Genetic Analysis of a Metazoan Pathway using Transcriptomic Phenotypes

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By definition, phenotypes are measurable traits that are related to genotypes via a formal mapping function. A requirement for a genetic relationship to exist is that two genes must act on the same phenotype; however, the converse, that two genes share a phenotype, does not imply that these genes interact. One way to prove genetic interaction is to measure epistasis in a double mutant. Epistasis refers to changes in a phenotype that are not additive. Epistatic analysis remains an important cornerstone of genetics today Phillips (2008).

Previous work in *S. cerevisiae* and *D. discoideum* using microarrays has shown that transcriptomes contain sufficient information to infer genetic relationships in a simple eukaryote Hughes et al. (2000; Van Driessche et al. 2005). Developments in the area of transcriptomics have brought forward new protocols for measuring global gene expression, such as RNA-seq Mortazavi et al. (2008), and have also made important progress towards cheaper sequencing Metzker (2010), better and faster abundance quantification Patro, Mount, and Kingsford (2014; N. L. Bray et al. 2015; Patro, Duggal, and Kingsford 2015) and improved differential analysis H. J. Pimentel et al. (2016; C. Trapnell et al. 2013). As a result, RNA-seq has been used to identify key regulatory modules involved in a variety of processes, including T-cell regulation Singer et al. (2016; A. K. Shalek et al. 2013), the *C. elegans* linker cell migration Schwarz, Kato, and Sternberg (2012), or planarian stem cell maintenance Van Wolfswinkel, Wagner, and Reddien (2014; Scimone et al. 2014). However, even in these novel applications, transcriptomes largely serve a descriptive role.

To investigate the ability of transcriptomes to serve as quantitative phenotypes, we selected mutants in the *C. elegans* hypoxia pathway for transcriptome sequencing. The hypoxia pathway is a conserved pathway that is found in all metazoans Gregg L. Semenza (2012). It plays an important role in oxygen and iron homeostasis and in the immune response among others Nizet and Johnson (2009; Ackerman and Gems 2012; N. V. Kirienko et al. 2013), and it is believed to play an important role in carcinogenesis and cancer progression, making it an attractive therapeutic target for disease Gregg L Semenza (2003). Under normoxic conditions, HIF-1 is constitutively degraded by a futile cycle that involves hydroxylation by the EGLN1 ortholog EGL-9, followed by ubiquitination by the von Hippel-Lindau Suppressor 1, VHL-1 Bishop et al. (2004; Shao, Zhang, and Powell-Coffman 2009; Tanimoto et al. 2000; Jaakkola et al. 2001). Inhibition of hydroxylation leads to accumulation of HIF-1 Bishop et al. (2004). In *C. elegans, egl-9* is known to be positively regulated at the protein level by *rhy-1*, via inhibition of *cysl-2*, which is an inhibitor of *egl-9* In turn, *rhy-1* and *egl-9 are* known to be induced in *a* HIF-1dependent manner. Shen, Shao, and Powell-Coffman (2006; Dengke K. Ma et al. 2012).

Here, we show that transcriptomes contain robust signals that are sufficient to reconstruct the hypoxia pathway in *C. elegans*. Our goal is not to generate a high-quality database of hypoxia-related genes, but rather to perform a quantitative genetic analysis analogous to classical genetics. Using this experimental setup, we show that various techniques, including pairwise transcriptome comparisons, expression clustering or *in silico* qPCR can be used to generate a testable model of genetic interactions. A complete, interactive version of the analysis is also available at <www.wormlabcaltech.github.io/mprsq>.

# Results

Transcriptomes of 4 single mutants, 2 single mutants

## Clustering visualizes epistatic relationships between genes

As a first step in our analysis, we analyzed our data using a general linear model with a genotype term (see [methods]) on logarithm-transformed counts. Genes that are significantly altered between wild-type and a given mutant have a genotype coefficient that is statistically significantly different from 0. We refer to these coefficients through the greek letter . These coefficients are not identical to the average log-fold change per gene, although they are loosely related to this quantity. In general, larger magnitudes correspond to larger perturbations. These coefficients can be used to study the RNA-seq data in question.

Clustering is a well-known technique in bioinformatics that is used to identify relationships between high dimensional data points Yeung, Medvedovic, and Bumgarner (2003). We wanted to make sure that clustering by differential expression yielded genetically relevant information. *hif-1* exhibits no obvious phenotypes under normoxic conditions, in contrast to *egl-9*, which exhibits an egg-laying (*egl*) phenotype in the same environment. *egl-9*; *hif-1* mutants suppress the *egl* phenotype. If transcriptomic phenotypes behave similarly to their macroscopic counterparts, *hif-1* should cluster with the *egl-9*; *hif-1* double mutant, whereas *egl-9* should cluster away from the *hif-1* mutant. Indeed, when unsupervised clustering was performed on the data, three clusters emerged. *hif-1* and *egl-9*;*hif-1* clustered together; whereas *egl-9*, *egl-9*;*vhl-1*, *vhl-1* and *rhy-1* all clustered separately. Finally, our negative control *fog-2* was in its own cluster (see Fig. [fig:dendrogram]). We conclude that expression data contains enough signal to cluster genes in a meaningful manner in complex metazoans.



