Analysis of allelic series with transcriptomic phenotypes

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Although transcriptomes have recently been used to perform epistasis analyses, they are not yet used to study intragenic function/structure relationships. We developed a theoretical framework to study allelic series using transcriptomic phenotypes. As a proof-of-concept, we apply our methods to an allelic series of dpy-22, a highly pleiotropic Caenorhabditis elegans gene orthologous to the human gene MED12, which is a subunit of the Mediator complex. Our methods identify functional regions within dpy-22 that modulate Mediator activity upon various genetic modules.

Introduction

Mutations of a gene can yield a series of alleles with different phenotypes that reveal multiple functions encoded by that gene, regardless of the alleles’ molecular nature. Homozygous alleles can be ordered by their phenotypic severity; then, phenotypes of trans-heterozygotes carrying two alleles can reveal which alleles are dominant for each phenotype. Together, the severity and dominance hierarchies show intragenic functional regions. In Caenorhabditis elegans, these series have helped characterize genes such as let-23/EGFR, lin-3/EGF and lin-12/NOTCH1,2,3.

Biological variation in expression measurements of single genes towards genome-wide measurements. Expression profiling via RNA-seq enables simultaneous measurement of transcript levels for all genes in a genome, yielding a transcriptome. These measurements can be made on whole organisms, isolated tissues, or single cells. Transcriptomes have been successfully used to identify new cell or organismal states. For mutant genes, transcriptomic states can be used for epistasis analysis9,10, but have not been used to characterize allelic series.

We have devised methods for characterizing allelic series with RNA-seq. To test these methods, we selected three alleles of a C. elegans Mediator complex subunit gene, dpy-22. Mediator is a macromolecular complex with ∼25 subunits that globally regulates RNA polymerase II (Pol II)14,15.

The Mediator complex has at least four biochemically distinct modules: the Head, Middle and Tail modules and a CDK-8-associated Kinase Module (CKM). The CKM associates reversibly with other modules, and appears to inhibit transcription16,17. In C. elegans development, the CKM promotes both male tail formation11 (through interactions with the Wnt pathway), and vulval formation18 (through inhibition of the Ras pathway). Homozygotes of allele dpy-22(bx93), which encodes a premature stop codon Q2549Amber11, appear grossly wild-type. In contrast, animals homozygous for a more severe allele, dpy-22(sy622) encoding another premature stop codon, Q1698Amber12, are dumpy (Dpy), have egg-laying defects (Egl), and have multiple vulvae (Muv). (see Fig. 1A). In spite of its causative role in a number of neurodevelopmental disorders19, the structural and functional features of this gene are poorly understood. In humans, MED12 is known to have a proline-, glutamine- and leucine-rich domain that interacts with the Wnt pathway20. However, many disease-causing variants fall outside of this domain21.

To study these variants and how they interfere with the functionality of MED12, quantitative and efficient methods are necessary. RNA-seq phenotypes have the potential to reveal functional regions within genes, but their phenotypic complexity makes this difficult. We developed a method for determining allelic series from transcriptomic phenotypes and used the C. elegans dpy-22 gene as a test case. Our analysis revealed functional regions that act to modulate Mediator activity at thousands of genetic loci.
Results and Discussion

We adapted the allelic series method, previously used for individual phenotypes, for use with expression profiles as multidimensional phenotypes (see Fig. 1). As a proof of principle, we carried out RNA-seq on biological triplicates of mRNA extracted from dpy-22(sy622) homozygotes, dpy-22(bx93) homozygotes and wild type controls, along with quadruplicates from trans-heterozygotes of both alleles. Sequencing was performed at a depth of 20 million reads per sample. Reads were pseudoaligned using Kallisto\textsuperscript{22}. We performed a differential expression using a general linear model specified using Sleuth\textsuperscript{23} (see Methods). Differential expression with respect to the wild type control for each transcript in a genotype \(g\) is measured via a coefficient \(\beta_{g,i}\), which can be loosely interpreted as the natural logarithm of the fold-change. Transcripts were considered to have differential expression between wild-type and a mutant if the false discovery rate, \(q\), was less than or equal to 10%. Supplementary File 1 contains all the beta values associated with this project. We have also generated a website containing complete details of all the analyses available at the following URL: \url{https://wormlabcaltech.github.io/med-cafe/analysis}.

