TITLE: Selection at the pathway level drives the evolution of gene-specific transcriptional noise

AUTHORS: Gustavo Valadares Barroso[1]; Natasa Puzovic[1] and Julien Y Dutheil[1,2]

Affiliations:

1) Max Planck Institute for Evolutionary Biology. Department of Evolutionary Genetics. August-Thienemann-Straße 2 24306 Plön – GERMANY

2) ISEM – Institut des Sciences de l'Évolution. UMR 5554, Université de Montpellier, Place Eugène Bataillon 34095 Montpellier cedex 05 – FRANCE

Corresponding Author:

Gustavo V. Barroso, Max Planck Institute for Evolutionary Biology. Department of Evolutionary Genetics. August-Thienemann-Straße 2, 24306 Plön – GERMANY.

ABSTRACT:

Because biochemical processes within individual cells involve a small number of molecules, they are subject to random fluctuations. As a result, isogenic cell populations show different concentrations of the same mRNA and protein, even in homogeneous conditions. The extent and consequences of this stochastic gene expression have only recently been assessed on a genome-wide scale, in particular thanks to the advent of single cell transcriptomics. Yet the evolutionary forces shaping this stochasticity remain to be unraveled. We took advantage of recently published data sets of the single cell transcriptome of the domestic mouse *Mus musculus* to characterize the genomic patterns of transcriptional stochasticity. We show that noise levels in the mRNA distributions (*a.k.a.* transcriptional noise) significantly correlate with nuclear domain organization, gene function and gene age. Position of the encoded protein in biological pathways, however, is the main factor that explains observed levels of transcriptional noise. We argue that these results are consistent with models of noise propagation within gene networks. Altogether, transcriptional noise appears to be under widespread selection and therefore constitutes an important of the phenotypical component. Differences in variance of expression – not only in mean expression level – potentially constitute a mechanism of adaptation and should be considered by functional and evolutionary studies of gene expression.

Introduction

Isogenic cell populations display phenotypic variability even in homogeneous environments (Spudich and Koshland 1976). This observation challenged the clockwork view of the intra-cellular molecular machinery and led to the recognition of the stochastic nature of gene expression. Because biochemical reactions result from the interactions of individual molecules in small numbers (Gillesple 1977), the inherent stochasticity of binding and diffusion processes generates noise along the biochemical cascade leading to the synthesis of a protein from its encoding gene (**Figure 1**). The study of stochastic gene expression (SGE), also referred to as expression noise, classically recognizes two sources of noise. Following the definition introduced by Elowitz et al (Elowitz et al. 2002), extrinsic noise results from variation in concentration, state and location of shared key molecules involved in the reaction cascade from transcription initiation to protein folding. This is because molecules that are shared among genes are typically present in low copy numbers relative to the number of genes actively transcribed (Shahrezaei and Swain 2008). Extrinsic factors also include physical properties of the cell such as size and growth rate, likely to impact the diffusion process of all molecular players. Extrinsic factors therefore affect every gene in a cell equally. Conversely, intrinsic factors generate noise in a gene-specific manner. They involve, for example, the strength of cis-regulatory elements (Suter et al. 2011) as well as the stability of the mRNA molecules that are transcribed (Mcadams and Arkin 1997; Thattai and Oudenaarden 2001). Every gene is affected by both sources of stochasticity and the relative importance of each has been discussed in the literature (Becskei et al. 2005; Raj and Oudenaarden 2008). Shahrezaei and Swain (Shahrezaei and Swain 2008) proposed a more general and explicit definition for any system, where intrinsic stochasticity is “generated by the dynamics of the system from the random timing of individual reactions” and extrinsic stochasticity is “generated by the system interacting with other stochastic systems in the cell or its environment”. This generic definition therefore includes Raser and O’Shea's (Raser and O’Shea 2005) suggestion to further distinguish extrinsic noise occurring

56 "within pathways" and "between pathways". Other intermediate organization levels of gene
57 expression are also likely to affect expression noise, such as chromatin structure (Blake et al. 2003;
58 Hebenstreit 2013), and three-dimensional genome organization (Pombo and Dillon 2015).
59 Pioneering work by Fraser et al (Fraser et al. 2004) has shown that SGE is an evolvable trait which
60 is subject to natural selection. First, genes involved in core functions of the cell are expected to
61 behave more deterministically (Barkai and Leibler 1999) because temporal oscillations in the
62 concentration of their encoded proteins are likely to have a deleterious effect. Second, genes
63 involved in immune response (Arkin et al. 1998; Norman et al. 2015) and response to
64 environmental conditions can benefit from being unpredictably expressed in the context of selection
65 for bet-hedging (Thattai and Oudenaarden 2004). As the relation between fitness and stochasticity
66 depends on the function of the underlying gene, selection on SGE is expected to act mostly at the
67 intrinsic level (Newman et al. 2006; Lehner 2008; Wang and Zhang 2011). The molecular
68 mechanisms by which natural selection operates to regulate expression noise, however, remain to be
69 elucidated.
70 Due to methodological limitations, seminal studies on SGE (both at the mRNA and protein levels)
71 have focused on only a handful of genes (Elowitz et al. 2002; Ozbudak et al. 2002; Chubb et al.
72 2006). The canonical approach consists in selecting genes of interest and recording the change of
73 their noise levels in a population of clonal cells as a function of either (1) the concentration of the
74 molecule that allosterically controls affinity of the transcription factor to the promoter region of the
75 gene (Blake et al. 2003; Bar-even et al. 2006) or (2) mutations artificially imposed in regulatory
76 sequences (Ozbudak et al. 2002). In parallel with theoretical work (Kepler and Elston 2001;
77 Kaufmann and van Oudenaarden 2007; Sánchez and Kondev 2008), these pioneering studies have
78 provided the basis of our current understanding of the proximate molecular mechanisms behind
79 SGE, namely complex regulation by transcription factors, architecture of the upstream region
80 (including the presence of TATA box), translation efficiency and mRNA / protein stability (Eldar
81 and Elowitz 2010). Measurements at the genome scale are however needed in order to go beyond

gene idiosyncrasies and particular histories and test hypotheses about the evolutionary forces shaping SGE (Sauer et al. 2007).

The recent advent of single-cell RNA sequencing makes it possible to sequence the transcriptome of each individual cell in a collection of cell clones and to observe the variation of gene-specific mRNA quantities across cells. This gives access to a genome-wide assessment of transcriptional noise. While not accounting for putative noise resulting from the process of translation of mRNA into protein, transcriptional noise accounts for both noise generated by the transcription process and noise resulting from the degradation of mRNA molecules (**Figure 1**). Previous studies, however, have shown that transcription is a limiting step in gene expression, and that transcriptional noise is therefore a good proxy for expression noise (Newman et al. 2006; Taniguchi et al. 2011). Here, we used publicly available single-cell transcriptomics data sets to quantify gene-specific transcriptional noise and relate it to other genomic factors, including protein conservation and position in the interaction network, in order to uncover the molecular basis of selection on stochastic gene expression.

Material and Methods

Single-cell gene expression data set

We used the dataset generated by Sasagawa et al. (Sasagawa et al. 2013) retrieved from the Gene Expression Omnibus repository (accession number GSE42268). We analyzed expression data corresponding to embryonic stem cells in G1 phase, for which more individual cells were sequenced. A total of 17,063 genes had non-zero expression in at least one of the 20 single cells. Similar to Shalek et al (Shalek et al. 2014), a filtering procedure was performed where only genes whose expression level satisfied $\log(\text{FPKM}+1) > 1.5$ in at least one single cell were kept for further analyses. This filtering step resulted in a total of 13,660 appreciably expressed genes for which transcriptional noise was evaluated, compared to 11,640 genes present in the filtered dataset of Shalek et al (2014).

Measure of transcriptional noise

The mean (μ) and variance (σ^2) of each gene over all single cells were computed. A linear model was fitted on the log-transformed means and variances in order to estimate the coefficients of the power law regression:

$$\sigma^2 = a.\mu^b \quad \text{(eqn 1)}$$

$$\log(\sigma^2) = \log(a) + b.\log(\mu) \quad \text{(eqn 2)}$$

We defined F* as the ratio of the observed variance and the predicted variance:

$$F^* = \frac{\sigma^2}{a.\mu^b} \quad \text{(eqn 3)}$$

F* can be seen as a general expression for the Fano factor (a = b = 1) and noise measure (a = 1, b = 2). F* is the stochasticity measure unit with which we produced our results, after estimating the a and b parameters from the data.

Genome architecture

The mouse proteome from Ensembl (genome version: mm9) was used in order to get coordinates of all genes. The Hi-C dataset for embryonic stem cells (ES) from Dixon et al (Dixon et al. 2012) was used to get three-dimensional domain information. Two genes were considered in proximity in one dimension (1D) if they are on the same chromosome and no protein-coding gene was found between them. The primary distance (in number of nucleotides) between their midpoint coordinates was also recorded as 1D a distance measure between the genes. Two genes were considered in proximity in three dimensions (3D) if the normalized contact number between the two windows the genes belong was non-null. Two genes belonging to the same window were considered in proximity. We further computed the relative difference of stochastic gene expression between two genes by computing the ratio $(F_2^* - F_1^*)/(F_2^* + F_1^*)$. For each chromosome, we independently tested if there was a correlation between the primary distance and the relative difference in stochastic gene expression with a Mantel test, as implemented in the ade4 package (Dray and Dufour, 2007). In

order to test whether genes in proximity (1D and 3D) had more similar transcriptional noise than distant genes, we contrasted the relative differences in transcription noise between pairs of genes in proximity and pairs of distant genes. As we test all pairs of genes, we performed a randomization procedure in order to assess the significance of the observed differences by permuting the rows and columns in the proximity matrices 1,000 times. Linear models accounting for spatial interactions with genes were fitted using the generalized least squares (GLS) procedure as implemented in the "nlme" package for R (Pinheiro et al 2016). A correlation matrix between all tested genes was defined as $G=\{g_{i,j}\}$, where $g_{i,j}$ is the correlation between genes i and j. We defined $g_{i,j}=1-\exp(-\lambda\delta_{i,j})$, where $\delta_{i,j}$ takes 1 if genes i and j are in proximity, 0 otherwise. Parameter λ was estimated jointly with other model parameters, it measures the strength of the genome "spatial" correlation. Parameters were estimated using the maximum likelihood (ML) procedure, instead of the default restricted maximum likelihood (REML) in order to perform model comparison using Akaike's information criterion (AIC).

Biological pathways and network topology

The 13,660 Ensembl ids in our dataset were mapped to 13,136 Entrez ids. We kept only genes with unambiguous mapping, resulting in 11,032 Entrez ids for the Reactome pathway analysis. We defined genes either in the top 10% least noisy or in the top 10% most noisy as candidate sets and used the Reactome PA package (Yu and He 2015) to search the mouse Reactome database for overrepresented pathways with a 1% false discovery rate.

Thirteen thousand six hundred and sixty Ensembl ids mapped to a total of 29,859 UniProt ids. For network analyses, we removed UniProt ids which were not annotated to the Reactome database, resulting in a total of 4,929 UniProt ids after this first step. We then removed genes that mapped ambiguously from Ensembl to UniProt, retaining 3,959 Ensembl / UniProt ids for which we computed centrality measures. At the network level, size, transitivity and diameter could be calculated for every pathway using a combination of three R packages ("pathview" (Luo 2013),

156 "igraph" (Csardi 2015**)** and "graphite" (Sales et al 2016)). As the calculation of assortativity does
157 not handle missing data (that is, nodes of the pathway for which no value could be computed), we
158 computed assortativity on the sub-network with nodes for which data were available. A principal
159 component analysis was conducted on all network centrality measures using the ade4 package for R
160 (Dray et al 2007). Models of F* assortativity measures were fitted and compared using Multivariate
161 Adaptive Regression Splines, as implemented in the "earth" package in R (Milborrow 2016).

