7 \\ \mu_8 
\end{pmatrix} , \Sigma(\beta_{ij} , A,B) \right) \\
\end{split}
\]

where

\[
\Sigma(\beta_{ij} , A,B) = \begin{pmatrix}
\sigma^2_1 & \sigma_1 \sigma_2 \rho(\beta_{ij} , A,B) \\
\sigma_1 \sigma_2 \rho(\beta_{ij} , A,B) & \sigma^2_2
\end{pmatrix}
\]

is the covariance matrix, and

\[
\rho(\beta_{ij} , A,B) = \sum_{i \in I , j \in T} \beta_{ij} I(A = i)I(B = j)
\]

where \( I \) is the indicator function, i.e. \( I(A = i) = 1 \) if \( A = i \) and 0 otherwise, and \( T \) is the set of genotypes. For example, in the yeast dataset \( T = \{0,1\} \). In this study, we focus on associations that cannot be detected using 1D-trait (expression level), i.e. we assume that \( \mu_4 \) and \( \mu_5 \) are independent of \( A \) and \( B \). This simple model may have overall good statistical power to detect Epistasis-2D modules as discussed in detail in the supplementary materials (Text S1, Figures S6 and S7). Without loss of generality, we let \( \mu_1 = \mu_2 = 0 \), which approximately hold after applying the following transformation to the expression data:

- For each gene, calculate the rank of the expression for each sample, denoted as \( R_1, ..., R_n \);
- Calculate the transformed expression level for each gene as \( \Phi^{-1}(\frac{R_1}{n+1}), ..., \Phi^{-1}(\frac{R_n}{n+1}) \), where \( \Phi(.) \) is the cumulative normal distribution;

The normal quantile transformation based on individual genes is a means to “normalize” the sample observations so that our procedure is robust to the effects of extreme observations and/or highly skewed distributions [12,15] (Text S1, Figures S1 and S2). In our model, we assume that \( \sigma_1 \) and \( \sigma_2 \) are independent of the genotype because we found this specification achieved a good balance between model adequacy and simplicity. We illustrate this through the analysis of simulated data and a subset of the real data in the supplementary materials (Text S1; Figures S3, S4, S5). We found that although it was feasible to fully consider genotype dependent variances, there may be overall power loss due to additional model parameters, especially when the sample size is limited.

Filtering process

Since MLE needs to solve a numerical optimization problem, applying the tests above to all possible modules is computationally expensive. Therefore, we introduce a statistic “PA-score” (Potential of Association) to estimate the lower bound of the p-value for each module. The PA-score is defined as

\[
PA = n \log(1 - \hat{\rho}^2) - \sum_{i,j \in T} n_{ij} \log(1 - \hat{\rho}^2_{ij})
\]

where \( n_{ij} \) is the number of individuals with genotypes \( A = i \) and \( B = j \), \( \hat{\rho}_{ij} \) is the Pearson correlation coefficient of the expression levels among the \( n_{ij} \) individuals and \( \hat{\rho} \) is the correlation coefficient among all the individuals.

We prove in the Text S1 that the expectation of PA corresponds to the lower bound of p-value for each module. In this case, we could control the sensitivity by choosing the cutoff for PA to filter out modules before performing the LR tests. In this paper, we chose a cutoff value of 45 for PA which has an estimated sensitivity > 0.995. The sensitivity here is one minus the percentage of the significant LR test modules which will be filtered out by PA score filtering. The details of the sensitivity estimation are provided in the supplementary materials (Text S1, Figure S8). After the PA-score filtering, more than 10^6 modules remained for each condition. Using the LR tests, we identified 225 and 224 2D-traits whose correlation patterns were under the epistatic control of pairs of markers. Therefore we estimated that \( 225 \times 0.005 < 1 \) epistatic controlled 2D-traits was filtered out by PA score in each condition. Since PA can be directly calculated from the data, the filtering process can reduce the total computing time by a factor of 16 from our experiments with the data (Text S1).

