Supplementary Methods

Genetic Disruption of WASHC4 Drives Endo-lysosomal Dysfunction and Cognitive-Movement Impairments in Mice and Humans

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Abstract

In the review of this manuscript, significant concerns were raised by the reviewers about the validity of our statistal approach to perform protein- and module-level inference from our `WASH-BioID` and `SWIP-TMT` proteomics datasets. Our previous statistical approach relied upon the R package `edgeR`, which utilizes a negative binomial generalized linear model (NBGLM) framework. Previously, we failed to fully consider the validity of the NBGLM model used by `edgeR` for proteomics data. In response to this criticism, we explore the goodness-of-fit of the NBGLM model for TMT proteomics data, and find evidence of a lack of fit. Thus we revised our statistical approach, and reanlyzed our data making use of the recently published tool `MSstatsTMT`. `MSstatsTMT` uses a flexible linear mixed model (LMM) framework to model major sources of variation in a proteomics experiment. We extend the LMM framework used by edgeR to re-evaluate both protein- and module-level statistical comparisions. Despite evidence of a lack-of-fit for the NBGLM method used by `edgeR`, we find that the inferences we derived from our previous analysis are largely preserved in our reanalysis using `MSstatsTMT`.

Reanalysis of SWIP^{P1019R} Spatial Proteomics

Our previous approach used the `edgeR` package to assess differential abundance of individual proteins as well as protein-groups or modules between SWIP^{P1019R} 'Mutant' (MUT) 'Control' mice.

As signal intensity in protein mass spectrometry is fundamentally related to the number of ions generated from a ioninized, fragmented protein, we incorrectly infered that TMT mass spectrometry data can be adequetly modeled as NB count data. Based on this assumption, we justified the use of `edgeR` and its NBGLM framework.

Statisitical inference in `edgeR` is built on a negative binomial (NB) model framework in which the data are assumed to be adequately described by a NB distribution parameterized by a dispersion parameter, ϕ. Practically, the dispersion parameter accounts for mean-variance relationships observed in proteomics and transcriptomics data.

Additionally, `edgeR` employs empirical Bayes methods that allow for the estimation of feature-specific (i.e. gene or protein) biological variation, even for experiments with small numbers of biological replicates, as is common in transcriptomics and proteomics experiments. This EB strategy is a strength of the `edgeR` approach as it reduces the uncertainty of the estimates and improves testing power.

We evaluated the overall adequacy of the `edgeR` model by plotting the residual protein deviance statistics of all proteins against their theoretical, normal quantiles in a quantile-quantile plot. A linear relationship between the observed and theoretical values is an indicator of the goodness-of-fit of a model. Deviation from this linear trend is evidence of a lack-of-fit.

Our previous approach is summarized as the 'Sum + IRS' appraoch by Huang et al. We drew precidence for use of `edgeR` from previous work by Plubell and Khan, et al. As a starting point, we evaluated the overall adequacy of the `edgeR` NBGLM model for the Khan et al., TMT proteomics dataset. The data were processed using the 'Sum + IRS' approach in which proteins are summarized as the sum of their constituent peptides and the protein-level data are normalized using internal reference standards (IRS normalization).

In an MS experiment, proteins are typically quantified by different peptides in each MS run due to the inherent random sampling of peptides at the MS level. IRS normalization accounts for this source of variability using internal reference standards, adjusting protein measurements by a scaling factor such that the geometric mean of all internal reference standards for a given protein are equal across all experiments (Plubell et al., 2017).

Following protein summarization and normalization, the data were fit with a simple NBGLM of the form `Abundance` $\sim$ `Condition` using `edgeR's` `glmFit` function which fits a NBGLM model to each protein or gene (sub-subplot summaries) in the data. For all three dispersion parameters, we observe deviation from the expected linear trend.

Figure 1 illustrates the divergence of the observed deviance statistics for data fit with the NBGLM model. These plots emphasize the overall lack of fit of proteomics data fit by the `edgeR` model.