162 Sequence divergence

163 The Ensembl's Biomart interface was used to retrieve the proportion of non-synonymous (Ka) and
164 synonymous (Ks) divergence estimates for each mouse gene relative to the human ortholog. This
165 information was available for 13,136 genes.

166 Gene Age

167 The relative taxonomic ages of the mouse genes have been computed and is available in the form of
168 20 Phylostrata (Neme and Tautz 2013). Each Phylostratum corresponds to a node in the
169 phylogenetic tree of life. Phylostratum 1 corresponds to "All cellular organisms" whereas
170 Phylostratum 20 corresponds to "*Mus musculus*", with other levels in between. We used this
171 published information to assign each of our genes to a specific Phylostratum and used this as a
172 relative measure of gene age: Age = 21 - Phylostratum, so that an age of 1 corresponds to genes
173 specific to *M. musculus* and genes with an age of 20 are found in all cellular organisms.

174 Linear modeling

175 The first axis (43.324% of the total variance) of the principal component analysis of centrality
176 measures was used as a synthetic measure of centrality (variable SynthNet, see **Figure 5**). We built
177 a linear model with F* as a response variable and the three predictor variables SynthNet, Ka / Ks
178 ratio and gene age, as well as their double and triple interactions. As the fitted model displayed
179 significant departure to normality, it was further transformed using the Box-Cox procedure

("boxcox" function from the MASS package for R (Venables and Ripley 2002)). The Box-Cox transformed model was then subject to backward model selection in order to discard extra-numerous parameters. The selected model according to Akaike's information criterion only contains single effects and the pairwise interaction between Ka / Ks and age. Residues of the selected model had independent residue distributions (Ljung-Box test, p-value = 0.09402) but still displayed slight departure to normality (Shapiro-Wilk test, p-value = 1.22e-7), and heteroscedasticity (Harrison-McCabe test, p-value = 0.001333). In order to assess whether these departures from the Gauss-Markov assumptions could bias our results, we used two complementary approaches. First we used the "robcov" function of the "rms" package in order to get robust estimates of the effect significativity (Harrel 2015). Second, we performed a quantile regression using the "rq" function (parameter tau set to 0.5, equivalent to a median regression) of the "quantreg" package for R (Koenker, 2016).

Gene Ontology Enrichment

Eight thousand three hundreds and twenty five out of the 13,660 genes were associated with Gene Ontology (GO) terms. We tested genes at both ends of the F* spectrum for GO terms enrichment using the same threshold percentile of 10% low / high noise genes as we did for the Reactome analysis. We carried out GO enrichment analyses using two different algorithms: "Parent-child" (Grossmann et al. 2007) and "Weight01", a mixture of two algorithms developed by Alexa et al (Alexa et al. 2006). We kept only the terms that appeared simultaneously on both Parent-child and Weight01 under 10% significance level, controlling for multiple testing using the FDR method (Benjamini and Hochberg 1995).

Additional data sets

The aforementioned analyses were additionally conducted on the data set of Shalek et al (Shalek et al. 2014). Following the filtering procedure established by the authors in the original paper, genes which did not satisfied the condition of being expressed by an amount such that log(TPM+1) > 1 in

at least one of the 95 single cells were further discarded, where TPM stands for transcripts per million. This cut-off threshold resulted in 11,640 genes being kept for investigation. The rest of the analyses was conducted in the same way as in Sasagawa's data set.

All datasets and scripts to reproduce the results of this study are available at Figshare, under the DOI 10.6084/m9.figshare.4587169.

Results

A new measure of noise to study genome-wide patterns of stochastic gene expression

We analyzed the dataset generated by Sasagawa et al (2013), which quantifies gene-specific amounts of mRNA as fragments per kilobase of transcripts per million mapped fragments (FPKM) values for each gene and each individual cell. Among these, we selected all genes in a subset containing 20 embryonic stem cells in G1 phase in order to avoid recording variance that is due to different cell types or cell-cycle phases. The Quartz-Seq sequencing protocol captures every poly-A RNA present in the cell at one specific moment, allowing to assess transcriptional noise. Following Shalek et al (2014) we first filtered out genes that were not appreciably expressed in order to reduce the contribution of technical noise to the total noise. For each gene we further calculated the mean μ in FPKM units and variance σ^2 in FPKM^2 units, as well as two previously published measures of SGE: *Fano factor*, usually referred to as the bursty parameter, defined as σ^2/μ and *Noise*, defined as the coefficient of variation squared (σ^2/μ^2). Both the variance and the *Fano factor* are monotonically increasing functions of the mean (**Figure 2A**). *Noise* is inversely proportional to mean expression (**Figure 2A**), in agreement with previous observations at the protein level (Bar-even et al. 2006; Taniguchi et al. 2011). While this negative correlation was theoretically predicted (Tao et al. 2007), it may confound the analyses of transcriptional noise at the genome level, because mean gene expression is under specific selective pressure (Pál et al. 2001). In order to disentangle

these effects, we developed a new quantitative measure of noise, independent of the mean expression level of each gene. To achieve this we fitted a linear model in the log-space plot of variance *versus* mean and extracted the slope (a) and intercept (b) of the regression line. We defined F* as $\sigma^2/(a.\mu^b)$ (see Material and Methods) that is, the ratio of the observed variance over the variance component predicted by the mean expression level. Genes with F* < 1 have a variance lower than expected according to their mean expression whereas genes with F* > 1 behave the opposite way **(Figure 2A**). As expected, F* displays no significant correlation with the mean (Kendall's tau = -0.009, p-value = 0.106**, Figure 2B**). We therefore use F* as a measure of SGE throughout this study.

Stochastic gene expression correlates with the three-dimensional, but not one-dimensional, structure of the genome

We first sought to investigate whether genome organization significantly impacts the patterns of stochastic gene expression. We assessed whether genes in proximity along chromosomes display more similar amount of transcriptional noise than distant genes. We tested this hypothesis by computing for each pair of genes their primary distance on the genome, as well as their relative difference in transcriptional noise (see Methods). We found no significant association between the two distances (Mantel tests, each chromosome tested independently). Neighbor genes in one dimension, however, have significantly more similar transcriptional noise that non-neighbor genes (permutation test, p-value < 1e-3, **Figure 3**). Using Hi-C data from mouse embryonic cells (Dixon et al. 2012), we report that genes in proximity in three-dimensions have significantly more similar transcriptional noise than genes not in contact (permutation test, p-value < 1e-3, **Figure 3**). Most neighbor genes in one-dimension also appear to be close in three-dimensions and the effect of 3D contact is stronger than that of 1D contact**.** These results therefore suggest that the three-dimensional structure of the genome has a stronger impact on stochastic gene expression than the

position of the genes along the chromosomes. We further note that while highly significant, the size of this effect is small, with a difference in relative expression of -1.12% (**Figure 3**).

Low noise genes are enriched for housekeeping functions

We investigated the function of genes at both ends of the F* spectrum. We defined as candidate gene sets the top 10% least noisy or the top 10% most noisy genes in our data set, and tested for enrichment of GO terms and Reactome pathways (see Methods). It is expected that genes encoding proteins participating in housekeeping pathways are less noisy because fluctuations in concentration of their products might have stronger deleterious effects (Pedraza and van Oudenaarden 2005). On the other hand, stochastic gene expression could be selectively advantageous for genes involved in immune and stress response, as part of a bet-edging strategy (eg Arkin et al. 1998; Shalek et al. 2013). While we do not find any significantly enriched Reactome pathway in the high noise gene set, a total of 37 pathways were significantly over-represented in the low-noise gene set (false discovery rate set to 1%). Interestingly, the top most significant pathways belong to modules related to translation (initiation, elongation, termination as well as ribosomal assembly), as well as several modules relating to gene expression, including chromatin regulation and mRNA splicing (**Figure 4**). GO terms enrichment tests lead to similar results (**Table 1**): we found the molecular functions “nucleic acid binding” and “structural constituent of ribosome”, the biological processes “nucleosome assembly”, “innate immune response in mucosa” and “translation”, as well as the cellular component “nuclear nucleosome” to be enriched in the low noise gene set. All these terms but one relate to gene expression.

The lack of significantly enriched Reactome pathways by high noise genes can potentially be explained by the nature of the data set: as the original experiment was based on unstimulated cells, genes that directly benefit from high SGE might not be expressed in these experimental conditions. In accordance, high-noise genes are not found to be enriched for any GO term.

Highly connected proteins are synthesized by low-noise genes

The structure of the interaction network of proteins inside the cell can greatly impact the evolutionary dynamics of genes (Jeong et al. 2000; Barabási and Oltvai 2004). Furthermore, the contribution of each constitutive node within a given network varies. This asymmetry is largely reflected in the power-law-like degree distribution that is observed in virtually all biological networks (Barabási and Albert 1999) with a few genes displaying a lot of connections and a majority of genes displaying only a few. The individual characteristics of each node in a network can be characterized by various measures of centrality (**Newmann 2003**). Following previous studies on protein evolutionary rate (Fraser et al. 2002; Hahn et al. 2004; Jovelin and Phillips 2009) we asked whether, at the gene level, there is a link between centrality of a protein and the amount of transcriptional noise as measured by F*, using five centrality metrics measured from the graphs provided by the Reactome database (**Croft et al. 2014**). Our data set encompasses 13,660 genes for which both gene expression data and pathway annotations were available.

We first estimated the pleiotropy index of single genes by counting in how many different pathways the corresponding proteins are involved in. We then computed centrality measures as averages over all pathways in which each gene is involved. A principal component analysis revealed two groups of measures (**Figure 5**). The first measures are related to the number of interacting partners of a given protein. These measures are all negatively correlated with transcriptional noise: the more central a protein is, the less transcriptional noise it displays (**Table 2**). The most simple measure of centrality of a node is its degree, that is, the number of nodes it is directly connected with (Kendall tau = -0.071, p-value = 6.27e-11; **Table 2**): the more connections a protein makes, the less noisy its synthesis is. The hub score and authority score are both calculated from the adjacency matrix of a graph, which describes the distribution of edges among the nodes. The hub score estimates the extent to which a node links to other influent nodes and the authority score estimates the importance of a node by assessing how many hubs link to it. Both scores negatively correlate similarly with F* (Hub score: Kendall's tau = -0.073, p-value = 1.474e-11; Authority score: Kendall's tau = -0.068, p-

value = 3.652e-10). We also observed that pleiotropy is negatively correlated with F* (Kendall's tau = -0.049, p-value = 1.149e-05; **Table 2**), although to a lesser extent. This effect is most likely explained by the fact that pleiotropic genes are themselves more central (e.g. correlation of pleiotropy and node degree: Kendall's tau = 0.229, p-value < 2.2e-16). Altogether, these results suggest that natural selection acts to reduce expression noise in genes encoding highly connected proteins.

The two measures of centrality "closeness" and "betweenness" are highly correlated with each other, but are independent of the degree measures (**Figure 5**). Closeness is a measure of the topological distance between a node and every other reachable node. The fewer steps (edge hops) it takes for a protein to reach every other protein in a network, the higher its closeness. We do not find any significant relation between F* and the closeness value of genes (Kendall's tau = -0.005, p-value = 0.663). Similarly, betweenness is proportional to the frequency with which a protein belongs to the shortest path between every pair of nodes. In modular networks (Hartwell et al. 1999) nodes that connect different modules are extremely important to the cell (Guimera and Amaral 2005) and are implied to show high betweenness scores. The same was pointed out by Joy et al (Joy et al. 2005) who showed that in yeast, high betweenness proteins tend to be older and more essential, which we also see in our data set (Betweenness *vs* gene age, Kendall's tau = 0.077, p-value = 7.569e-10; Betweenness *vs* Ka/Ks, Kendall's tau = -0.077, p-value = 7.818e-12). It has been argued, however, that in protein-protein interaction networks high betweenness proteins are less essential due to the lack of directed information flow, compared to, for instance, regulatory networks (Yu et al. 2007). In agreement with this latter hypothesis, we do not find any significant correlation between betweenness and transcriptional noise (Kendall's tau = -0.014, p-value = 0.206), and report that degree measures are better predictors of constraints in SGE than betweenness.