Yeast dataset

We analyzed the yeast dataset collected by Kruglyak and colleagues [3]. The expression data were downloaded comparable to the yeast data and the number of differentially expressed transcripts was around 8000 [4]. In this case, the search space is on the order of \( 10^{33} \), which can be readily handle by parallelizing our algorithm. In the human eQTL data, up to 5,000,000 SNPs may be genotyped and up to 50,000 transcripts may be profiled. This will dramatically increase the computation time. We may reduce the computational burden by focusing only on those transcripts of interest (e.g., those known to be relate to diseases) or setting more stringent cutoffs in the filtering process to accelerate the processing. However, more computationally efficient methods need to be developed to identify Epistasis-2D modules for these data if we want to consider all the traits and markers. One possible direction is to jointly consider multiple markers within a region as those done for GWAS data [68,69].
from http://www.plosbiology.org/article/info%3Adoi%2F10.1571%2F10.1371%2Fjournal.pbio.0060083, with 4,482 genes measured in 109 segregants derived from a cross between BY and RM. The experiments were performed under two conditions, glucose and ethanol. We removed 63 genes with more than 10 missing values in either condition for a total of 4,419 genes analyzed. The authors provided genotypes at 2,956 loci. We combined neighboring loci having fewer than 5 discordant calls among the 109 samples, leading to 829 merged markers. In this manuscript, we still call these merged markers as markers to simplify the discussion. For each marker pair, an individual can have four joint genotypes, (0,0), (0,1), (1,0), and (1,1). We only considered marker pairs where there were at least 15 individuals for each joint genotype. There were a total of 305,301 such pairs. Therefore, we tested 305,301 × C²_{4150} ≈ 3 × 10^{12} modules. The algorithm was implemented in R. Applying our procedure to one condition took one week of one CPU on a Linux cluster with 2.40 GHz CPU.

False discovery rate estimation

We estimate the false discovery rate (FDR) through a permutation technique similar to previous study [8]. Specifically, we randomly permuted the expression data across all the genes and applied our procedure to the permuted data set using exactly the same setting as the real dataset. That is we used the same cutoff 45 for PA, and the same cutoff 10^{-12} for p-values of the LR tests (also select the best model). For a given threshold for LR tests, we counted the number of unique 2D-trait involved in modules with their p-values lower than the threshold. Note that we did not use the number of modules to calculate FDR because a 2D-trait may be mapped to multiple neighboring marker pairs since neighboring markers tended to have similar genotypes. Hence, we use 2D-trait to label the modules for FDR estimation. We performed ten permutations for each condition to yield ten sets of 3 × 10^{12} simulated null modules, and the results were consistent across these ten permuted data sets (Table S2). For example, at the threshold value of 10^{-12}, the average number of unique 2D-trait involved in modules with a statistical significance level less than 10^{-12} in the permuted dataset was 36.4 (SD = 4.9) and 38.5 (SD = 5.1), respectively. Therefore, with a total of 225 and 224 significant 2D-trait-identified for the observed data under the two conditions, the estimated FDR was <0.2 for both conditions (Text S1, Table S3).

Merging of Epistasis-2D modules

Among the inferred Epistasis-2D modules, neighboring markers tended to show similar patterns of interactions as discussed previously [70]. We merged neighboring markers with fewer than 15 individuals showing discordant genotypes among all samples, leading to 266 merged markers for clustering analysis. Table S4 listed all detected Epistasis-2D modules after the merging.

Clustering in the epistasis map

We define an epistasis map E under a specific condition as

\[ E_{ij} = \begin{cases} 1 & \text{if there is at least one Epistasis-2D module containing Locus i and Locus j}, \\ 0 & \text{otherwise}. \end{cases} \]

We performed hierarchical clustering on this map using Cluster 3.0.

Functional enrichment analysis for all gene pairs in the modules

For each gene, we used GO slim to annotate its function. The gene pair in each Epistasis-2D module were annotated with a pair of functions. To investigate whether a particular pair of functions were over-represented among the Epistasis-2D modules, we performed the following hypergeometric test,

\[ p = \frac{\sum_{i=k}^{n} C_{M}^{i} C_{N-M}^{n-i}}{C_{N}^{n}} \]

(9)

where N is the total number of gene pairs, M is the number of gene pairs with two specific functions, n is the number of gene pairs from Epistasis-2D modules, and k is the number of Epistasis-2D gene pairs having the specific two functions. The p-values were Bonferroni corrected for multiple testing.

Before analyzing the results, we note that many genes involved in these function categories are overlapped. Under the glucose condition, 18 genes annotated as “precursor metabolites/energy” actually consist of carbohydrate metabolic genes (7/18) and cellular respiration genes (11/18). Genes annotated as “cofactor metabolic” are also highly overlapped with these two processes (7/13). In addition, genes annotated as “RNA metabolic process” are mainly involved in ribosome biogenesis (33/80). Under the ethanol condition, the most genes annotated as “transcription” and “chromosome organization” are involved in the RNA metabolic process (32/34, 9/19). According to these overlaps, the main differences between the two conditions can be summarized as shown in Figure 4.