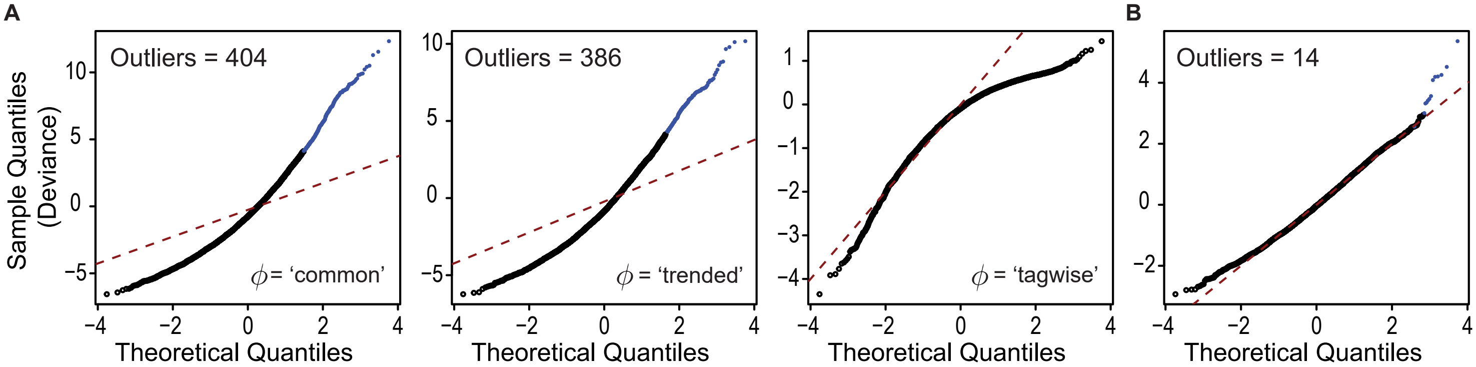


Figure 1. Goodness-of-fit of `edgeR` (A), and `MSstatsTMT` (B) statistical approaches. The overall adequacy of the linear models fit to the data were assessed by plotting the residual deviance for all proteins as a quantile-quantile plot (McCarthy *et al.*, (2012)). **(A)** For analysis with `edgeR`, The normalized protein data from `MSstatsTMT` were fit with a negative binomal generalized linear model (NBGLM) of the form: `Abundance` ∼ `Mixture + Condition`. Where `Mixure` is an additive blocking factor that accounts for variablity between experiments. The NB framework used by edgeR utilizes a dispersion parameter to account for mean-variance relationships in the data. The dispersion parameter can take several forms. `edgeR` supports three dispersion models: 'common', 'trended', and 'tagwise'. However, when using `edgeR's` robust quasi-likelihood test methods, only global (i.e. 'common' or 'trended') dispersion metrics are appropriate (see `edgeR::glmQLFit's` documentation). We plot the protein-wise deviance from the data fit withe ach of the disperions parameters. Protein-wise deviance statistics were transformed to normality and plotted against theoretical normal quantiles using the `edgeR::gof` function. **(B)** For analysis with `MSstatsTMT`, the normalized protein data were fit with a linear mixed-effects model (LMM) of the form: `Abundance` ∼ `0 + Condition + (1|Mixture)`. Where `Mixture` represents the random effect of `Mixture`. The residual deviance and degrees of freedom were extracted from the fitted models, z-score normalized, and plotted as in (A). Proteins with a significantly poor fit are indicated as outliers in blue (Holm-adjusted P-value < 0.05).

Of note, most tools for analysis of protein mass spectromety data are derived from tools originally developed for analysis of genomics and transcriptomics data. An exception to this norm is `MSstatsTMT`, an extension of `MSstats` for analysis of TMT proteomics experiments.

`MSstatsTMT` utilizes a linear mixed-model framework. The strength of linear mixed models (LMMs) is in their ability to account for complex sources of variation in an experimental design. **Figure ??** shows the proportion of variance attributable to major covariates for each protein.

Linear mixed models are an extension of linear models which include both fixed and random effects. The response (protein abundance) is a function of both fixed and mixed effects. In a mixed model, one or more of the covariates are a categorical variable representing experimental or observational "units" in the data set.

Thus random effects in mixed models often reflect heirarchically organized data such as repeated measurments of individual subjects. In mixed models we account for the variation occurring among all of the lower level units of a particular upper level unit.

The distinction between fixed and mixed effects is often subtle. By definition if the set of possible levels of the covariate is fixed and reproducible then the factor is modeled as a fixed-effect parameter. In contrast, if the levels of an observation reflect a random sampling of the set of all pssible levels then the covariate is modeled as a random effect.