It was previously shown that centrality negatively correlates with evolutionary rate (Hahn and Kern 2004). Our results suggest that central genes are selectively constrained for their transcriptional noise such that centrality also influences the regulation of gene expression. Interestingly, it has been

reported that central genes tend to be more duplicated (Vitkup et al. 2006). The authors proposed that such duplication events would have been favored as they would confer greater robustness to deleterious mutations in proteins. Our results suggest another, non exclusive, possible advantage: having more gene copies could reduce transcriptional noise by averaging the amount of transcripts produced by each gene copy (Raser and O'Shea 2005).

Network structure impacts transcriptional noise of constitutive genes

Whereas estimators of node centrality highlight gene-specific properties inside a given network, measures at the whole-network level enable the comparison of networks with distinct properties. We computed the size, diameter and transitivity for each annotated network in our data set (1,364 networks, Supplementary Material), as well as average measures of node scores (degree, hub score, authority score, closeness, betweenness) which we compare with the average F* measure of all constitutive nodes. The size of a network is defined as its total number of nodes, while diameter is the length of the shortest path between the two most distant nodes. Transitivity is a measure of connectivity, defined as the average of all nodes' clustering coefficients, itself defined for each node as the proportion of its neighbors that also connect to each other. Interestingly, while network size is positively correlated with average degree and transitivity (Kendall's tau = 0.372, p-value < 2.2e-16 and Kendall's tau = 0.119, p-value = 2.807, respectively), diameter displays a positive correlation with average degree (Kendall's tau = 0.202, p-value < 2.2e-16) but a negative correlation with transitivity (Kendall's tau = -0.115, p-value = 2.237e-08). This is because diameter increases logarithmically with size, that is, addition of new nodes to large networks do not increase the diameter as much as additions to small networks. This suggests that larger networks are relatively more compact than smaller ones, and their constitutive nodes are therefore more connected. We find that average transcriptional noise correlates negatively with network size (Kendall's tau = -0.0594, p-value = 0.001376), while being independent of the diameter (Kendall's tau = 0.0125, p-value = 0.5366). Transcriptional noise is also strongly negatively correlated with all averaged centrality

354 measures (**Table 3**). These results are in line with the node-based analyses, and show that the more
355 connections a network has, the less stochastic the expression of the underlying genes is. This
356 supports the view of Raser and Oshea (Raser and O'Shea 2005) that the gene-extrinsic, pathway-
357 intrinsic level is functionally pertinent and needs to be distinguished from the globally extrinsic
358 level.
359 We further asked whether genes with similar transcriptional noise tend to synthesize proteins that
360 connect to each other (positive assortativity) in a given network, or on the contrary, tend to avoid
361 each other (negative assortativity). We considered all Reactome pathways annotated to the mouse
362 and estimated their respective F* assortativity. We found the mean assortativity to be significantly
363 negative, with a value of -0.131 (one sample Wilcoxon rank test, p-value < 2.2e-16), meaning that
364 proteins with different F* values tend to connect with each other (**Figure 6**). Maslov & Sneppen
365 (Maslov and Sneppen 2002) reported a negative assortativity between hubs in protein-protein
366 interaction networks, which they hypothesized to be the result of selection for reduced vulnerability
367 to deleterious perturbations. In our data set, however, we find the assortativity of hub scores to be
368 slightly but significantly positive (average of 0.060, one sample Wilcoxon rank test, p-value =
369 0.0002702, **Figure 6**), although with a large distribution of assortativity values. As we showed that
370 hub scores correlates negatively with F* (**Table 2**), we asked whether the negative assortativity of
371 hub proteins can at least partly explain the negative assortativity of F*. We found a significantly
372 positive correlation between the two assortativity measures (Kendall's tau = 0.338, p-value < 2.2e-
373 16). The relationship between the measures, however, is not linear. A Multivariate Adaptive
374 Regression Spline was fitted to the two assortativity measures and resulted in a selected model with
375 a strong positive correlation for hub score assortativity below -0.16, and virtually no correlation
376 above (**Figure 6**), suggesting a distinct relationship between hub score and F* for negative and
377 positive hub score assortativity. Negative assortativity of hub proteins contributes to a negative
378 assortativity of SGE (Kendall's tau = 0.381, p-value < 2.2e-16), while for pathways with positive
379 hub score assortativity the effect disappears (Kendall's tau = 0.052, p-value = 0.06282). While

assortativity of F* is closer to 0 for pathways with positive assortativity of hub score, we note that it is still significantly negative (average = -0.047, one sample Wilcoxon test with p-value < 2.2e-16). This suggests the existence of additional constraints that act on the distribution of noisy proteins in a network.

Transcriptional noise is positively correlated with the evolutionary rate of proteins

Evolutionary divergence between orthologous coding sequences in yeast has been shown to correlate negatively with fitness effect on knock-out strains of the corresponding genes (Hirsh and Fraser 2001) demonstrating that protein functional importance is reflected in the strength of purifying selection acting on it. Fraser et al (Fraser et al. 2004) studied transcription and translation rates of genes in the yeast *Saccharomyces cerevisiae*, and classified genes in distinct noise categories according to their expression strategies. They reported that genes with high fitness effect display lower expression noise than the rest. Following these early observations, we hypothesized that genes under strong purifying selection at the protein sequence level should also be highly constrained for their expression and therefore display a lower transcriptional noise. To test this hypothesis, we correlated F* with the ratio of non-synonymous (Ka) to synonymous substitutions (Ks), as measured by sequence comparison between mouse genes and their human orthologs, after discarding genes with evidence for positive selection (n = 5). In agreement with our prediction, we report a significantly positive correlation between the Ka / Ks ratio and F* (**Figure 7**, Kendall's tau = 0.0619, p-value < 2.2e-16), that is, highly constrained genes display less transcriptional noise than fast evolving ones. These results demonstrate that purifying selection is acting on expression noise in addition to the protein sequence and mean expression level.

Older genes are less noisy than younger ones

Evolution of new genes was long thought to occur via duplication and modification of existing genetic material ("evolutionary tinkering", (Jacob 1977)). Evidence for *de novo* gene emergence is however becoming more and more common (Tautz and Domazet-Lošo 2011; Xie et al. 2012). *De novo* created genes undergo several optimization steps, including their integration into a regulatory network (Neme and Tautz 2013). We tested whether this historical process of incorporation into pathways impacts the evolution of transcriptional noise. As older genes tend to be more conserved (Wolf et al. 2009), we further controlled for sequence conservation, as measured by the Ka / Ks ratio of the gene. We used the phylostratigraphic approach of Neme & Tautz (Neme and Tautz 2013), which categorizes genes into 20 strata, to compute gene age and tested for a correlation with F*, correcting for sequence divergence as a putative covariate (**Figure 7**, Kendall's tau = -0.047, p-value = 3.001e-13; partial correlation controlling for gene sequence conservation). This negative correlation still holds when we discard very recent *de novo* genes (belonging to Phylostratum 20) to minimize influence of putative annotation errors (Kendall's tau = -0.047, p-value = 3.534e-13). These results suggest that older genes are more deterministically expressed while younger genes are more noisy, independently of the selective pressure acting on them.

Biological network growth is currently thought to occur by preferential attachment (Jeong et al. 2001): the more edges a node has, the more likely this node is to make yet another edge with a newly arrived protein. This would lead to older genes playing more central roles in more pathways, and therefore explain the correlation of F* and gene age. Under this hypothesis, we expect the centrality of a gene to positively correlate with gene age. However, we observe the opposite trend (average degree *vs* gene age, Kendall's tau = -0.090, p-value = 3.578e-13), indicating that older proteins actually tend to have fewer edges. A possible explanation to this trend is that older genes are under stronger purifying selection (gene age *vs* Ka / Ks, Kendall's tau = -0.139; p-value < 2.2e-16) preventing them from linking to many younger proteins, indicating that the preferential attachment model is an oversimplification of how intra-cellular network growth is achieved

(Barabási and Oltvai 2004; Kim et al. 2013). In the same vein, gene age is not associated with higher pleiotropy (pleiotropy *vs* gene age: Kendall's tau = -0.012, p-value = 0.353). To see if this inverted preferential attachment could be explained by distinct constraints on more ancient housekeeping genes, we tested for the same correlations using only younger genes (*i.e.*, genes from Phylostrata 7, Bilateria, to 20, *Mus musculus*, which are not enriched for any particular housekeeping function [n = 1048]). This time we observe a positive, albeit non-significant correlation (average degree vs. gene age: Kendall's tau = 0.053, p-value = 0.2953), indicating that more ancient genes evolve their connectivity differently from younger ones. The effect of gene age on transcriptional noise therefore appears independent of the effect of selective constraints and position of genes in the network. While we cannot rule out that functional constraints not fully accounted for by the Ka / Ks ratio or unavailable functional annotations explain at least partially the correlation of gene age and transcriptional noise, a possible hypothesis is that ancient gene have acquired more complex regulation schemes through time. Higher order interaction in the regulation network involve for instance negative feedback loops, which have been shown to stabilize gene expression and reduce expression noise (Becskei and Serrano 2000; Thattai and Oudenaarden 2001).

Position in the protein network is the main driver of transcriptional noise

Since network topology measures, Ka / Ks ratio and gene age are correlated variables, we sought at disentangling potential confounding effects by modeling the patterns of transcriptional noise as a function of all predictive factors, as well as their interactions. Because network centrality measures are themselves not independent from each other, we used the first axis of the principal component analysis of network variables (**Figure 5A**) as a synthetic measure of node centrality. This measure essentially captures the effect of nodes degree, hub and transitivity scores (**Figure 5A**) and is negatively correlated with F* (Kendall’s tau = -0.075, p-value = 2.858e-12, **Figure 7**). We then

453 constructed a linear model with F* as a response variable, and synthetic network centrality
454 (SynthNet, explaining 43.32% of the total inertia), sequence conservation (Ka / Ks) and gene age as
455 explanatory variables, as well as all their possible interactions. We conducted a model selection
456 procedure where we allowed for interactions between variables of up to three degrees and tested
457 significance of coefficients on the selected model, controlling for various model departures (see
458 Methods and **Table 4** for results). All individual variables are retained, as well as the interaction
459 term between Ka / Ks and gene age. When taken together, only the network centrality measure and
460 gene age are significant (**Table 4**), and the coefficients in the multiple regression have the same sign
461 as the non-parametric correlation coefficients observed for F*. All variables explain 2.98% of
462 variance together. This small value indicates either that gene idiosyncrasies largely predominate
463 over general effects, or that our estimates of transcriptional noise have a large measurement error, or
464 both. An analysis of variance shows that the three individual variables explain a significant part of
465 the variance, with centrality measures explaining the largest part (SynthNet variable, 1.62%
466 variance explained, Fisher's test p-value = 9.552e-15). Gene age only explains 0.99% of the
467 variance (Fisher's test p-value = 1.386e-09) and functional constraints 0.31% (Ka / Ks variable,
468 Fisher's test p-value = 0.0006567). This suggests that position in protein network is the main driver
469 of the evolution of gene-specific stochastic expression. It also suggests that gene age has an effect
470 on F* independent of the strength of purifying selection on the genes.
471 We further included the effect of three-dimensional organization of the genome in order to assess
472 whether it could be a confounding factor. We developed a correlation model allowing for genes in
473 contact to have correlated values of transcriptional noise. The correlation model was fitted together
474 with the previous linear model in the generalized least square (GLS) framework. This model allows
475 for one additional parameter, λ, which captures the strength of correlation due to three-dimensional
476 organization of the genome (see Methods). The estimate of λ was found to be 0.0029, which means
477 that the spatial autocorrelation of transcriptional noise is low on average. While this estimate is
478 significantly higher than zero, model comparison using Akaike's information criterion favors the

linear model without three-dimensional correlation. Consistently, accounting for this correlation does not change significantly our estimates (**Table 4**), confirming network centrality measures as the main factor explaining the distribution of transcriptional noise.