By these criteria, we found 481 genes differentially expressed in dpy-22(bx93) homozygotes, and 2,863 differentially expressed genes in dpy-22(sy622) homozygotes (see Basic Statistics Notebook). Trans-heterozygotes with the genotype dpy-6[el4]/dpy-22(bx93)/+ dpy-22(sy622) had 2,214 differentially expressed genes with respect to the wild type.

We used a false hit analysis to identify four non-overlapping phenotypic classes. We use the term genotype-specific to refer to groups of transcripts that were perturbed in one mutant. We use the term genotype-associated to refer to groups of transcripts whose expression was significantly altered in two or more mutants with respect to the wild type control. The \textit{dpy-22(sy622)}-associated phenotypic class consisted of 720 genes differentially expressed in dpy-22(sy622) homozygotes and in trans-heterozygotes, but which had wild-type expression in dpy-22(bx93) homozygotes. The \textit{dpy-22(bx93)}-associated phenotypic class contains 403 genes differentially expressed in all genotypes. We also identified a \textit{dpy-22(sy622)}-specific phenotypic class (1,841 genes) and a \textit{trans-heterozygote-specific} phenotypic class (1,226 genes; see the Phenotypic Classes Notebook). All genotype-associated phenotypes had Spearman rank correlations > 0.8, indicating that transcripts within these classes changed in the same direction amongst the genotypes studied.

We measured allelic dominance for each class using a dominance coefficient (see Methods). The dominance coefficient is a measure of the contribution of each allele to the total expression level in trans-heterozygotes. By definition, the \textit{dpy-22(sy622)} allele is completely recessive to \textit{dpy-22(bx93)} for the \textit{dpy-22(sy622)}-specific phenotypic class. The \textit{dpy-22(sy622)} and \textit{dpy-22(bx93)} alleles are semidominant (\(d_{bx93} = 0.51\)) to each other for the \textit{dpy-22(sy622)}-associated phenotypic class. The \textit{dpy-22(bx93)} allele is largely dominant over the \textit{dpy-22(sy622)} allele (\(d_{sy622} = 0.81\); see Table 1) for the \textit{dpy-22(bx93)}-associated phenotypic class.

| Phenotypic Class                  | Dominance   |
|----------------------------------|-------------|
| \textit{dpy-22(sy622)}-specific  | 1.00 ± 0.00 |
| \textit{dpy-22(sy622)}-associated| 0.51 ± 0.01 |
| \textit{dpy-22(bx93)}-associated | 0.81 ± 0.01 |

Table 1. Dominance analysis for the \textit{dpy-22/MDT12} allelic series. Dominance values closer to 1 indicate \textit{dpy-22(bx93)} is dominant over \textit{dpy-22(sy622)}, whereas 0 indicates \textit{dpy-22(sy622)} is dominant over \textit{dpy-22(bx93)}.

Because the mutations we used are truncations, our results suggest the existence of various functional regions in \textit{dpy-22/MDT12} (see Fig. 2). The \textit{dpy-22(sy622)}-specific phenotypic class is likely controlled by a single functional region, functional region 1 (FC1), and the \textit{dpy-22(sy622)}-associated phenotypic class is likely controlled by a second functional region, functional region 2 (FC2). It is unlikely that these regions are identical because their dominance behaviors are very different. The \textit{dpy-22(bx93)} allele was largely dominant over the \textit{dpy-22(sy622)} allele for the \textit{dpy-22(bx93)}-associated class, but gene expression in this class was perturbed in both homozygotes. The perturbations were greater for \textit{dpy-22(sy622)} homozygotes than for \textit{dpy-22(bx93)} homozygotes. This behavior can be explained if the \textit{dpy-22(bx93)}-associated class is controlled jointly by two distinct effectors, functional regions 3 and 4 (FC3, FR4, see Fig. 2). A rigorous examination of this model will require studying alleles that mutate the region between Q1689 and Q2549 using homozygotes and trans-heterozygotes.

We also found a class of transcripts that had perturbed levels in trans-heterozygotes only; its biological significance is unclear. Phenotypes unique to trans-heterozygotes are often the result of physical interactions such as homodimerization, or dosage reduction of a toxic product\textsuperscript{24}. In the case of
Allele a and b are semidominant at this class. Functional unit is sensitive to dosage. Determine which classes are real (5). Perform a dominance analysis for all classes present in at least two genotypes. Infer functional units and sequence requirements. Find the total number of observed intersections or classes (7). Determine differentially expressed transcripts relative to a wild type control.