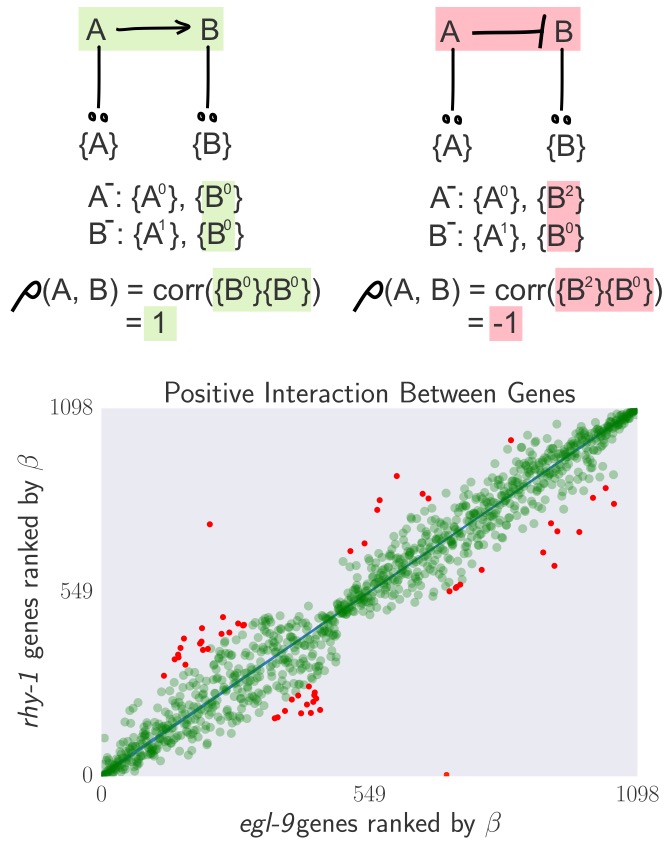
Blind unsupervised clustering of various C. elegans mutants. Genes cluster in a manner that is biologically intuitive. Genes that inhibit hif-1 (i.e, egl-9, vhl-1, and rhy-1) cluster far from hif-1. hif-1 clusters with the suppressed egl-9; hif-1 double mutant. A control gene, fog-2, clusters farthest away.

## Transcriptomic correlations can predict genetic regulation

Theoretically, two genes that share linear positive regulation should be positively correlated in their overlapping transcriptomes, whereas two genes that share linear negative regulation should be negatively correlated in their transcriptomes. Conversely, it follows that if two mutants have overlapping transcriptomes that are strongly positively correlated, it is likely that these two genes share a positive regulatory association. In other words, the existence of a transcriptomic correlation between two genes is a good predictor of genetic regulation between them. We have developed a formal genetic notation and logic to analyze and understand regulatory relationships between genes (see S.I.).

Although transcriptomic correlations could theoretically be used for the purposes of identifying genetic regulation, noise can cause serious interference with any inferences that are made. Additionally, genes sometimes experience multiple modes of regulation, including positive and negative regulation, from the same gene or pathway. Because we are measuring the system at steady state, both modes of regulation will be measured simultaneously. If a positive and a negative signal of equal signal are both present in a transcriptome, running a naive regression may result in a value close to zero. Therefore, we took steps to mitigate noise emanating from frequent outliers and to identify multiple regulatory signals.

To mitigate noise, we rank-tranformed the coefficients for each mutant. This has the effect of mitigating outliers by resetting the difference between adjacent coefficients to unity. Next, we performed robust Bayesian regressions using a Student-T distribution as a prior. A Student-T distribution decays less quickly than a normal distribution, which causes the model to consider outliers to be less informative than traditional regressions.



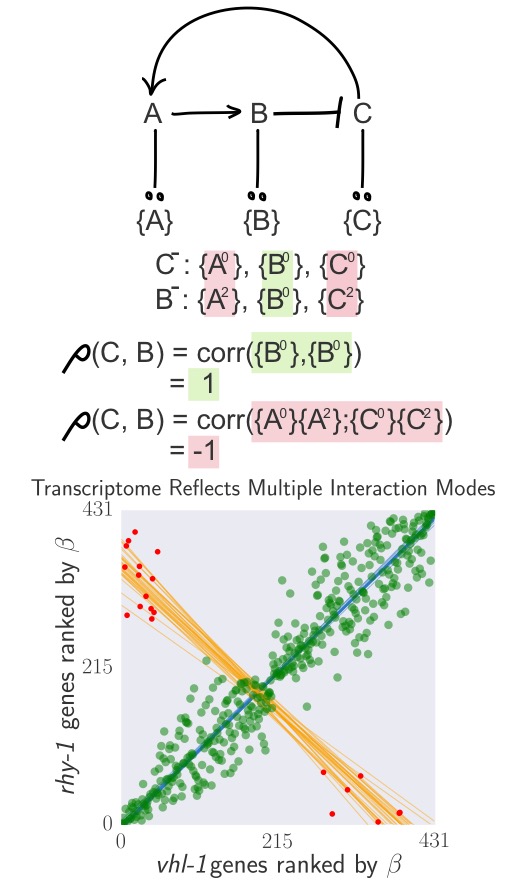
**Top** Schematic Diagram showing that genes that interact positively should have a positive transcriptomic correlation, whereas genes that interact negatively should have a negative correlation. Single genes are referred to by their names (A, B), and the transcriptome associated only with gene X is referred to as {X}. We use superscripts to denote expression level. In this case, 0 = no expression (knockout); 1 = WT level; 2 = Greater than WT level. **Bottom** Empirical demonstration that transcriptomes between two interacting genes can be extremely well correlated when genes are ranked by expression changes relative to a wild-type.