Analysis of bone marrow-derived dendritic cells supports the generality of the results to other cell types

We assessed the reproducibility of our results by analyzing an additional single-cell transcriptomics data set of 95 unstimulated bone marrow-derived dendritic cells (Shalek et al. 2014). After filtering (see Methods), the data set consisted of 11,640 genes. Using the same normalization procedure as for the Sasagawa data set, we nonetheless report a weak but significant negative correlation between F* and the mean expression (-0.068, p-value < 2.2e-16). Despite this correlation, the patterns we observed with the bone marrow-derived dendritic cells dataset are qualitatively and quantitatively consistent with the ones obtained with embryonic stem cells (**Table S1**), supporting the generality of our observations to other cell types. This dataset further revealed a significant negative correlation of F* with closeness and betweenness.

Biological, not technical noise is responsible for the observed patterns

The variance in gene expression measured from single-cell transcriptomics is a combination of biological and technical variance. While the two sources of variance are a priori independent, gene-specific technical variance has been observed in micro-array experiments (Pozhitkov et al. 2007) making a correlation of the two types of variance plausible. If similar effects also affect RNA-Seq experiments, technical variance could be correlated to gene function and therefore act as a covariate in our analyses. In order to assess whether this is the case, we used the dataset of Shalek et al (Shalek et al. 2013), which contains both single-cell transcriptomics and 3 replicates of 10,000 pooled-cell RNA sequencing. In traditional RNA sequencing, which is typically performed on pooled populations of several thousands of cells, biological variance is averaged out so that the

resulting measured variance between replicates is essentially the result of technical noise. We computed the mean and variance in expression of each gene across the three populations of cells. By plotting the variance versus the mean in log-space, we were able to compute a "technical" F* (F_t^*) value for each gene (Methods). We conducted our correlation analyses using F_t^* instead of F*. We report no significant correlation between F_t^* and network centralities and gene age. There is a significant correlation between F* and sequence conservation (Kendall's tau = 0.036, p-value = 1.085e-06). However, this correlation is weaker than the one reported between F* and sequence conservation for the single-cell data set (Kendall's tau = 0.0619, p-value < 2.2e-16), thus not being sufficient to explain the latter finding. At the pathway level, correlations with F* are either non-significant or go in the opposite direction than the ones observed in single-cell datasets. In addition, there was no enrichment of the 10^{th} and 90^{th} F_t^* percentiles for any particular pathway or GO term. These results support our conclusion that the correlations we observe are due to variations that are biological, not technical.

Discussion

Throughout this work, we provided the first genome-wide evolutionary and systemic study of transcriptional noise, using a mouse cell as a model. We have shown that transcriptional noise correlates with functional constraints both at the level of the gene itself via the protein it encodes, but also at the level of the pathway(s) the gene belongs to. We further discuss here potential confounding factors in our analyses and argue that our results are compatible with selection acting to reduce noise-propagation at the network level.

In this study, we exhibited several factors explaining the variation in transcriptional noise between genes. While highly significant, the effects we report are of small size, and we only explain a few percent of the total observed variance. There are several possible explanations for this reduced explanatory power: (1) transcriptional noise is a proxy for noise in gene expression, at which

527 selection occurs (**Figure 1**). As transcriptional noise is not randomly distributed across the genome,
528 it must constitute a significant component of expression noise, in agreement with previous
529 observations (Blake et al. 2003; Newman et al. 2006). Translational noise, however, might
530 constitute an important part of the expression noise and was not assessed in this study. (2) Gene
531 expression levels were assessed on embryonic stem cells in culture. Such an experimental system
532 may result in gene expression that differs from that in natural conditions under which natural
533 selection acted. (3) Functional annotations, in particular pathways and gene interactions are still
534 incomplete, and network-based measures have most likely large estimation errors. (4) While the
535 newly introduced F* measure allowed us to assess the distribution of transcriptional noise
536 independently of the average mean expression – therefore constituting an improvement over
537 previous studies – it does not capture the full complexity of SGE. Explicit modeling, for instance
538 based in the Beta-Poisson model (Vu et al. 2016) is a promising avenue for the development of
539 more sophisticated quantitative measures.

540 In a pioneering study, Fraser et al, followed by Shalek et al, demonstrated that essential genes
541 whose deletion is deleterious and genes encoding subunits of molecular complexes (Fraser et al.
542 2004) as well as housekeeping genes (Shalek et al. 2013) display reduced gene expression noise.
543 Our findings go beyond these earlier observations as they reveal that network centrality measures
544 are the major explanatory factor of the distribution of transcriptional noise in the genome. This
545 suggests that selection at the pathway level is a widespread phenomenon that drives the evolution of
546 SGE at the gene level. This multi-level selection mechanism, we propose, can be explained by
547 selection against noise propagation within networks. It has been experimentally demonstrated that
548 expression noise can be transmitted from one gene to another gene with which it is interacting
549 (Pedraza and van Oudenaarden 2005). Large noise at the network level is deleterious (Barkai and
550 Leibler 1999) but each gene does not contribute equally to it, thus the strength of selective pressure
551 against noise varies among genes in a given network. We have shown that highly connected,
552 “central” proteins typically display reduced transcriptional noise. Such nodes are likely to constitute

key players in the flow of noise in intra-cellular networks as they are more likely to transmit noise to other components. In accordance with this hypothesis, we find genes with the lowest amount of transcriptional noise to be enriched for top-level functions, in particular involved in the regulation of other genes.

These results have several implications for the evolution of gene networks. First, this means that new connections in a network can potentially be deleterious if they link genes with highly stochastic expression. Second, distinct selective pressures at the “regulome” and “interactome” levels (**Figure 1**) might act in opposite direction. We expect genes encoding highly connected proteins to have more complex regulation schemes, in particular if their proteins are involved in several biological pathways. In the simplest scenario of open chromatin and absence of transcription factors and enhancers, each gene has a constant probability of being transcribed per time unit and the resulting amount of transcripts follows a Poisson distribution with *Fano factor* equal to 1, that is, with variance equal to mean expression (Raj and Oudenaarden 2008). The early evidence for widespread bursty transcription, leading to overdispersion (variance > mean expression, (Raj et al. 2006; So et al. 2011)) suggests that complex regulation leads to increased transcriptional noise. Subsequently, several studies demonstrated that expression noise of a gene positively correlates with the number of transcription factors controlling its regulation (Sharon et al. 2014). Central genes, while being under negative selection against stochastic behavior, are then more likely to be controlled by numerous transcription factors which will tend to increase transcriptional noise. As a consequence, if the number of connections at the interactome level is highly correlated with the number of connections at the regulome level, there must exist a trade-off in the number of connections a gene can make in a network. Alternatively, highly connected genes might evolve regulatory systems allowing them to uncouple these two levels: negative feedback loops, for instance, where the product of a gene down-regulates its own production have been shown to stabilize expression and significantly reduce stochasticity (Becskei and Serrano 2000; Dublanche et al. 2006; Tao et al.

2007). We therefore predict that negative feedback loops are more likely to occur at genes that are more central in protein networks, as they will confer a greater advantage in terms of SGE.

Our results enabled the identification of possible selective pressures acting on the level of stochasticity in gene expression. The mechanisms by which the amount of stochasticity can be controlled remain however to be elucidated. We evoked the existence of negative feedback loops which reduce stochasticity and the multiplicity of upstream regulator which increase it. Recent work by Wolf et al (Wolf et al. 2015) and Metzger et al (Metzger et al. 2015) add further perspective to this scheme. Wolf and colleagues found that in *Escherichia coli* noise is higher for natural than experimentally evolved promoters selected for their mean expression level. They hypothesized that higher noise is selectively advantageous in case of changing environments. On the other hand, the Metzger and colleagues found the signature of selection for reduced noise in natural populations of *Saccharomyces cerevisae*. Together, these results provide additional evidence that the amount of stochasticity in the expression of every single gene has an optimum, with higher values being less advantageous because of noise propagation in the network the gene belongs to and lower values being suboptimal in case of changing environment because of less phenotypic plasticity. Conclusion

Using a new measure of transcriptional noise, our results demonstrate that the position of the protein in the interactome is a major driver of selection against stochastic gene expression. As such, transcriptional noise is an essential component of the phenotype, in addition to the mean expression level and the actual sequence and structure of the encoded proteins. This is currently an under-appreciated phenomenon, and gene expression studies that focus only on the mean expression of genes may be missing key information about expression diversity. The study of gene expression must consider changes in noise in addition to change in mean expression level as a putative explanation for adaptation. Further work aiming to unravel the exact structure of the regulome is however needed in order to fully understand how transcriptional noise is generated or inhibited.

Acknowledgements

The authors would like to thank Rafiq Neme-Garrido, Frederic Bartels and Estelle Renaud for fruitful discussions about this work, as well as Diethard Tautz for comments on an earlier version of this manuscript. JYD acknowledges funding from the Max Planck Society. This work was supported by the German Research Foundation (DFG), within the priority program (SPP) 1590.

References

Alexa A, Rahnenführer J, Lengauer T. 2006. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics 22:1600–1607.

Arkin A, Ross J, Mcadams HH. 1998. Stochastic Kinetic Analysis of Developmental Pathway Bifurcation in Phage L-Infected Escherichia coli Cells. Genetics 149:1633–1648.

Barabási A-L, Albert R. 1999. Emergence of Scaling in Random Networks. Science 286:509–513.

Barabási A-L, Oltvai ZN. 2004. Network biology: understanding the cell's functional organization. Nature reviews. Genetics 5:101–113.

Bar-even A, Paulsson J, Maheshri N, Carmi M, Shea EO, Pilpel Y, Barkai N. 2006. Noise in protein expression scales with natural protein abundance. Nature genetics 38:636–643.

Barkai N, Leibler S. 1999. Circadian clocks limited by noise. Nature 403:267–268.

Becskei A, Kaufmann BB, van Oudenaarden A. 2005. Contributions of low molecule number and chromosomal positioning to stochastic gene expression. Nature Genetics 37:937–944.

Becskei A, Serrano L. 2000. Engineering stability in gene networks by autoregulation. Nature 405:590–593.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society 57:289–300.

Blake WJ, Kærn M, Cantor CR, Collins JJ. 2003. Noise in eukaryotic gene expression. Nature 422:633–637.

Chubb JR, Trcek T, Shenoy SM, Singer RH. 2006. Transcriptional Pulsing of a Developmental Gene. Current Biology 16:1018–1025.

Croft D, Mundo AF, Haw R, Milacic M, Weiser J, Wu G, Caudy M, Garapati P, Gillespie M, Kamdar MR, et al. 2014. The Reactome pathway knowledgebase. Nucleic Acids Research 42:472–477.

Csardi G, Nepusz T. 2006. The igraph software package for complex network research, InterJournal, Complex Systems 1695.

Dixon JR, Selvaraj S, Yue F, Kim A, Li Y, Shen Y, Hu M, Liu JS, Ren B. 2012. Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature 485:376–380.

Dray, S, Dufour, AB. 2007. The ade4 package: implementing the duality diagram for ecologists. Journal of Statistical Software. 22(4): 1-20.

Dublanche Y, Michalodimitrakis K, Kümmerer N, Foglierini M, Serrano L. 2006. Noise in transcription negative feedback loops: simulation and experimental analysis. Molecular systems biology 2:41–41.