Functional enrichment analysis for chromosome intervals

Since a chromosomal interval encompassing the markers may contain multiple candidate genes, we need to perform enrichment analysis to investigate whether there is statistically significant evidence for the enrichment of certain function. We performed hypergeometric test to investigate whether a particular function was over-represented among the genes located at the chromosomal intervals within one or several modules. The p-value was calculated as,

\[ p = \prod_{i=1}^{n} \frac{C_{M}^{n_{i}} C_{N-M}^{n-j_{i}}}{C_{N}^{n}} \]

(10)

where n is the total number of considered chromosome intervals, N is the total number of annotated genes, M is the number of genes with specific function, n_{i} is the number of genes in ith chromosome interval, and k_{i} is the number of genes having the specific function in ith chromosome interval. The gene function is defined by GO annotation at level 5 (downloaded from DAVID Knowledgebase [71, 72]). The calculation detail of the examples that discussed in the results section was listed in Table S6.

Supporting Information

Figure S1 Examples showing the effect of normal quantile transformation. (A) Example 1 with an outlier which overestimates the correlation. (B) Normal quantile transformation (NQT) of Example 1 can reduce the effect of the outlier. (C) Example 2 with an outlier which understimates the correlation. (D) Normal quantile transformation can reduce the effect of the outlier. (EPS)
Figure S2 Comparison of correlation coefficients calculated from the original data and transformed data. As shown in the figure, there is a high degree of correlation between gene expression correlation coefficients calculated from normalized and unnormalized data.

Figure S3 Comparison of estimating standard deviation from MLE and using SD = 0.97. (A) For the identified Epistasis-2D modules with large LR statistics, the LR statistics calculated from the two methods are highly correlated. (B) For randomly sampled modules with relative low LR statistics, the LR statistics calculated from the two methods are also highly correlated. (C) The distribution of the estimated standard deviations for the Epistasis-2D modules. (D) The distribution of the estimated standard deviations for random sampled modules.

Figure S4 Comparison of the statistical power of model (3) and model (6) using simulated data. (A) For simulated IMDVED modules with 100 samples, using model (6) resulted in higher power than model (3) for 54% of the modules. (B) For simulated IMIVED modules with 100 samples, using model (6) resulted in lower power than model (3) for 88% of the modules. (C) For simulated IMDVED modules with 500 samples, using model (6) resulted in higher power for 89% of the modules. (D) For simulated IMIVED modules with 500 samples, using model (6) resulted in lower power than model (3) for 94% of the modules.

Figure S5 Comparison of the statistical power of model (3) versus model (6) using real data. For modules with \( p < 10^{-10} \) based on the LR tests, using model (6) resulted in lower power than model (3) for 73% of the modules.

Figure S6 Comparison of the statistical power of model (3) and model (20) using simulated data. (A) For simulated DMIVED modules with 100 samples, using model (20) led to higher power than model (3) for 58% of the modules. (B) For simulated IMIVED modules with 100 samples, using model (20) resulted in lower power than model (3) for 68% of the modules. (C) For simulated DMIVED modules with 500 samples, using model (20) led to higher power for 92% of the modules. (D) For simulated IMIVED modules with 500 samples, using model (6) resulted in lower power than model (3) for 96% of the modules.

Figure S7 Comparison of the statistical power of model (3) and model (20) using real data. For modules with \( p < 10^{-10} \) in the LR tests, using model (20) resulted in lower power than model (3) for 96% of the modules.

Figure S8 Evaluation of PA score and sensitivity estimation. (A) For simulated IMIVED modules, the LR scores and PA scores are highly correlated. (B) For simulated IMDVED modules, the correlation between LR scores and PA scores is lower than that in IMIVED modules. (C) For simulated DMIVED modules, the correlation between LR scores and PA scores is also lower than that in IMIVED modules. (D) For simulated negative controls, the correlation between LR scores and PA scores is much lower than that in IMIVED modules. (E) The correlation between LR scores and PA scores in sampled modules from yeast dataset. (F) For simulated IMIVED modules and different threshold c, the fraction of modules with PA - LR > -c is relatively robust to the LR score level. (G) IMIVED modules have higher fraction of modules with PA - LR > -c for different threshold c. (H) Distribution of the difference between the PA scores and LR scores in yeast data. As shown in the figure, PA-LR = -5.8 (dash line) is the 0.005 quantile.

Table S1 Parameter settings.

Table S2 Number of unique 2D-traits in significant modules (\( p < 10^{-12} \)) in each permuted dataset.

Table S3 FDRs under different cutoffs.

Table S4 Epistasis-2D modules.

Table S5 “RNA metabolic-RNA metabolic” co-regulated modules.

Table S6 Functional enrichment analysis for Epistasis-2D modules.

Text S1 Supplementary methods and simulation study.

Author Contributions
Conceived and designed the experiments: LW HZ MD. Analyzed the data: LW. Wrote the paper: LW WZ HZ MD. Interpreted the results: LW WZ.

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