We saw that certain gene pairs correlated very well when genes were ranked by their expression changes (see Fig. [fig:genetic\_interactions]). We were able to identify a strong positive interaction between *egl-9* and *rhy-1*. The transcriptomes for these genes consisted of 1487 and 1816 significantly altered genes respectively and the overlap between both transcriptomes was extensive. On the other hand, none of the primary correlations between *hif-1* and its controlling genes were negative. We were unable to definitively determine the reason for behind this. The overlap between *hif-1* and all other genes was relatively small, and each overlap involved different sets of genes, which suggests that we did not sequence deeply enough to identify the nature of these positive interactions. See SI A for an exhaustive analysis of the expected and observed correlation between each gene pair in this circuit respectively. Next, we generated all pairwise correlations between transcriptomes and we weighted the correlations by the number of genes that participated in the correlation (that were not outliers) divided by the total number of differentially expressed genes in either transcriptome. The regression slopes recapitulated a network with three ‘modules’: A control module, a responder module and an uncorrelated module (see Fig. [fig:heatmap]).



**Top**: Heatmap showing pairwise regression values between all single mutants. **Bottom**: Correlation network drawn from the diagram. Edge width is directly proportional to the regression value.

Previous work in the hypoxia pathway has found extensive feedback loops in this pathway. Using the same genetic formalism as above, we realized that due to the fine-grained nature of interactomes, we can use them to measure two regulatory interactions of opposite sign simultaneously in a single gene pair. This should lead to a characteristic *X* pattern in the ranked data. Such cross patterns could be indicative of loops (see Fig [fig:xpattern]).



**Top**: A feedback loop can generate transcriptomes that are both correlated and anti-correlated. **Bottom**: hif-1 transcriptome correlated to the rhy-1 transcriptome. Green large points are inliers to the first regression. Red small points are outliers to the first regression. Only the red small points were used for the secondary regression. Blue lines are representative samples of the primary bootstrapped regression lines. Orange lines are representative samples of the secondary bootstrapped regression lines.

We investigated whether any pairwise comparisons between our single mutants generated this cross pattern. Indeed, we found that comparing *hif-1* with *rhy-1*, and *hif-1* with *egl-9* yielded negative correlations, as did *rhy-1* and *vhl-1*. While the number of genes that lead these negative correlations is small (10-30 genes in any comparison) and is not significantly different from random expectation as assessed by a hypergeometric test, these outliers are expected for this circuit (see SI). On the other hand, unweighted correlations were strong for most comparisons, and they are predicted by theory. Statistical information should be integrated holistically with genetic models to assess whether outliers are meaningful or not.

## *in silico* qPCR reveals extensive feedback in the hypoxia pathway

Our dataset enables us to perform *in silico* qPCR by selectively looking at expression of only a few genes at a time. To verify the quality of our data, we queried the changes in expression of *nhr-57*. This reporter has been shown to have *hif-*1 dependent expressionShen, Shao, and Powell-Coffman (2006; Shen et al. 2005; Ackerman and Gems 2012; E. C. Park et al. 2012). In our dataset, this gene is upregulated in *egl-9*, *rhy-1* and *vhl-1*, but it remains unchanged in *hif-1*. The egl-9;vhl-1 mutant had an expression level similar to egl-9, whereas the egl-9;hif-1 mutant showed suppression of the egl-9 reporter expression phenotype. All of these interactions reflect the literature accurately[cite].



**Top**: In silico qPCR results. nhr-57 is an expression reporter that has been used previously to identify hif-1 regulators Shen, Shao, and Powell-Coffman (2006; Shao, Zhang, and Powell-Coffman 2009). The nhr-57 mRNA levels replicate what is observed in the literature and serves as a quality control for our dataset. lam-3 is a negative control that should not be involved in this pathway. Changes in the hypoxia pathway suggest that hif-1 activates rhy-1, and possibly egl-9, when it is not hydroxylated. hif-1 also appears to autoactivate in a hydroxylation-dependent manner.

Next, we performed *in silico* qPCR of every gene under scrutiny to get a clearer idea of the relationships between them (see Fig. [fig:qpcr]). We found that *rhy-1* transcription levels were increased by mutations in *egl-9*, *rhy-1* and *vhl-1*. This suggests that *hif-1* is a positive regulator of *rhy-1*. *Egl-*9 was upregulated in response to mutations in *egl-*9. Although not statistically significant, *vhl-*1 and *rhy-*1 also showed levels of *egl-*9 that trended towards an increase. The *egl-*9;vhl-1 double mutant similarly exhibited increased levels *of egl-9* mRNA, whereas the *hif-1 and egl-9;hif-1* mutants did not have increased levels of *egl-*9 mRNA. These observations would establish *hif-1* as a positive regulator of *egl-9*, in agreement with the previous literature.