Eldar A, Elowitz MB. 2010. Functional roles for noise in genetic circuits. Nature 467:167–173.

Elowitz MB, Levine AJ, Siggia ED, Swain PS. 2002. Stochastic Gene Expression in a Single Cell. Science 297:1183–1186.

Fraser HB, Hirsh AE, Giaever G, Kumm J, Eisen MB. 2004. Noise Minimization in Eukaryotic Gene Expression. PLoS Biology 2:0834–0838.

Fraser HB, Hirsh AE, Steinmetz LM, Scharfe C, Feldman MW. 2002. Evolutionary Rate in the Protein Interaction Network. Science 296:750–752.

Gillesple DT. 1977. Exact Simulation of Coupled Chemical Reactions. The Journal of Physical Chemistry 81:2340–2361.

Grossmann S, Bauer S, Robinson PN, Vingron M. 2007. Improved detection of overrepresentation of Gene-Ontology annotations with parent – child analysis. Bioinformatics 23:3024–3031.

Guimera R, Amaral LAN. 2005. Functional cartography of complex metabolic networks. Nature 433:895–900.

Hahn MW, Conant GC, Wagner A. 2004. Molecular Evolution in Large Genetic Networks: Does Connectivity Equal Constraint? Journal of Molecular Evolution 58:203–211.

Hahn MW, Kern AD. 2004. Comparative Genomics of Centrality and Essentiality in Three Eukaryotic Protein-Interaction Networks. Molecular Biology and Evolution 22:7–10.

Harrell Jr, FE. 2016. rms: Regression Modeling Strategies. R package version 4.5-0.

Hartwell LH, Hopfield JJ, Leibler S, Murray AW. 1999. From molecular to modular cell biology. Nature 402:C47–C52.

Hebenstreit D. 2013. Are gene loops the cause of transcriptional noise? Trends in Genetics 29:333–338.

Hirsh A, Fraser H. 2001. Protein dispensability and rate of evolution. Nature 411:1046–1049.

Jacob F. 1977. Evolution and Tinkering. Science 196:1161–1166.

Jeong H, Mason SP, Barabási a L, Oltvai ZN. 2001. Lethality and centrality in protein networks. Nature 411:41–42.

Jeong H, Tombor B, Albert R, Oltvai ZN, Barabási A-L. 2000. The large-scale organization of metabolic networks. Nature 407:651–654.

Jovelin R, Phillips PC. 2009. Evolutionary rates and centrality in the yeast gene regulatory network. Genome biology 10:R35–R35.

Joy MP, Brock A, Ingber DE, Huang S. 2005. High-betweenness proteins in the yeast protein interaction network. Journal of Biomedicine and Biotechnology 2005:96–103.

Kaufmann BB, van Oudenaarden A. 2007. Stochastic gene expression: from single molecules to the proteome. Current opinion in genetics & development 17:107–112.

Kepler TB, Elston TC. 2001. Stochasticity in Transcriptional Regulation : Origins, Consequences, and Mathematical Representations. Biophysical Journal 81:3116–3136.

Kim PM, Lu LJ, Xia Y, Gerstein MB. 2013. Relating Three-Dimensional Structures to Protein Networks Provides Evolutionary Insights. Science 603:1938–1941.

Koenker, R. 2016. quantreg: Quantile Regression. R package version 5.29.

Lehner B. 2008. Selection to minimise noise in living systems and its implications for the evolution of gene expression. Molecular systems biology 4:170–170.

Luo, W, Brouwer C. 2013. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics, 29(14): 1830-1831.

Maslov S, Sneppen K. 2002. Specificity and Stability in Topology of Protein Networks. Science 296:910–913.

Mcadams HH, Arkin A. 1997. Stochastic mechanisms in gene expression. Proceedings of the National Academy of Sciences of the United States of America 94:814–819.

Metzger BPH, Yuan DC, Gruber JD, Duveau F, Wittkopp PJ. 2015. Selection on noise constrains variation in a eukaryotic promoter. Nature 521:344–347.

Milborrow, S. 2016 Derived from mda:mars by Trevor Hastie and Rob Tibshirani. Uses Alan Miller's Fortran utilities with Thomas Lumley's leaps wrapper. earth: Multivariate Adaptive Regression Splines. R package version 4.4.7.

Neme R, Tautz D. 2013. Phylogenetic patterns of emergence of new genes support a model of frequent de novo evolution. BMC genomics 14:117–117.

Newman JRS, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, Derisi JL, Weissman JS. 2006. Single-cell proteomic analysis of S . cerevisiae reveals the architecture of biological noise. Nature 441:840–846.

Newmann MEJ. 2003. The structure and function of complex networks. SIAM Review 45:167–256.

Norman TM, Lord ND, Paulsson J, Losick R. 2015. Stochastic Switching of Cell Fate in Microbes. Annual review of microbiology 69:381–403.

Ozbudak EM, Thattai M, Kurtser I, Grossman AD, Oudenaarden AV. 2002. Regulation of noise in the expression of a single gene. Nature genetics 31:69–73.

Pál C, Papp B, Hurst LD. 2001. Highly Expressed Genes in Yeast Evolve Slowly. Genetics 158:927–931.

Pedraza JM, van Oudenaarden A. 2005. Noise propagation in gene networks. Science 307:1965–1969.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2017. _nlme: Linear and Nonlinear Mixed Effects Models_. R package version 3.1-129.

Pombo A, Dillon N. 2015. Three-dimensional genome architecture: players and mechanisms. Nature Reviews Molecular Cell Biology 16:245–257.

Pozhitkov, Alex E., Tautz D, Noble, Peter A. 2007. Oligonucleotide microarrays: widely appliedçpoorly understood. Briefings in Functional Genomics and Proteomics. 6:141–148.

Raj A, Oudenaarden AV. 2008. Review Nature , Nurture , or Chance : Stochastic Gene Expression and Its Consequences. Cell 135:216–226.

Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S. 2006. Stochastic mRNA Synthesis in Mammalian Cells. PLoS Biology 4:e309–e309.

Raser JM, O'Shea EK. 2005. Noise in Gene Expression: Origins, Consequences, and Control. Science 309.

Sales, G, Calura, E, Romualdi, C. 2016. graphite: GRAPH Interaction from pathway Topological Environment. R package version 1.20.1.

Sánchez A, Kondev J. 2008. Transcriptional control of noise in gene expression. Proceedings of the National Academy of Sciences of the United States of America 105:5081–5086.

Sasagawa Y, Nikaido I, Hayashi T, Danno H, Uno KD, Imai T, Ueda HR. 2013. Quartz-Seq : a highly reproducible and sensitive single-cell RNA sequencing method , reveals non- genetic gene-expression heterogeneity. Genome Biology 14:R31–R31.

Sauer U, Heineman M, Zamboni N. 2007. Getting Closer to the Whole Picture. Science 316:550–551.

Shahrezaei V, Swain PS. 2008. The stochastic nature of biochemical networks. Curr. Opin. Biotechnol. 19:369–374.

Shalek AK, Satija R, Adiconis X, Gertner RS, Gaublomme JT, Raychowdhury R, Schwartz S, Yosef N, Malboeuf C, Lu D, et al. 2013. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498:236–240.

Shalek AK, Satija R, Shuga J, Trombetta JJ, Gennert D, Lu D, Chen P, Gertner RS, Gaublomme JT, Yosef N, et al. 2014. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature 510:363–369.

Sharon E, Van Dijk D, Kalma Y, Keren L, Manor O, Yakhini Z, Segal E. 2014. Probing the effect of promoters on noise in gene expression using thousands of designed sequences. Genome Research 24:1698–1706.

So L, Ghosh A, Zong C, Sepúlveda LA, Segev R, Golding I. 2011. General properties of transcriptional time series in Escherichia coli. Nature Genetics 43:554–560.

Spudich JL, Koshland DEJ. 1976. Non-genetic individuality: chance in the single cell. Nature:467–471.

Suter DM, Molina N, Gatfield D, Schneider K, Schibler U, Naef F. 2011. Mammalian Genes Are Transcribed with Widely Different Bursting Kinetics. Science 332:472–474.

Taniguchi Y, Choi PJ, Li G, Chen H, Babu M, Hearn J, Emili A, Xie XS. 2011. Quantifying E. coli Proteome and Transcriptome with Single-Molecule Sensitivity in Single Cells. Science (New York, N.Y.) 329:533–539.

Tao Y, Zheng X, Sun Y. 2007. Effect of feedback regulation on stochastic gene expression. J. Theor. Biol. 247:827–836.

Tautz D, Domazet-Lošo T. 2011. The evolutionary origin of orphan genes. Nature reviews. Genetics 12:692–702.

Thattai M, Oudenaarden AV. 2001. Intrinsic noise in gene regulatory networks. Proceedings of the National Academy of Sciences of the United States of America 98:8614–8619.

Thattai M, Oudenaarden AV. 2004. Stochastic Gene Expression in Fluctuating Environments. Genetics 167:523–530.

Venables, W N, Ripley, B D. 2002. Modern Applied Statistics with S. Fourth Edition. Springer, New York.

Vitkup D, Kharchenko P, Wagner A. 2006. Influence of metabolic network structure and function on enzyme evolution. Genome biology 7:R39–R39.

Vu TN, Wills QF, Kalari KR, Niu N, Wang L, Rantalainen M, Pawitan Y. 2016. Beta-Poisson model for single-cell RNA-seq data analyses. Bioinformatics:1–8.

Wang Z, Zhang J. 2011. Impact of gene expression noise on organismal fitness and the efficacy of natural selection. Proceedings of the National Academy of Sciences 108:E67–E76.

Wolf L, Silander OK, van Nimwegen EJ. 2015. Expression noise facilitates the evolution of gene regulation. eLife 4:1–48.

Wolf YI, Novichkov PS, Karev GP, Koonin EV, Lipman DJ. 2009. The universal distribution of evolutionary rates of genes and distinct characteristics of eukaryotic genes of different apparent ages. Proceedings of the National Academy of Sciences of the United States of America 106:7273–7280.

Xie C, Zhang YE, Chen JY, Liu CJ, Zhou WZ, Li Y, Zhang M, Zhang R, Wei L, Li CY. 2012. Hominoid-Specific De Novo Protein-Coding Genes Originating from Long Non-Coding RNAs. PLoS Genetics 8:e1002942.-e1002942.

Yu G, He, Q. 2016 ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. Molecular BioSystems 12(2):477-479.

Yu H, Kim PM, Sprecher E, Trifonov V, Gerstein M. 2007. The importance of bottlenecks in protein networks: Correlation with gene essentiality and expression dynamics. PLoS Computational Biology 3:713–720.

609 **Tables**

610

611 Table 1: GO terms significantly enriched in the 10% genes with lowest transcriptional noise.

Ontology	GO ID	GO term	FDR Fisher "parent-child"	FDR Fisher "weight01"
MF	GO:0003676	nucleic acid binding	2.406E-03	1.475E-08
MF	GO:0003735	structural constituent of ribosome	6.099E-03	1.708E-05
BP	GO:0006334	nucleosome assembly	3.816E-03	1.380E-02
BP	GO:0002227	innate immune response in mucosa	6.727E-03	2.018E-02
BP	GO:0006412	translation	1.257E-02	1.380E-02
CC	GO:0000788	nuclear nucleosome	3.493E-05	2.587E-05

612

613 Note: FDR: False Discovery Rate. MF: Molecular Function. BP: Biological Process. CC: Cellular

614 Compartment.

615

616 Table 2: Correlation of transcriptional noise with genes centrality measures and pleiotropy.

Measure	Correlation with F*	p-value
Degree	-0.071	6.271E-11
Hub score	-0.073	1.474E-11
Authority score	-0.068	3.652E-10
Closeness	-0.005	6.633E-01
Betweenness	-0.014	2.061E-01
Pleiotropy	-0.049	1.149E-05

617

618 Note: All correlations are computed using Kendall’s rank correlation test.

619 Table 3: Correlation of average transcriptional noise with pathway centrality measures.

Measure	**Correlation with average F***	**p-value**
Size	-0.059	1.376E-03
Diameter	0.012	5.366E-01
Average degree	-0.172	8.944E-21
Average hub score	-0.188	1.724E-24
Average authority score	-0.166	2.487E-19
Average closeness	0.050	6.500E-03
Average betweenness	-0.166	2.487E-19
Average pleiotropy	-0.137	1.276E-13

620

621 Note: All correlations are computed using Kendall's rank correlation test.

622

623 Table 4: Linear models with F* as the independent variable and SynthNet, gene age and Ka/Ks ratio

624 as explanatory variables.