Huang et al., describe a general linear mixed model framework for mass spectrometry experiments. A TMT proteomics experiment consists of the analysis of `m =1` ... `M` concatensions of isobarically labeled samples or `Mixtures`. Within a mixture, each TMT channel is dedicated to the analysis of `c = 1` ... `C` individual biological or treatment `Conditions` prepared from `b = 1` ... `B` biological replicates or `Subjects`. A single mixture may be profiled in `t = 1` ... `T` technical replicate mass spectrometry runs.

The following mixed-effects model describes the response, the abundance of protein Y_{mcbt}, in an experiment composed of `M` mixtures, `T` technical replicates of mixture, `C` conditions, and `B` biological subjects.

$$Y_{mcbt} = \mu + Mixture_m + TechRep(Mixture)_{m(t)} + Subject_b + Condition_c + \epsilon_{mcbt} \qquad (1)$$

The model's constraints distinguish fixed and random components of variation in the response. `Mixture` is a mixed effect and represents the variation between TMT mixtures which is assumed to be random and normally distributed. `TechRep(Mixture)` represents random variation between replicate mass spectrometry runs of a same mixture. The term `Subject` cooresponds to each biological replicate of a condition and represents biological variation among the levels of each `Condition`.

The term $\epsilon_m tcb$, is a random effect representing both biological and technical variation, quantifying any remaining error (σ^2), and is assumed to be independent and non-systematic (no mean variance).

If a component of the model is not estimable, it is removed. Thus, if there is no technical replication of Mixture, the model is reduced to:

$$Y_{mcbt} = \mu + Mixture_m + Condition_c + \epsilon_{mc} \tag{2}$$

In the reduced model, biological variation among individual subjects is captured by the term `Condition` and is thus ommited.

We prepared 7 `BioFractions` from 'Control' and SWIP$^{\mathrm{P1019R}}$ 'Mutant' mice. Thus, in our experiment, the fixed effect term condition represents interaction of `Genotype` and `BioFraction` and represents the 14 unique combinations of 7 subcellular `BioFractions` prepared from 'Control' and 'Mutant' mice.

In our experimental design, we made seven repeated measurments from each biological Subject. Thus, the term Subject represents the random error within a subject. However, in our design `Mixture` is confounded with the term `Subject` – in each mixture we analyzed all `BioFractions` from a single Control and Mutant mouse. Thus we can choose to account for the effect of `Mixture` or `Subject`, but not both. Assuming Mixture contributes greater to the variance, we drop the term Subject, and the reduced model is equivalent to 2. Our experimental design is summarized in **Figure** 2.