We also discovered that knockout of *hif-1* increased levels of *rhy-1*. This suggests that *hif-1* is also a negative regulator of *rhy-1*. One potential mechanism through which *hif-1* could be both a positive and a negative regulator would be for hydroxylation of HIF-1 to change its activity. Under this mechanism, loss of hydroxylated HIF-1 leads to activation of *rhy-1* and *egl-9* as a homeostatic mechanism, whereas excessive hydroxylation causes inhibition of these genes.



Top: Pairwise weighted correlations between transcriptomes can be used to infer functional distance between interacting genetic partners. Pairwise correlations are ordered by increasing network distance between genes. Correlations were weighted by the fraction of genes that overlapped between the two genes being compared. Notice that correlations involving the fog-2 negative control are very near zero. Error bars represent standard deviation of the weighted correlation. Bottom: Simplified schematic of the hypoxia pathway shown to illustrate functional distance between genes in the pathway.

In summary, the *in silico* qPCR recapitulate the previous results that show *egl-9* and *vhl-1* act in concert to inhibit *hif-1*. Moreover, these results taken together with the transcriptome-wide cross-patterns that emerge from pairwise comparisons between genes in the hypoxia pathway suggest that there are both positive and negative feedback loops feeding into *rhy-1* and possibly *egl-9*. These feedback loops could explain why *hif-1* is positively transcriptomically correlated with *egl-9*.

## Epistasis effects can be detected and quantified.

To quantify any epistasis between *egl-9* and *vhl-1* in our dataset, we identified the genes that were shared between each single mutant and the double mutant *egl-9*;*vhl-1*. If two genes act, for example, in a linear manner, then the double mutant should exhibit an identical phenotype to each single mutant. To test such a relationship, we can plot the difference between the log-fold change in expression between the two mutants, , versus the log-fold change in the single mutant. We can then fit a weighted linear regression to measure the slope of best fit. Genes that act in a linear pathway should yield lines with a slope of 0. Genes that have some additive flavor should have slopes greater than 0. Suppression, a hallmark of inhibition, should yield a slope less than 0.

We observe that the *egl-9*;*vhl-1* mutant has an identical phenotype to the *egl-9* single mutant (slope = 0; see Fig. [tab:double\_mutant\_comparison]). On the other hand, *vhl-1* has a positive slope, indicating that *egl-9* is additive to *vhl-1*. Such partial additivity can be explained if *egl-9* is inhibiting *hif-1* in a *vhl-1*-dependent as well as a *vhl-1*-independent manner Shao, Zhang, and Powell-Coffman (2009).

Response Modeling of Double Mutants to Single Mutants

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Double Mutant | Single Mutant |  | SEM | p-value |
| 1. *egl-9*;*vhl-1* | *egl-9* |  |  |  |
| 2. *egl-9*;*vhl-1* | *vhl-1* |  |  |  |
| 3. *egl-9*;*hif-1* | *egl-9* |  |  |  |
| 4. *egl-9*;*hif-1* | *hif-1* |  |  |  |

[tab:double\_mutant\_comparison]

On the other hand, comparison of the *egl-9*;*hif-1* double mutant showed suppression of the *egl-9* transcriptomic phenotype. This suppression is expressed in various ways. First, the double mutant shows less statistically significantly differentially expressed genes than either single mutant. Secondly, the genes that are common to the *egl-9* and *egl-9;hif-1* transcriptomes show decreased expression in the egl-9;hif-1 mutant than they do in *egl-9* on average. Likewise, the genes that are common to *hif-1* and *egl-9*;hif-1 show no change in expression on average between these two mutants.

## Transcriptomic decorrelation can be used to infer functional distance

We were interested in figuring out whether RNA-seq could be used to identify functional interactions within a genetic pathway. Although there is no *a priori* reason why global gene expression should reflect functional interactions, the strength of the unweighted correlations between genes in the hypoxia pathway made us wonder how much information can be extracted from this dataset.

We investigated the possibility that transcriptomic signals might contain relevant information about the degrees of separation by weighting the robust bayesian regression of each pairwise analysis by . We then plotted the weighted correlation of each gene pair, ordered by increasing functional distance (see Fig. [fig:decorrelation]). In every case, we see that the weighted correlation decreases monotonically due mainly, but not exclusively, to decreasing . We believe that this result is not due to random noise or insufficiently deep sequencing. Instead, we propose a framework in which every gene is regulated by multiple different molecular species. Even in unbranched pathways, this would induce progressive decorrelation between genes.

## Identification of a new pathway controlled by *egl-9* and *vhl-1*

nog-1. Quick verification of nog-1.