Effect	Estimate	P-value		Estimate	P-value	
	OLS			OLS + robust estimates		
SynthNet1	-0.051315	8.06E-16	***	-0.0513	<0.0001	***
Age	-0.028263	7.97E-05	***	-0.0283	<0.0001	***
Ka/Ks	-0.340854	0.474	NS	-0.3409	0.4523	
Age : Ka/Ks	0.040627	0.131	NS	0.0406	0.1164	
	Quantile regression			GLS		
SynthNet1	-0.04359	<0.00001	***	-0.0511684	<0.0001	***
Age	-0.02616	0.01016	*	-0.0283132	0.0001	***
Ka/Ks	-0.18344	0.75452	NS	-0.3370668	0.4789	NS
Age : Ka/Ks	0.03638	0.27612	NS	0.0404483	0.1330	NS

625

626 Note: OLS: Ordinary Least Squares. GLS: Generalized Least Squares.

627 **Figures**

628 Figure 1: A systemic view of gene expression.

629 Figure 2: Transcriptional noise and mean gene expression. A) Measures of noise plotted against the
630 mean gene expression for each gene, in logarithmic scales together with corresponding regression
631 lines: variance, Fano factor (variance / mean), noise (square of the coefficient of variation,
632 variance / mean^2) and F* (this study). B) Distribution of F* over all genes in this study. Vertical
633 line corresponds to F* = 1.

634 Figure 3: Impact of genome organization on the distribution of transcriptional noise. The x-axis
635 shows the mean relative difference in transcriptional noise .Vertical lines show observed values and
636 histograms the distribution over 1,000 permutations (see Methods). Left panel: distribution for
637 neighbor genes along the genome. Right panel: distribution for genes in contact in three-
638 dimensions.

639 Figure 4: Enriched pathways in the 10% genes with lowest transcriptional noise.

640 Figure 5: Correlation of network measures. A) Correlation circle of netork centrality measures. B)
641 Proportion of total inertia explained by each principal component (bars) and cumulative proportion
642 of inertia explained (lines).

643 Figure 6: Assortativity in networks. For the 101 pathways for which data was available, assortativity
644 for F* and hub score were plotted against each other. Orange line: simple linear model. Blue line:
645 “breakpoint” model. Vertical dashed line show the minimal value of hub score assortativity from
646 which it has no effect on F* assortativity.

647 Figure 7: Correlation of F* with synthetic centrality measure, gene age and Ka / Ks ratio.

648

649 Sup mat:

650 Table S1: Correlation analyses with Shalek (2013) data set.

651

652 Table S2: Correlation analyses with pooled RNA-Seq data.

Figure 1

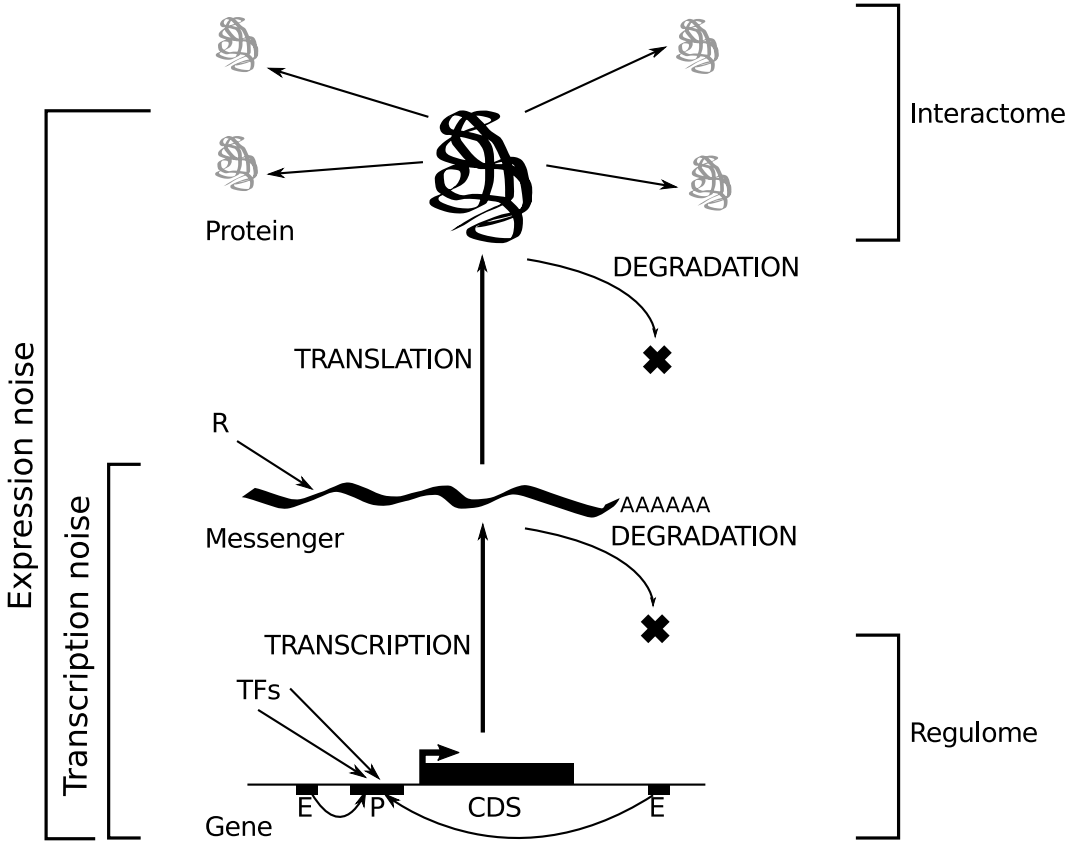
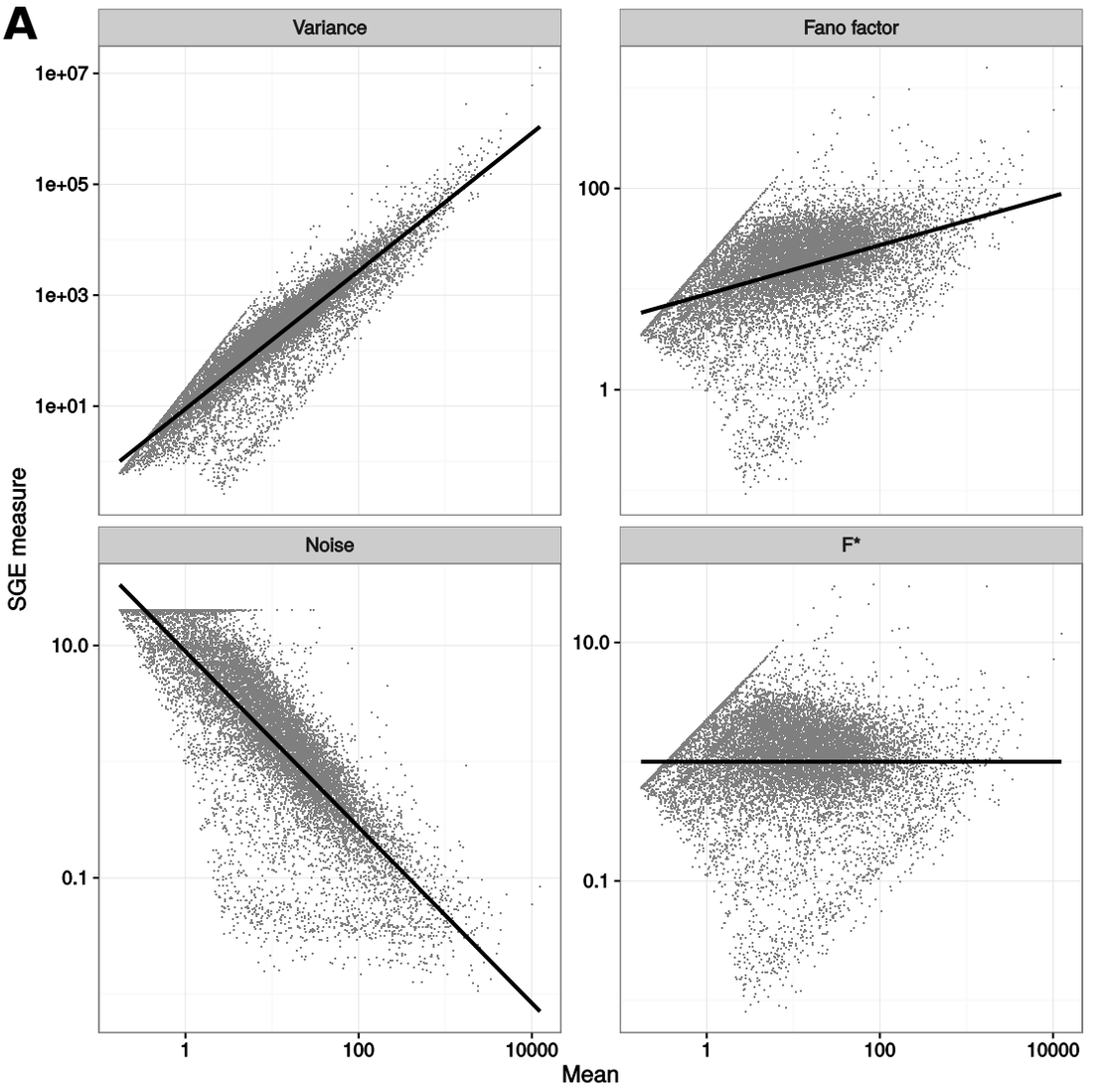

Interactome
Protein
DEGRADATION
TRANSLATION
Expression noise
R
AAAAAA
Messenger
DEGRADATION
Transcription noise
TRANSCRIPTION
TFs
Regulome
E
P
CDS
E
Gene