Figure 1. A Protein sequence of dpy-22. The positions of the nonsense mutations used are shown. B Flowchart for an analysis of arbitrary allelic series. A set of alleles is selected, and the corresponding genotypes are sequenced. Independent phenotypic classes are then identified. For each phenotypic class, the alleles are ordered in a dominance/complementation hierarchy, which can then be used to infer functional regions within the genes in question.


Figure 2. The functional regions associated with each phenotypic class can be mapped intragenically. The number of genes associated with each class is shown. The dpy-22(bx93)-associated class may be controlled by two functional regions. FR2 and FR3 could be redundant if FR4 is a modifier of FR2 functionality at dpy-22(bx93)-associated loci. Note that the dpy-22(bx93)-associated phenotypic class is actually three classes merged together. Two of these classes are DE in dpy-22(bx93) homozygotes and one other genotype. Our analyses suggested that these two classes are likely the result of false negative hits and genes in these classes should be differentially expressed in all three genotypes, so they were merged all classes together (see Methods).

dpy-22/MDT12 orthologs, how either mechanism could operate is not obvious, since DPY-22 is expected to assemble in a monomeric manner into the CKM. Massive single-cell RNA-seq of C. elegans has recently been reported. When this technique becomes cost-efficient, single-cell profiling of these genotypes may provide information that complements the whole-organism expression phenotypes, perhaps explaining the origin of this phenotype.

Intragenic mapping of functional regions associated with phenotypic classes is important, but their biological meaning remains unclear. To assign biological functionality to phenotypic classes, we extracted transcriptomic signatures associated with a Dumpy (Dpy) phenotype using transcriptomes from dpy-7 and dpy-10 mutants (DAA, CPR and PWS unpublished), and a hif-1-dependent hypoxia response from a previously published analysis and asked whether any phenotypic class was enriched in either response. The sy622-specific and -associated classes were enriched in genes that are transcriptionally associated with a Dpy phenotype (fold-change enrichment = 3, p = 2 \times 10^{-40}, 167 genes observed; fold-change = 1.9, p = 9 \times 10^{-9}, 82 genes observed). The bx93-associated class also showed significant enrichment (fold-change = 2.2, p = 4 \times 10^{-10}, 68 genes observed). The class that showed the most extreme deviation from random was the sy622-specific class. dpy-22(sy622) homozygotes are severely Dpy, whereas dpy-22(bx93) homozygotes and trans-heterozygotes have a slight Dpy phenotype. Plotting the changes in gene expression for sy622 homozygotes versus the changes in expression in dpy-7 mutants revealed that 75% of the transcripts were strongly correlated in both genotypes (see Figure 3). Therefore, the sy622-specific phenotypic class contains a transcriptional signature associated with morphological Dpy phenotype (see the Enrichment Notebook).

dpy-22 is not known to be upstream of the hif-1 dependent hypoxia response in C. elegans. Enrichment tests revealed that the hypoxia response was significantly enriched in the bx93-associated (fold-change = 2.1, p = 10^{-8}, 63 genes observed), the sy622-associated (fold-change = 1.9, p = 4 \times 10^{-8}, 78 genes observed) and the sy622-specific classes (fold-change = 2.4, p = 9 \times 10^{-55}, 186 genes observed). However, there was no correlation between the expression levels of these genes in dpy-22 genotypes and the expression levels expected from the hypoxia response. Although the hypoxia gene battery can be found in dpy-22 mutants, these genes are not used to deploy a hif-1-dependent hypoxia phenotype. Taken together, our results suggest that transcriptomic signatures can be used to understand the biological func-
tionality of phenotypic classes, and they may be useful in associating phenotypic classes with other phenotypes. This highlights the importance of generating an index set of mutants that can be used to derive a gold standard of transcriptional signatures with which to test future results.

Transcriptomic phenotypes generate large amounts of differential gene expression data, so false positive and false negative rates can lead to spurious phenotypic classes whose putative biological significance is badly misleading. Such artifacts are particularly likely for small phenotypic classes, which should be viewed with skepticism. Notably, errors of interpretation cannot be avoided by setting a more stringent q-value cut-off: doing so will decrease the false positive rate, but increase the false negative rate, which will in turn produce smaller phenotypic classes than expected. Our method avoids this pitfall by using total error rate estimates to assess the plausibility of each class. These conclusions are of broad significance to research where highly multiplexed measurements are compared to identify similarities and differences in the genome-wide behavior of a single variable under multiple conditions.