## Identification of novel targets and biological processes in the hypoxia response

So far, our analysis has focused mainly on extracting genetic relationships between the set of mutants we sequenced. Our dataset also provides us with a unique view of the *hif-1*-dependent response in *C. elegans*. In total, we identified 3211 differentially expressed genes that are altered in any of the hypoxia pathway mutants. Of these 3211 genes, 53 genes were differentially expressed in all the hypoxia mutants. Because of the extensive feedback between *hif-1* and *egl-9*, we expected to identify a small subset of genes that were up-regulated or down-regulated consistently in every hypoxia mutant except the *egl-9*;*hif-1* double mutant. We identified 10 genes that were up-regulated in this manner, and 13 genes that were down-regulated (see SI for gene identities). These genes likely constitute a core response around the circuit in question, and their behaviour should reflect the genetic relationships the best. Indeed, graphing these genes shows beautiful agreement with predictions (see <www.wormlabcaltech.github.io/mprsq> for interactive graphics).

In order to identify affected biological processes, we performed an in-house gene ontology enrichment analysis using annotations provided by WormBase, followng the procedure shown in TEA Angeles-Albores et al. (2016). Top enriched terms included ‘hydrolase activity’ (869 observed hits; 7.8 fold change; p-value ); ‘organic anion transport’ (803 hits; 7.5; p-value ); ‘spliceosomal complex’ (647 hits; 8.2 p-value ); ‘SAM-depdendent methyltransferase activity’ (1215 hits; 6.6; p-value ); and ‘cell division’ (1251 hits; 7.9; p-value ). In mammals, the mammalian target of rapamycin pathway, which is intimately associated with the hypoxia pathway, has been previously linked to osmotic stress responses B. Zhou et al. (2007). Our findings also suggest that the *hif-1*-dependent response causes important changes in chromatin structure via activation or recruitment of chromatin remodelling factors, as well as changes in isoform processing.

We identified downstream targets of the genes we studied that were not associated with other genes in the *hif-1* circuit. *vhl-1* targets were particularly easy to isolate because *vhl-1* does not seem to participate in the *rhy-1*, *egl-9*, *hif-1* feedback circuit, and as a result it is easy to isolate targets for that are *hif-1*-independent. We found 36 genes that are putative candidates for *vhl-1*-targeted degradation. These 36 genes include *pole-1*, an ortholog of human polymerase catalytic subunit; *F33H2.6*, an ortholog of the human regulator of microtubule dynamics 1 (RMDN1)**???**; and many solute carriers. Reflecting this, enriched GO terms were ‘ion binding’, ‘growth’, ‘cell division’, ‘cell projection assembly’ as well as ‘ion binding’ and ‘divalent metal ion transport’. *vhl-1* has been previously implicated as a controller of mitotic fidelity in renal cell carcinoma Hell et al. (2014). Our findings support a role of *vhl-1* in chromosomal integrity and mitotic fidelity. Furthermore, recent reports suggest that solute carriers may be associated with poor prognosis in clear-cell renal carcinoma Liu et al. (2015), which highlights the biological relevance of our predictions.

We identified 133 genes that are activated by HIF-1. We verified that the genes we identified are actually *hif-1* targets by searching for a set of 20 gold-standard genes from the literature **???** in our gene-set (see SI), and found that *hif-1* targets were significantly enriched in these genes (). GO term enrichment indicated that this list was associated with ‘cell division’, ‘SAM methyltransferase activity’ and ‘cellular modified amino acid metabolic processes’. A full list of *hif-1* targets can be found in S.I..

# Discussion



Top: Previous model. Bottom: Current model derived from RNA-seq data.

Previous work has established a circuit in which *rhy-1* activates *egl-9* in a linear pathway, and *egl-9* inhibits *hif-1* in an oxygen-dependent manner. Hydroxylated HIF-1 can then be degraded in a *vhl-1*-dependent manner. There is also evidence that *egl-9* and *rhy-1* are in turn activated by *hif-1* Bishop et al. (2004; Powell-Coffman 2010). Finally, there is evidence that although the interaction between *egl-9* and *vhl-1* is important for *hif-1* repression, *egl-9* can also act in a non-*vhl-1* dependent manner (see Fig. [fig:newmodel] top).

We were able to impute the positive regulatory relationship between *egl-9* and *rhy-1*. We would not have been able to infer the order of the regulation without additional information. Using clustering as a proxy for phenotype, we were able to infer the relationship between *egl-9* and *hif-1*. We were also able to infer a positive (linear or AND) relationship between *egl-9* and *vhl-1* using clustering. Alternatively, we gained the same information by performing *in silico* qPCR on the genes under study. *In silico* qPCR also revealed that *hif-1* has two states with different activities: Non-hydroxylated HIF-1 increases levels of *rhy-1*, and hydroxylated HIF-1 inhibits *rhy-1* and possibly *egl-9* as well, although the double mutant did not recapitulate that interaction. We also revealed that *hif-1* is an autoregulator.

These discoveries are consistent with a homeostatic circuit. By autoregulating itself, *hif-1* can mantain appropriate protein levels both in normoxic and hypoxic conditions. Inhibition of *rhy-1*, and possibly of *egl-9*, ensures that an appropriate equilibrium is maintained between hydroxylated and non-hydroxylated protein, which may have functional consequences for the cell if both forms are active.