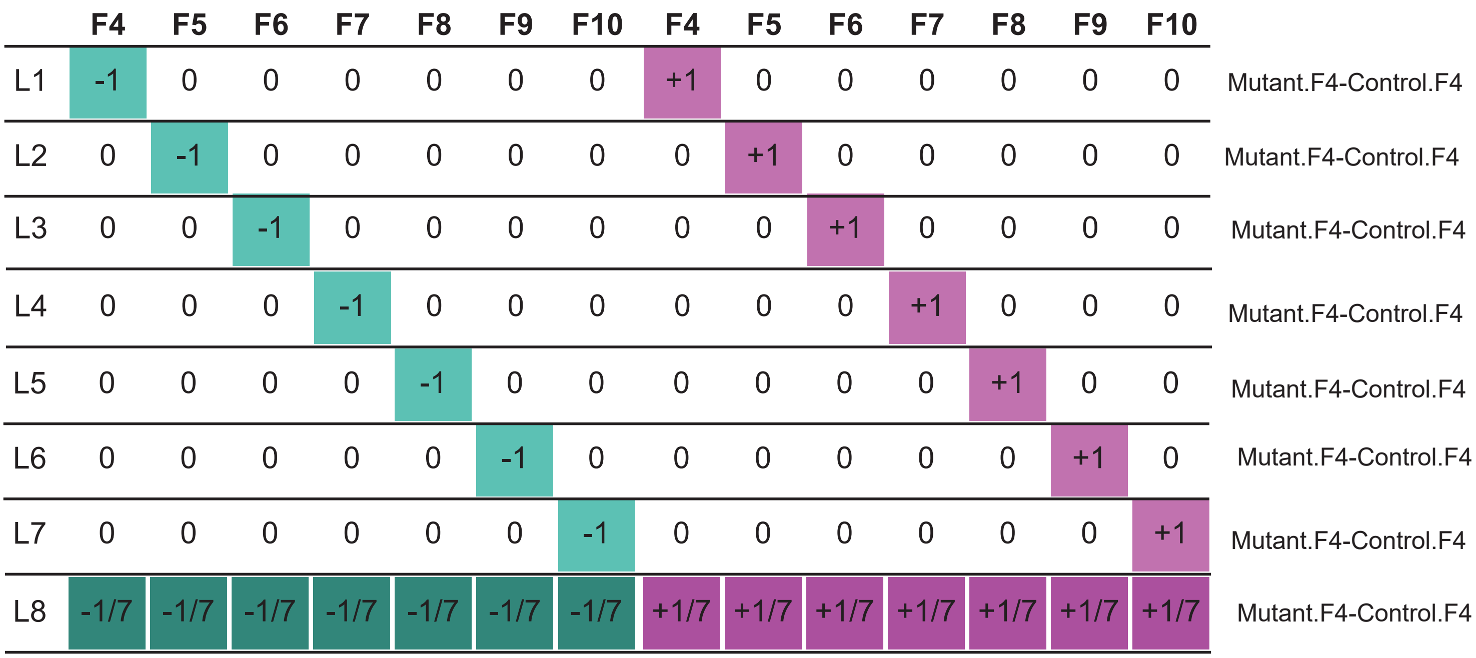
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
Mix1	WT-5K	WT-9K	WT-12K	WT-15K	WT-30K	WT-79K	WT-129K	QC1	MUT-5K	MUT-9K	MUT-12K	MUT-15K	MUT-30K	MUT-79K	MUT-129K	QC2
Mix2	WT-5K	WT-9K	WT-12K	WT-15K	WT-30K	WT-79K	WT-129K	QC1	MUT-5K	MUT-9K	MUT-12K	MUT-15K	MUT-30K	MUT-79K	MUT-129K	QC2
Mix3	WT-5K	WT-9K	WT-12K	WT-15K	WT-30K	WT-79K	WT-129K	QC1	MUT-5K	MUT-9K	MUT-12K	MUT-15K	MUT-30K	MUT-79K	MUT-129K	QC2

Figure 2. Experimental Design. We performed three 16-plex TMT experiments. Each TMT mixture is a concatenation of 16 labeled samples. In each experiment we analyzed 7 subcellular `BioFractions` prepared from the brain of a 'Control' or 'Mutant' mouse. In all we analyzed 3 `Subjects` from each Condition. Each `Mixture` includes two `Channels` dedicated to the analysis of a common quality control sample.

Model based testing of differential abundance between pairs of conditions is assessed through contrast of conditioned means estimated by fitting the parameters of the model by REML to obtain $\hat{\beta}$, σ^2 and $\hat{V}$. A contrast or comparison between coefficients in the model is specified by a vector of sum 1 which indicates the positive and negative coefficients of the comparison **Figure** 3.

The degrees of freedom are determined by the Satterthwaite approximation and the T-statistic for the contrast is taken to be (lmerTest ref):

$$t = \frac{l^T * \hat{\beta}}{sqrt(l * \sigma^2 * \hat{V} * l^T)} \tag{3}$$



	F4	F5	F6	F7	F8	F9	F10	F4	F5	F6	F7	F8	F9	F10	
L1	-1	0	0	0	0	0	0	+1	0	0	0	0	0	0	Mutant.F4-Control.F4
L2	0	-1	0	0	0	0	0	0	+1	0	0	0	0	0	Mutant.F4-Control.F4
L3	0	0	-1	0	0	0	0	0	0	+1	0	0	0	0	Mutant.F4-Control.F4
L4	0	0	0	-1	0	0	0	0	0	0	+1	0	0	0	Mutant.F4-Control.F4
L5	0	0	0	0	-1	0	0	0	0	0	0	+1	0	0	Mutant.F4-Control.F4
L6	0	0	0	0	0	-1	0	0	0	0	0	0	+1	0	Mutant.F4-Control.F4
L7	0	0	0	0	0	0	-1	0	0	0	0	0	0	+1	Mutant.F4-Control.F4
L8	-1/7	-1/7	-1/7	-1/7	-1/7	-1/7	-1/7	+1/7	+1/7	+1/7	+1/7	+1/7	+1/7	+1/7	Mutant.F4-Control.F4

Figure 3. Statistical Comparisons. We assessed two types of contrasts. Each row of the matrix specifies a contrast between positive and negative coefficients in the mixed effects model fit to each protein. Contrasts1-7 are 'intra-BioFraction' contrasts that specify the pairwise comparisons of Control and Mutant groups for a single fraction. In Contrast 8 we compare 'Mutant-Control' and asses the overall difference of 'Control' and 'Mutant' conditions. Each contrast is a vector of sum 1.