Figure 2



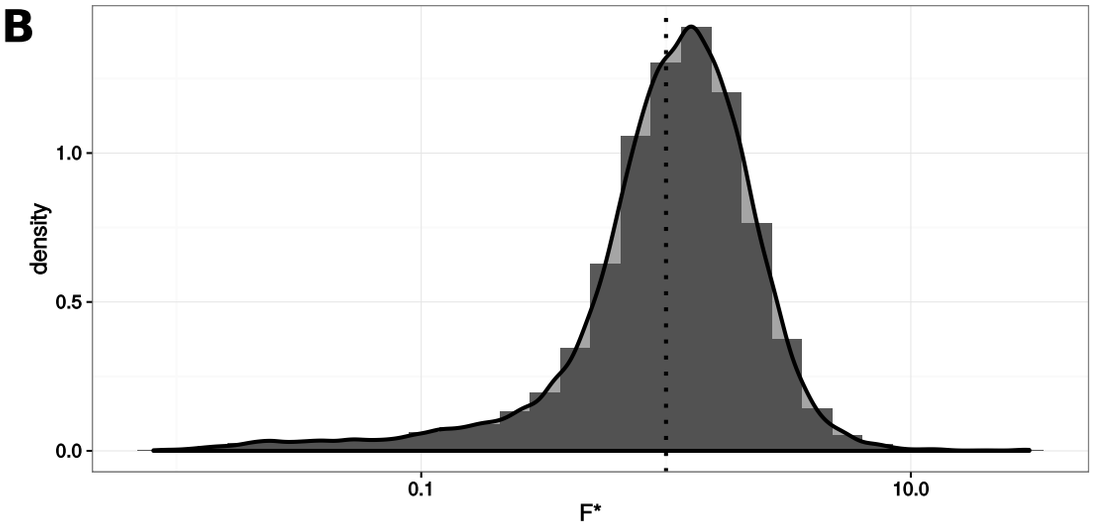


Figure 3

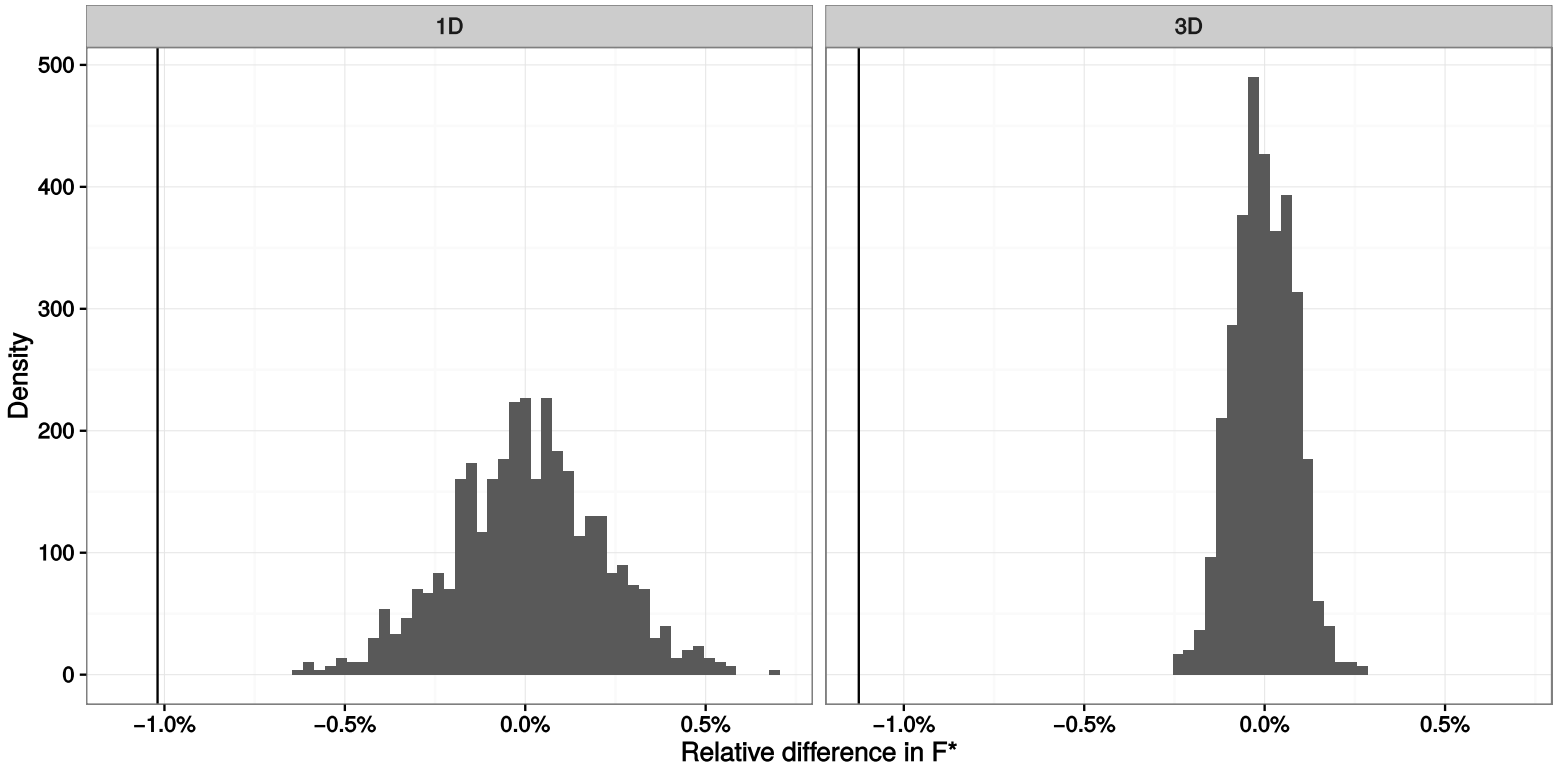

1D
3D
Density
500
400
300
200
100
0
−1.0%
−0.5%
0.0%
0.5%
Relative difference in F*

Figure 4

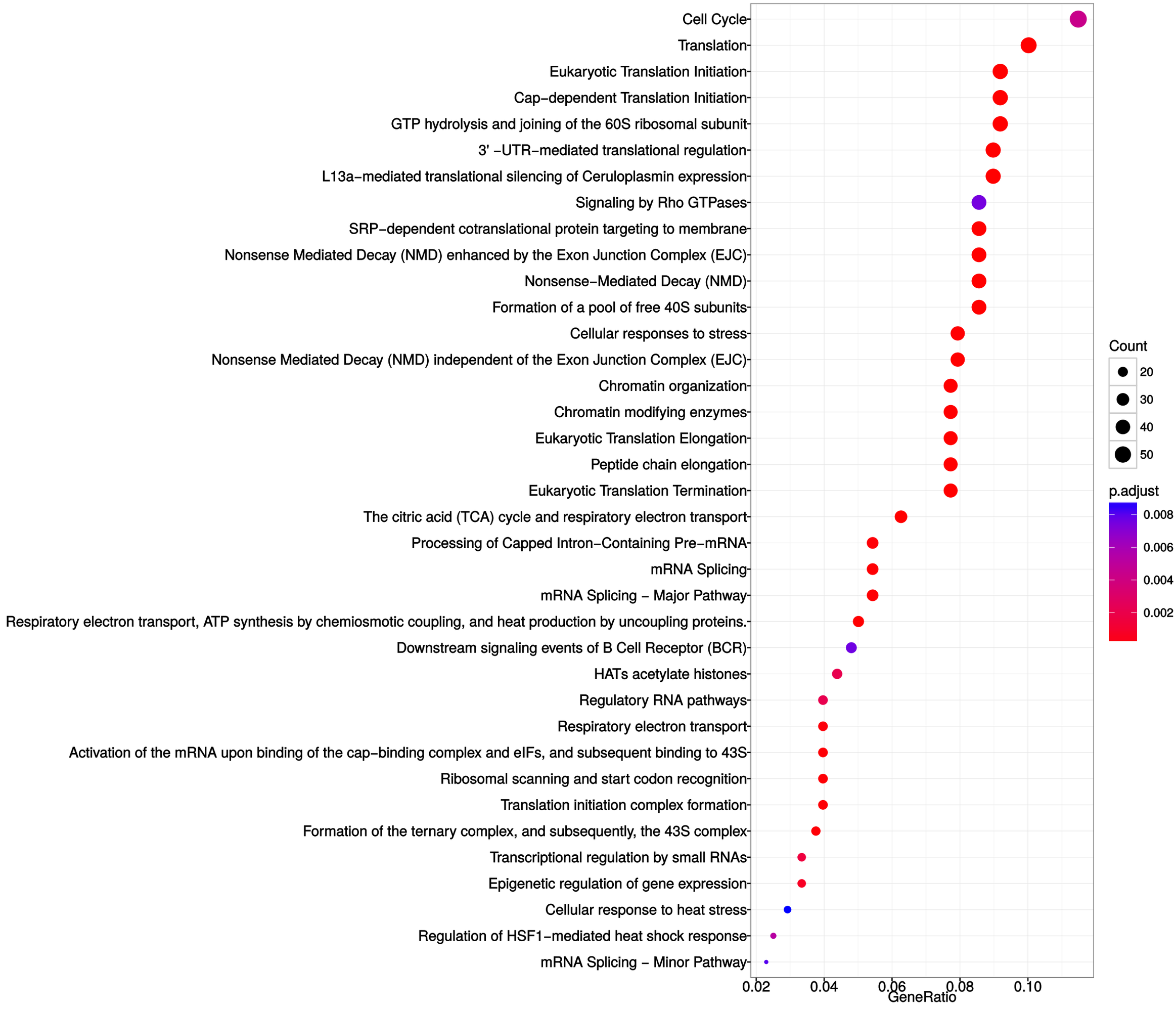
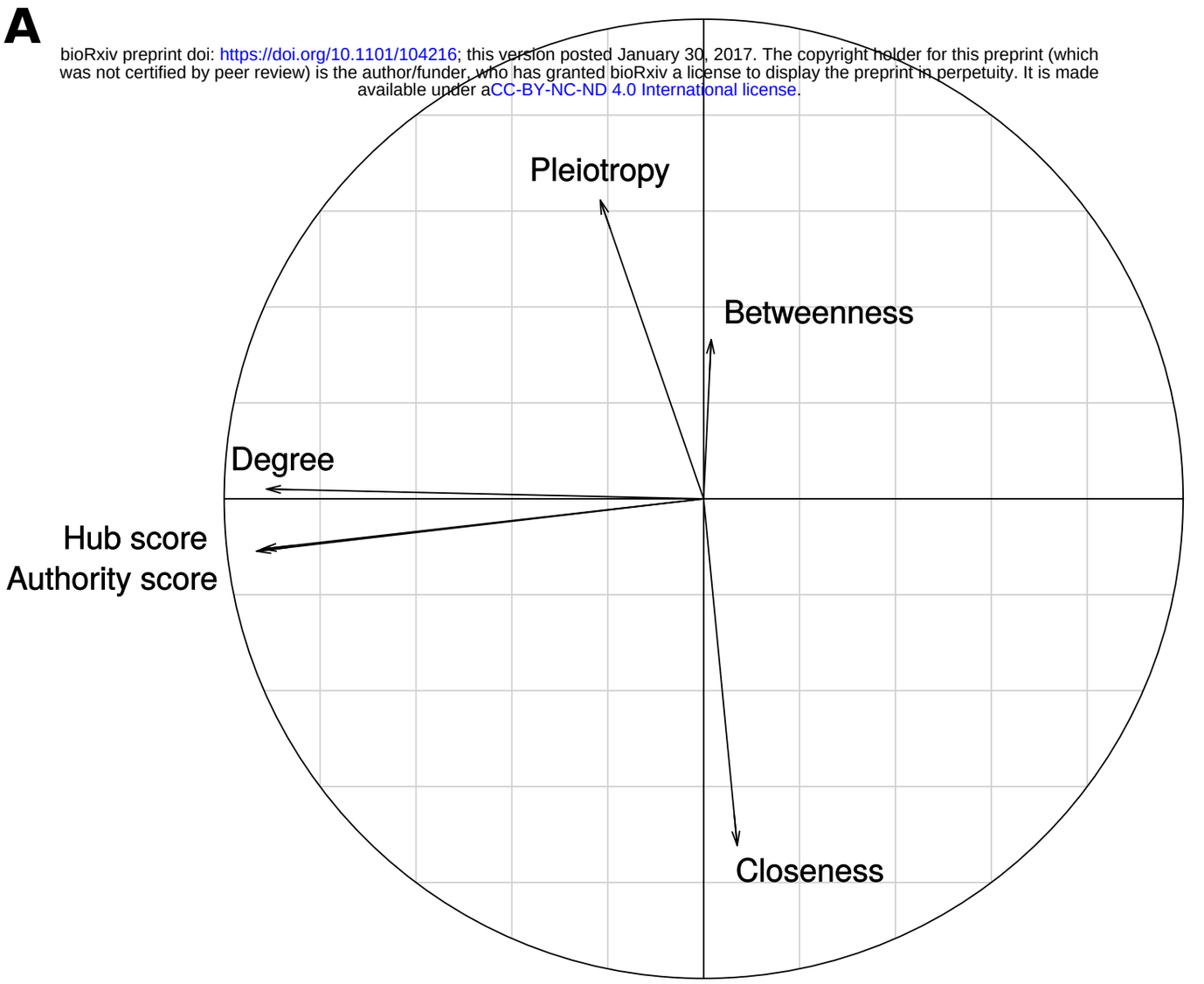