We have shown that transcriptomes can be used to study allelic series in the context of a large, pleiotropic gene. We identified separable phenotypic classes that would otherwise be obscured by other methods, correlated each class to a functional region, and identified sequence requirements for each region. Given the importance of allelic series for characterizing gene function and their roles in specific genetic pathways, we are optimistic that this method will be a useful addition to the geneticist’s arsenal.

Methods

Strains used

Strains used were N2 wild-type (Bristol), PS4087 dpy-22(sy622), PS4187 dpy-22(bx93), and PS4176 dpy-6(e14) dpy-22(bx93)/ + dpy-22(sy622). Lines were grown on standard nematode growth media (NGM) Petri plates seeded with OP50 E. coli at 20°C.

Strain synchronization, harvesting and RNA sequencing

Strains were synchronized by bleaching P0’s into virgin S. basal (no cholesterol or ethanol added) for 8–12 hours. Arrested L1 larvae were placed in NGM plates seeded with OP50 at 20°C and grown to

Figure 3. sy622 homozygotes show a transcriptional response associated with the Dpy phenotype. A We obtained a set of transcripts associated with the Dpy phenotype from dpy-7 and dpy-10 mutants. We identified the transcripts that were differentially expressed in sy622 homozygotes. We ranked the β values of each transcript in sy622 homozygotes and plotted them against the ranked β values in dpy-7 mutants. A significant portion of the genes are correlated between the two genotypes, showing that the signature is largely intact. 25% of the genes are anti-correlated. B We performed the same analysis using a set of transcripts associated with the hif-1-dependent hypoxia response as a negative control. Although sy622 is enriched for the transcripts that make up this response, there is no correlation between the β values in sy622 homozygotes and the β values in egl-9 homozygotes.
the young adult stage (assessed by vulval morphology and lack of embryos). RNA extraction and sequencing was performed as previously described by Angeles-Albores et al.\(^{10,7}\).

**Read pseudo-alignment and differential expression**

Reads were pseudo-aligned to the *C. elegans* genome (WBcel235) using Kallisto\(^{22}\), using 200 bootstraps and with the sequence bias (--seqBias) flag. The fragment size for all libraries was set to 200 and the standard deviation to 40. Quality control was performed on a subset of the reads using FastQC, RNaseQC, BowTie and MultiQC\(^{27,28,29,30}\).

Differential expression analysis was performed using Sleuth\(^{21}\). We used a general linear model to identify genes that were differentially expressed between wild-type and mutant libraries. To increase our statistical power, we pooled young adult wild-type replicates from other published\(^{10,7}\) and unpublished analyses adjusting for batch effects.

**False hit analysis**

To accurately count phenotypes, we developed a false hit algorithm (Algorithm 1). We implemented this algorithm for three-way comparisons in Python. Although experimentally restricted, a three-way comparison can result in >5,000 possible sets (ignoring size). This large number of models necessitates an algorithmic approach that can at least restrict the possible number of models. Our algorithm uses a noise function that assumes false hit events are non-overlapping (i.e. the same gene cannot be the result of two false positive events in two or more genotypes) to determine the average noise flux between phenotypic classes. These assumptions break down rapidly if false-positive or negative rates exceed 20%.

To benchmark our algorithm, we generated one thousand Venn diagrams at random. For each Venn diagram, we calculated the average false positive and false negative flux matrices. Then, we added noise to each phenotypic class in the Venn diagram, assuming that fluxes were normally distributed with mean and standard deviation equal to the flux coefficient calculated. We input the noised Venn diagram into our false hit analysis and collected classification statistics. For a given signal-to-noise cut-off, \(\lambda\), classification accuracy varied significantly with changes in the total error rate. In the absence of false negative hits, false hit analysis can accurately identify non-empty genotype-associated phenotypic classes, but identifying genotype-specific classes becomes difficult if the experimental false positive rate is high. On the other hand, even moderate false negative rates (>10%) rapidly degrade signal from genotype-associated classes. For classes that are associated with three genotypes, an experimental false negative rate of 30% is enough on average to prevents this class from being observed.

We selected \(\lambda = 3\) because classification using this threshold was high across a range of false positive and false negative combinations. A challenge to applying this algorithm to our data is the fact that the false negative rate for our experiment is unknown. Although there has been significant progress in controlling and estimating false positive rates, we know of no such attempts for false negative rates. It is unlikely that the false negative rate for our study is lower than the false positive rate, because all genotypes except the controls are likely underpowered. We used false negative rates between 10–20% for false hit analysis. When the false negative rate was set at 15% or higher, the algorithm converged on the same five classes shown above. For false negative rates between 10–15%, the algorithm output the same five classes, but also accepted the \((dpy-22(bx93))-associated class. We selected the model corresponding to false negative rates of 15–20% because this model had lower \(\chi^2\) values than the model selected with a false negative rate of 10–15% (4,212 versus 100,650).