Where σ^2 is the error from **Equation** 1. l^T is a vector specifying a contrast between positive and negative coefficients in the model.

Together, the denominator $\sqrt{(l * \sigma^2 * \hat{V} * l^T)}$ is the standard error of the contrast.

We reanalyzed the data with `MSstatsTMT` starting with PSM-level data exported from ProteomeDiscoverer. The intial dataset was composed of 8,590 unique proteins. The PSM level data are converted to MSstatsTMT's format and protein summarization and normalization is performed using the QC samples.

Reprodcible quantification of these samples is essential. Thus we examined PSM features for potential outliers, accounting for the mean-variance relationship in PSM quantification using the method described by Plubell et al. (REF).

We removed PSM with incomplete observations from each experiment (n=186, 132, and 174 respectively from each of the three mixtures).

A small number of QC outliers were identified and removed (M1=259; M2=169; M3=159).

We performed protein-level normalization and sumamrization using `MSstatsTMT`. This step is computationally expensive as each protein is fit with a linear model. We increased the efficiency of this computation by employing 23 parallel processors, in all taking approximately 11.094 minutes.

MSstatsTMT takes care to impute missing values within each Run. But missing values still exist at the protein level. In order to avoid discarding a large number or proteins we impute these missing values using the KNN algorithm for MNAR data. This does not affect the statistical testing, but helps retain proteins used to build the covariation network. Porieins with more than 50% missingness cannot be reliably imputed and are removed (66 rows). In all we retained 6,910 of the inital 8,590 proteins in the final normalized dataset.

We assess protein-level comparisons at two different levels using MSStatsTMT. We asses 'intra-BioFraction' comparisions and moderated these test statistics for small sample size (n=3 for each condition). This step is computationally expensive as a linear-mixed model is fit to each protein in the data. The time to perform intra-Biofraction comparisons for all proteins was approximately 17.834 minutes.

We assed overall differnces between 'Mutant' and 'Control' conditions using MSstatsTMT (Moderated=FALSE).

There were 163 instances of signficant differential abundance for 'intra-BioFraction' comparisons (FDR < 0.05). The following table summarizes the number of significantly differential abundant proteins for each of the seven intra-BioFraction comparisons.

There were 785 proteins with an overall significant change for the 'Mutant-Control' comparison.

Prior to building the protein covariation network, we removed the effect of Mixture using limma::RemoveBatchEffect. This is necessary as we wish to identify modules that covary together across subcellular space (BioFraction) and not batch or Mixture. These adjusted data are used for network construction and plotting but not statistical modeling. It is preferable to include these factors in the statistical model.

The final, tidy normalized protein data is avaialbe as an R object, `msstats_prot` in `SwipProteomics/data`.

We extracted MSstatsTMT's core model-fitting and statistical testing steps. It is usefull to allow investiagors flexiblity and transparency.

We evaluate the percent variance explained by each factor in the LMM using the `variancePartition` package.

We assessed the goodness-of-fit of each protein-wise LMM using Nagagawa's coefficient of determination, as implemented by the `r.squaredGLMM.merMod` function forked from the `MuMin` package.

Prior to network construction, we removed models with poor fit. We removed proteins whose model explained less than 0.7 of the variation for that protein.

Number of proteins with poor fit: 791

Removing this small number of proteins facilitates clustering.

Removing 791 proteins with poor fit before building network. The final network was constructed using both 'Control' and 'Mutant' samples. The data adjsuted for batch (Mixture). It contained 42 samples and 6,119 proteins. We found that the pearson correlatioin statistic outperformed the bicor statistic we previously used.

We performed network enhancement to remove biological noise from the datset. This step is essential for module detection. Our approach borrows many of the conceptual ideas utilized in the WGCNA or WPCNA analysis workflows. Network enhancement is analogous to the weighting step performed by WGCNA and analogous emthods in which the network correlation network is transformed by a power in order to re-weight the network. Network enhancment has the effect of making the network sparse and facilitates the identification of network structure.