Figure 5



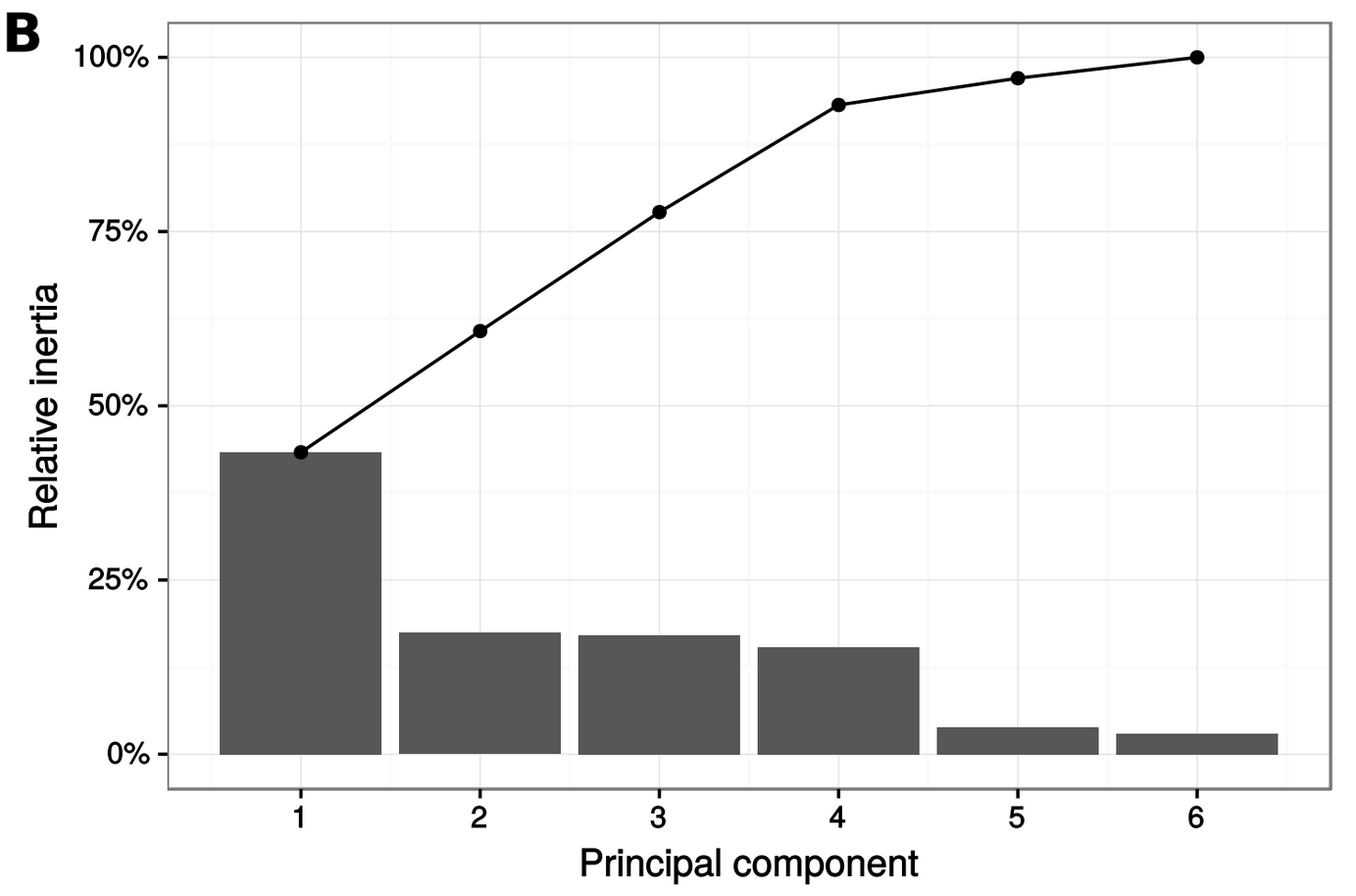


Figure 6

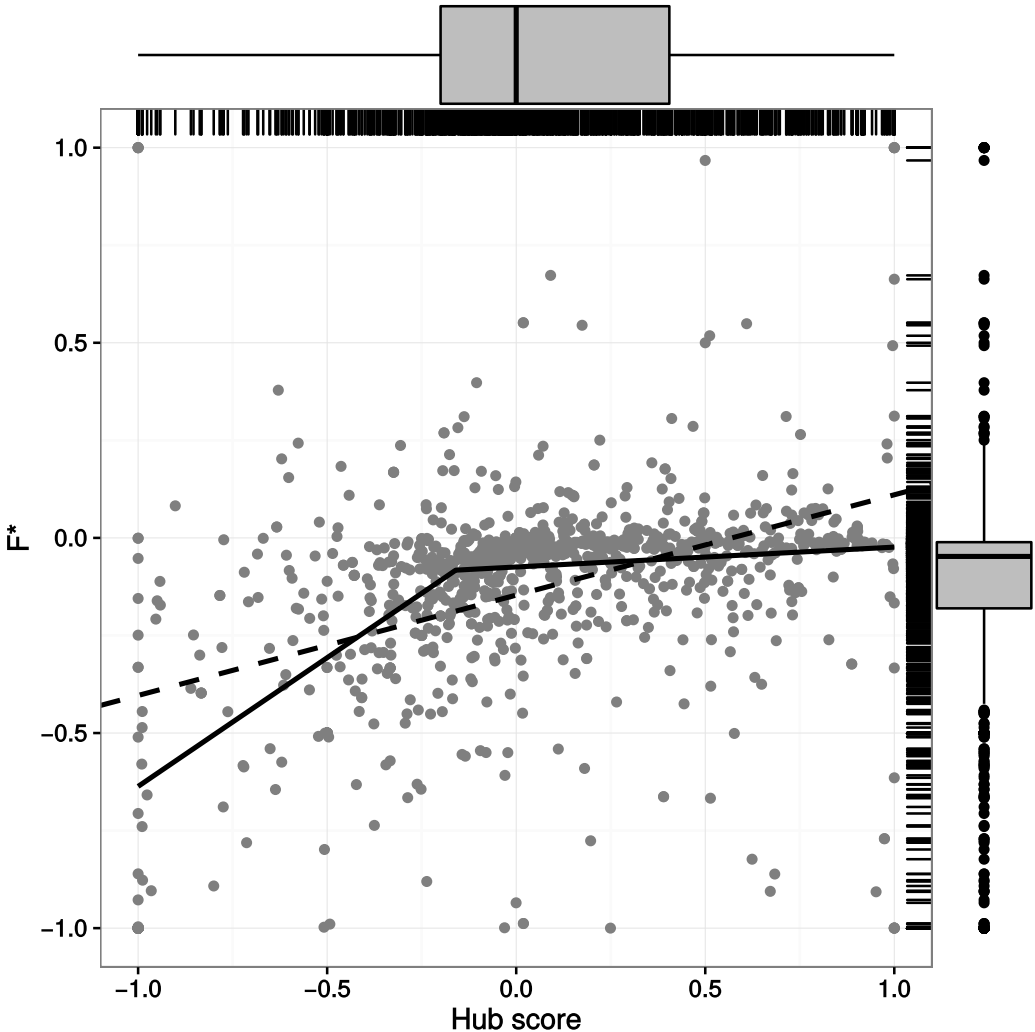
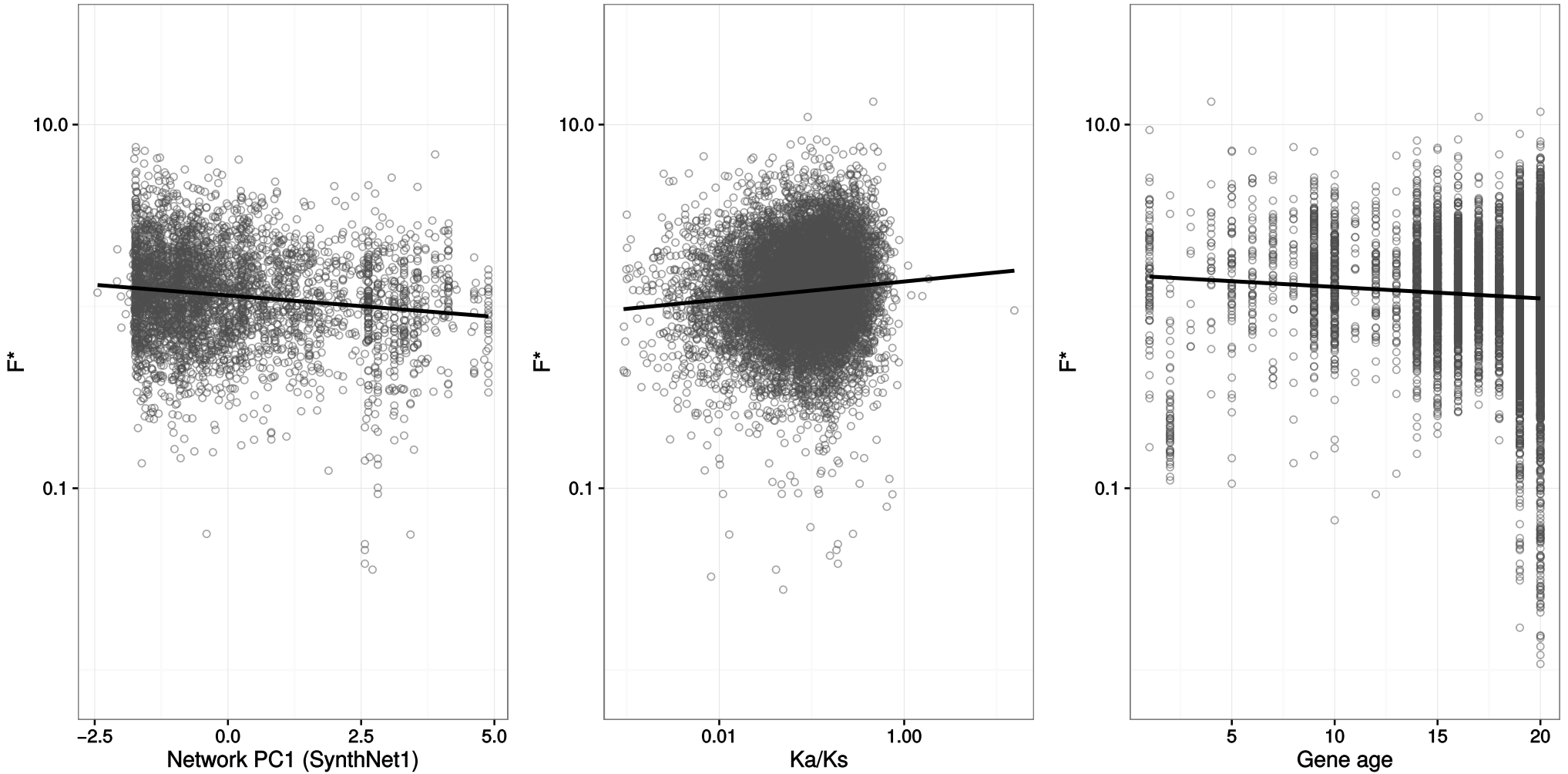


Figure 7


10.0
0.1
F*
−2.5
0.0
2.5
5.0
Network PC1 (SynthNet1)
10.0
0.1
F*
0.01
1.00
Ka/Ks
10.0
0.1
F*
5
10
15
20
Gene age