We asked whether re-classification of some classes into others could improve model fit. We manually re-classified the \((dpy-22(sy622),dpy-22(bx93))-associated and the \((dpy-22(bx93),transheterozygote)-associated classes into the \(bx93\)-associated class (which is associated with all genotypes), and we compared \(\chi^2\) statistics between a re-classified reduced model and a reduced model. The re-classified model had a lower \(\chi^2\) (181). Thus, we concluded that the re-classified reduced model is
the most likely model to give rise to our data.

Data: $M_{obs} = \{N_i\}$, an observed set of classes, where each class is labelled by $l \in L$ and is of size $N_i$. $f_p, f_n$, the false positive and negative rates respectively. $\alpha$, the signal-to-noise threshold for acceptance of a class.

Result: $M_{reduced}$, a reduced model that fits the data.

\begin{algorithm}
\begin{algorithmic}
\STATE Define a minimal set to initialize the reduced model
\STATE $K = \{\min_{l \in L} N_i\}$
\STATE \textbf{while} ($i < i_{\text{max}}$) \textbf{do}
\STATE \hspace{1em} Define a noise function to estimate error flows in $K$
\STATE \hspace{1em} $F \leftarrow \text{noise}(K, f_p, f_n)$
\STATE \hspace{1em} \textbf{for} $l \in L$ \textbf{do}
\STATE \hspace{2em} Calculate signal to noise for each labelled class
\STATE \hspace{2em} False negatives can result in $\lambda < 0$
\STATE \hspace{2em} $\lambda_l \leftarrow M_{obs,l} / F_l$
\STATE \hspace{2em} \textbf{if} ($\lambda > \alpha$) \textbf{then}
\STATE \hspace{3em} $K_l \leftarrow M_{obs,l}$
\STATE \hspace{1em} end
\STATE \hspace{1em} end
\STATE \hspace{1em} $i++$
\STATE \textbf{end}
\STATE \textbf{end}
\STATE Return the reduced model
\STATE $M_{reduced} = K$
\STATE return $M_{reduced}$
\end{algorithmic}
\end{algorithm}

Algorithm 1: False Hit Algorithm. Briefly, the algorithm initializes a reduced model with the phenotypic class or classes labelled by the largest number of genotypes. This reduced model is used to estimate noise fluxes, which in turn can be used to estimate a signal-to-noise metric between observed and modelled classes. Classes that exhibit a high signal-to-noise are incorporated into the reduced model.

Dominance analysis

We modeled allelic dominance as a weighted average of allelic activity:

$$\beta_{a/b,i,\text{Pred}}(d_a) = d_a \cdot \beta_{a/a,i} + (1 - d_a) \cdot \beta_{b/b,i}$$

where $\beta_{k/k,i}$ refers to the $\beta$ value of the $i$th isoform in a genotype $k/k$, and $d_a$ is the dominance coefficient for allele $a$.

To find the parameters $d_a$ that maximized the probability of observing the data, we found the parameter, $d_a$, that maximized the equation:

$$P(d_a|D, H, I) \propto \prod_{i \in S} \exp \left( \beta_{a/b,i,\text{Obs}} - \beta_{a/b,i,\text{Pred}}(d_a) \right)^2 / 2\sigma_i^2$$

where $\beta_{a/b,i,\text{Obs}}$ was the coefficient associated with the $i$th isoform in the trans-het $a/b$ and $\sigma_i$ was the standard error of the $i$th isoform in the trans-heterozygote samples as output by Kallisto. $S$ is the set of isoforms that participate in the regression (see main text). This equation describes a linear regression which was solved numerically.

Data Availability

Raw and processed reads were deposited in the Gene Expression Omnibus. Scripts for the entire analysis can be found with version control in our Github repository, \url{https://github.com/WormLabCaltech/med-cafe}. A user-friendly, commented website containing the complete analyses can be found at \url{https://wormlabcaltech.github.io/med-cafe/}. Raw reads and quantified abundances for each sample were deposited at the NCBI Gene Expression Omnibus (GEO) under the accession code GSE107523 (\url{https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107523}).

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