We constructed a protein-protein interaction graph which was not used to guide clukstering, but as an additional layer of information in the final network graphs. The PPI graph for all proteins contained 93,573 edges.

We sought to identify groups, aka clusters or modules, of proteins that strongly covary together across subcellular space. Intuitively, we wish to identify a partition of the graph which maximizes intra-module connectivity and minimizes inter-module connectivity. Numerous quality statistics descriving the overall quality of a network partition exist, and numerous heuristical algorithms.

Identification of communities in a graph by optimization of a quality function is NP-hard5, and consequentially many heuristic algorithms exist. One of the most well known algorithms, is the Louvain algorithm (Traag2010ref10).

We utilized a recent improvement of the Louvain algorith, the Leiden algorithm to identify optimal partitions of the graph. The Leiden algorithm is implemented in Python and supports several quality statistics, including Modularity, CPM, Surprise, RBER, and RBConfiguration.

We aim to cluster the protein-covariation network in order to identify modules which cohesive protein abundance profiles.

For each model fit the module-level data, we asses the proprotion of variance explained by BioFraction, Genotype, Mixture, and Protein using the `VariancePartition` R package. We also compute Nagakawa's coefficient of determination for mixed models, as implemented by the R `MuMin` package. Inspection of these variance explained by the components of our model we realize a natural description of a modules quality.

R2(fixef) aka R2c (conditional) – interpretation: the total variance explained by fixed effects (Genotype:BioFraction). We wish to maximize this quantity.

PVE(protein) – the percent of the modules variation explained by protein variability. An ideal module is a perfect summary of its constituent proteins and this quantity is 0. We aim to minimize the PVE(protein).

An ideal module is a perfect summary of its constituent proteins. Thus, we seek to minimize the variation arising from Protein within a module. While minimizing this quantity, we aim to retain clusters whose variation attributed to fixed effects of BioFraction and Genotype is maximized. Thus, a simple quality metric for a module may be the ratio of variance attributable to fixed effects and the random effect of protein:

The overall quality of a partition is the average all module quality.

All things being equal, an increase in the number of clusters results in a decrease in overall quality. (Imagine splitting a perfect module,into two; for each the variance attributed to Protein is 0, but when this module is split the variance attributable to each modules fixed effects is halved).

Performing Leidenalg clustering utilizing the SurpriseVertexPartition method to find optimal partition(s).

Recursively splitting modules larger than 100 nodes with 'Surprise'. We find that recursive splitting of large modules is necessary to resolve significant heterogenity which exists in large modules and this also improves recovery of biological signal.

We split modules with more than 100 nodes. While this threshold is arbitrary, we found that recursively spliting large modules resulted in higher quality modules. and facilitates biological indference.

Final partition: Clustering with 6119 elements and 502 clusters We removed small mdoules of less than 5 nodes.

Module statistic(s) used to evaluate module preservation: avg.weight, avg.cor, avg.contrib. Criterion for module preservation: strong.

We enforced module quality by module preservation using a permutation appraoch. Modules with random toplogy we discarded.

Evaluating preservation of Swip modules in the Swip network... ... 296 of 329 Swip modules are preserved in the Swip network.

In all there were 296 modules.

Nakagawa coefficient of determination R2m: Marginal; variation explained by fixed effects. R2c: Conditional; total variation explained by the model.

Assessing module-level contrasts with lmerTest.

Time to analyze 296 modules: Time difference of 5.151603 secs

Final number of modules : 296

Final percent clustered : 0.908

Final Median module size: 13

Washc4 assigned to module: M17

All significant (Padjust < 0.05) modules:

Number of significant modules (Bonferroni<0.05): 61

Evaluating goodness-of-fit of modules. There were problems fitting 0 models.

Partition Quality: 2.14995 (mean module quality).

The Columns BioFraction Genotype, Mixture, Protein, and Residuals describe the percent variance attributable to that term for the mixed-effect model fit to each module. R2.fixef is the overall variance explained by fixed effects. R2.total is the overall variance explained by the model. An intuitive measure of module quality is the ratio of variance explained by fixed effects and Protein. We wish to maximize the variance explained by fixed effects and minimize the random effect of Protein. An ideal module is a perfect summary of its protein constituents and thus PVE(Protein) = 0.

Number of modules with something interesting going on: 116

Significant Modules with significant gse: (23 of 61 significant modules.)