In addition to these biological findings, our dataset allows us to generate predictions of genes that may be under direct *hif-1* regulation. Assuming that non-hydroxylated HIF-1 has different activities from hydroxylated HIF-1, we identified 5 genes that are candidates for activation by hydroxylated HIF-1. These genes have been implicated in the *C. elegans* immune response, or have behavioural phenotypes, underscoring the importance of *hif-1* in neurobiology and immunology Gray et al. (2004; Cheung et al. 2005; Chang and Bargmann 2008; Dengke K Ma et al. 2013). We have shown that transcriptomes contain sufficient information to be used as semi-quantitative phenotypes in metazoans. These phenotypes can be interpreted globally via correlation tests, clustering or other probabilistic methods; alternatively, they can be used to query single reporter genes in a manner similar to qPCR today. Transcriptomic phenotypes have distinct advantages over physical traits. Firstly, due to their increased complexity, the genotype-phenotype mapping degeneracy ought to be greatly reduced, which facilitates predictions of genetic interaction. Secondly, genes that result in subtle or no visible traits when mutated may have strong (detectable), reproducible phenotypes at the transcriptomic level, which would make the study of small-effect genes significantly easier.

RNA-seq and microarray datasets have been used previously by bioinformaticians to generate high-throughput predictions of genetic interactions and consortiums such as the The Cancer Genome Atlas have sequenced RNA from many different cancers in the hope of identifying clinically or biologically relevant interactions **???**. By correlating many different datasets in many different conditions, it is possible in theory to predict genetic interaction. Our approach differs from these high-throughput methods in that we are not attempting to generate large scale networks. Rather, the strength in our analysis derives from our experimental design, which allows us to ask and answer a large number of questions about the functional interactions between genes. As a by-product, we are also able to identify genes related to the core circuit studied in question, but our main goal is not to generate databases or predict large numbers of interactions between a large number of genes. We have shown that transcriptomic phenotypes can capture distinct interaction modes in a single experiment, making it possible to infer complex regulatory relationships between genes. By measuring these transcriptomes under a rigorous experimental design, it is possible to identify many relationships simultaneously. With the advent of fast pseudo-alignment tools and ever cheaper sequencing techniques, biologists should consider using global transcriptomes as a tool beyond hypothesis generation or target acquisition.

Ackerman, Daniel, and David Gems. 2012. “Insulin/IGF-1 and hypoxia signaling act in concert to regulate iron homeostasis in Caenorhabditis elegans.” *PLoS Genetics* 8 (3). doi:[10.1371/journal.pgen.1002498](https://doi.org/10.1371/journal.pgen.1002498).

Angeles-Albores, David, Raymond Y. N. Lee, Juancarlos Chan, and Paul W. Sternberg. 2016. “Tissue enrichment analysis for C. elegans genomics.” *BMC Bioinformatics* 17 (1). BMC Bioinformatics: 366. doi:[10.1186/s12859-016-1229-9](https://doi.org/10.1186/s12859-016-1229-9).

Bishop, Tammie, Kah Weng Lau, Andrew C R Epstein, Stuart K. Kim, Min Jiang, Delia O’Rourke, Christopher W. Pugh, et al. 2004. “Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans.” *PLoS Biology* 2 (10). doi:[10.1371/journal.pbio.0020289](https://doi.org/10.1371/journal.pbio.0020289).

Bray, Nicolas L, Harold Pimentel, Páll Melsted, and Lior Pachter. 2015. “Near-optimal RNA-Seq quantification.” *ARxiv*. doi:[arXiv:1505.02710](https://doi.org/arXiv:1505.02710).

Chang, Andy J, and Cornelia I Bargmann. 2008. “Hypoxia and the HIF-1 transcriptional pathway reorganize a neuronal circuit for oxygen-dependent behavior in Caenorhabditis elegans.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (20): 7321–6. doi:[10.1073/pnas.0802164105](https://doi.org/10.1073/pnas.0802164105).

Cheung, Benny H H, Merav Cohen, Candida Rogers, Onder Albayram, and Mario De Bono. 2005. “Experience-dependent modulation of C. elegans behavior by ambient oxygen.” *Current Biology* 15 (10): 905–17. doi:[10.1016/j.cub.2005.04.017](https://doi.org/10.1016/j.cub.2005.04.017).

Gray, Jesse M, David S Karow, Hang Lu, Andy J Chang, Jennifer S Chang, Ronald E Ellis, Michael a Marletta, and Cornelia I Bargmann. 2004. “Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue.” *Nature* 430 (6997): 317–22. doi:[10.1038/nature02714](https://doi.org/10.1038/nature02714).

Hell, Michael P., Maria Duda, Thomas C. Weber, Holger Moch, and Wilhelm Krek. 2014. “Tumor suppressor vhl functions in the control of mitotic fidelity.” *Cancer Research* 74 (9): 2422–31. doi:[10.1158/0008-5472.CAN-13-2040](https://doi.org/10.1158/0008-5472.CAN-13-2040).

Hughes, Timothy R, Matthew J Marton, Allan R Jones, Christopher J Roberts, Roland Stoughton, Christopher D Armour, Holly a Bennett, et al. 2000. “Functional Discovery via a Compendium of Expression Profiles.” *Cell* 102 (1): 109–26. doi:[10.1016/S0092-8674(00)00015-5](https://doi.org/10.1016/S0092-8674(00)00015-5).

Jaakkola, P, D R Mole, Y M Tian, M I Wilson, J Gielbert, S J Gaskell, A von Kriegsheim, et al. 2001. “Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation.” *Science* 292 (5516): 468–72. doi:[10.1126/science.1059796](https://doi.org/10.1126/science.1059796).

Kirienko, Natalia V., Daniel R. Kirienko, Jonah Larkins-Ford, Carolina Wählby, Gary Ruvkun, and Frederick M. Ausubel. 2013. “Pseudomonas aeruginosa disrupts Caenorhabditis elegans iron homeostasis, causing a hypoxic response and death.” *Cell Host and Microbe* 13 (4): 406–16. doi:[10.1016/j.chom.2013.03.003](https://doi.org/10.1016/j.chom.2013.03.003).

Liu, Yidong, Liu Yang, Huimin An, Yuan Chang, Weijuan Zhang, Yu Zhu, Le Xu, and Jiejie Xu. 2015. “High expression of Solute Carrier Family 1, member 5 (SLC1A5) is associated with poor prognosis in clear-cell renal cell carcinoma.” *Scientific Reports* 5 (October): 16954. doi:[10.1038/srep16954](https://doi.org/10.1038/srep16954).

Ma, Dengke K, Michael Rothe, Shu Zheng, Nikhil Bhatla, Corinne L Pender, Ralph Menzel, and H Robert Horvitz. 2013. “Cytochrome P450 drives a HIF-regulated behavioral response to reoxygenation by C. elegans.” *Science (New York, N.Y.)* 341 (6145): 554–8. doi:[10.1126/science.1235753](https://doi.org/10.1126/science.1235753).

Ma, Dengke K., Roman Vozdek, Nikhil Bhatla, and H. Robert Horvitz. 2012. “CYSL-1 Interacts with the O 2-Sensing Hydroxylase EGL-9 to Promote H 2S-Modulated Hypoxia-Induced Behavioral Plasticity in C. elegans.” *Neuron* 73 (5). Elsevier Inc.: 925–40. doi:[10.1016/j.neuron.2011.12.037](https://doi.org/10.1016/j.neuron.2011.12.037).

Metzker, Michael L. 2010. “Sequencing technologies - the next generation.” *Nature Reviews. Genetics* 11 (1). Nature Publishing Group: 31–46. doi:[10.1038/nrg2626](https://doi.org/10.1038/nrg2626).

Mortazavi, Ali, Brian A Williams, Kenneth McCue, Lorian Schaeffer, and Barbara Wold. 2008. “Mapping and quantifying mammalian transcriptomes by RNA-Seq.” *Nature Methods* 5 (7): 621–28. doi:[10.1038/nmeth.1226](https://doi.org/10.1038/nmeth.1226).

Nizet, Victor, and Randall S Johnson. 2009. “Interdependence of hypoxic and innate immune responses.” *Nature Reviews. Immunology* 9 (9): 609–17. doi:[10.1038/nri2607](https://doi.org/10.1038/nri2607).

Park, Eun Chan, Piya Ghose, Zhiyong Shao, Qi Ye, Lijun Kang, Xz Shawn Xu, Jo Anne Powell-Coffman, and Christopher Rongo. 2012. “Hypoxia regulates glutamate receptor trafficking through an HIF-independent mechanism.” *The EMBO Journal* 31 (6): 1618–9. doi:[10.1038/emboj.2012.44](https://doi.org/10.1038/emboj.2012.44).

Patro, Rob, Geet Duggal, and Carl Kingsford. 2015. “Salmon: Accurate, Versatile and Ultrafast Quantification from RNA-seq Data using Lightweight-Alignment.” *BioRxiv*, 021592. doi:[10.1101/021592](https://doi.org/10.1101/021592).

Patro, Rob, Stephen M Mount, and Carl Kingsford. 2014. “Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms.” *Nature Biotechnology* 32 (5). Nature Publishing Group: 462–64. doi:[10.1038/nbt.2862](https://doi.org/10.1038/nbt.2862).

Phillips, Patrick C. 2008. “Epistasis — the essential role of gene interactions in the structure and evolution of genetic systems.” *Nat Rev Genet* 9 (11): 855–67. doi:[10.1038/nrg2452](https://doi.org/10.1038/nrg2452).

Pimentel, Harold J, Nicolas Bray, Suzette Puente, Páll Melsted, and Lior Pachter. 2016. “Differential analysis of RNA-Seq incorporating quantification uncertainty.” *BioRxiv*, 058164. doi:[10.1101/058164](https://doi.org/10.1101/058164).

Powell-Coffman, Jo Anne. 2010. “Hypoxia signaling and resistance in C. elegans.” *Trends in Endocrinology and Metabolism* 21 (7). Elsevier Ltd: 435–40. doi:[10.1016/j.tem.2010.02.006](https://doi.org/10.1016/j.tem.2010.02.006).

Schwarz, Erich M., Mihoko Kato, and Paul W. Sternberg. 2012. “Functional transcriptomics of a migrating cell in Caenorhabditis elegans.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (40): 16246–51. doi:[10.1073/pnas.1203045109](https://doi.org/10.1073/pnas.1203045109).

Scimone, M. Lucila, Kellie M. Kravarik, Sylvain W. Lapan, and Peter W. Reddien. 2014. “Neoblast specialization in regeneration of the planarian schmidtea mediterranea.” *Stem Cell Reports* 3 (2). The Authors: 339–52. doi:[10.1016/j.stemcr.2014.06.001](https://doi.org/10.1016/j.stemcr.2014.06.001).

Semenza, Gregg L. 2003. “Targeting HIF-1 for cancer therapy.” *Nature Reviews. Cancer* 3 (10): 721–32. doi:[10.1038/nrc1187](https://doi.org/10.1038/nrc1187).

Semenza, Gregg L. 2012. “Hypoxia-inducible factors in physiology and medicine.” *Cell* 148 (3). Elsevier Inc.: 399–408. doi:[10.1016/j.cell.2012.01.021](https://doi.org/10.1016/j.cell.2012.01.021).

Shalek, Alex K, Rahul Satija, Xian Adiconis, Rona S Gertner, Jellert T Gaublomme, Raktima Raychowdhury, Schraga Schwartz, et al. 2013. “Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells.” *Nature* 498 (7453). Nature Publishing Group: 236–40. doi:[10.1038/nature12172](https://doi.org/10.1038/nature12172).

Shao, Zhiyong, Yi Zhang, and Jo Anne Powell-Coffman. 2009. “Two distinct roles for EGL-9 in the regulation of HIF-1-mediated gene expression in Caenorhabditis elegans.” *Genetics* 183 (3): 821–29. doi:[10.1534/genetics.109.107284](https://doi.org/10.1534/genetics.109.107284).

Shen, Chuan, Daniel Nettleton, Min Jiang, Stuart K. Kim, and Jo Anne Powell-Coffman. 2005. “Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in Caenorhabditis elegans.” *Journal of Biological Chemistry* 280 (21): 20580–8. doi:[10.1074/jbc.M501894200](https://doi.org/10.1074/jbc.M501894200).

Shen, Chuan, Zhiyong Shao, and Jo Anne Powell-Coffman. 2006. “The Caenorhabditis elegans rhy-1 gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include vhl-1.” *Genetics* 174 (3): 1205–14. doi:[10.1534/genetics.106.063594](https://doi.org/10.1534/genetics.106.063594).

Singer, Meromit, Chao Wang, Le Cong, Nemanja D. Marjanovic, Monika S. Kowalczyk, Huiyuan Zhang, Jackson Nyman, et al. 2016. “A Distinct Gene Module for Dysfunction Uncoupled from Activation in Tumor-Infiltrating T Cells.” *Cell* 166 (6). Elsevier Inc.: 1500–1511.e9. doi:[10.1016/J.CELL.2016.08.052](https://doi.org/10.1016/J.CELL.2016.08.052).

Tanimoto, K, Y Makino, T Pereira, and L Poellinger. 2000. “Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein.” *Embo J* 19 (16): 4298–309. doi:[10.1093/emboj/19.16.4298](https://doi.org/10.1093/emboj/19.16.4298).

Trapnell, Cole, David G Hendrickson, Martin Sauvageau, Loyal Goff, John L Rinn, and Lior Pachter. 2013. “Differential analysis of gene regulation at transcript resolution with RNA-seq.” *Nature Biotechnology* 31 (1). Nature Publishing Group: 46–53. doi:[10.1038/nbt.2450](https://doi.org/10.1038/nbt.2450).

Van Driessche, Nancy, Janez Demsar, Ezgi O Booth, Paul Hill, Peter Juvan, Blaz Zupan, Adam Kuspa, and Gad Shaulsky. 2005. “Epistasis analysis with global transcriptional phenotypes. TL - 37.” *Nature Genetics* 37 VN - r (5): 471–77. doi:[10.1038/ng1545](https://doi.org/10.1038/ng1545).

Van Wolfswinkel, Josien C., Daniel E. Wagner, and Peter W. Reddien. 2014. “Single-cell analysis reveals functionally distinct classes within the planarian stem cell compartment.” *Cell Stem Cell* 15 (3). Elsevier Inc.: 326–39. doi:[10.1016/j.stem.2014.06.007](https://doi.org/10.1016/j.stem.2014.06.007).

Yeung, Ka Yee, Mario Medvedovic, and Roger E Bumgarner. 2003. “Clustering gene-expression data with repeated measurements.” *Genome Biology* 4 (5): R34. doi:[10.1186/gb-2003-4-5-r34](https://doi.org/10.1186/gb-2003-4-5-r34).

Zhou, B, D K Ann, X Li, K J Kim, H Lin, P Minoo, E D Crandall, and Z Borok. 2007. “Hypertonic induction of aquaporin-5: novel role of hypoxia-inducible factor-1alpha.” *Am J Physiol Cell Physiol* 292 (4): C1280–90. doi:[10.1152/ajpcell.00070.2006](https://doi.org/10.1152/ajpcell.00070.2006).