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Human Gene Expression Variability and its Dependence on Methylation and Aging

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Abstract

Background: Phenotypic variability of human populations is partly the result of gene polymorphism and differential gene expression. As such, understanding the molecular basis for diversity requires identifying genes with both high and low population expression variance and identifying the mechanisms underlying their expression control. Key issues remain unanswered with respect to expression variability in human populations. For example, the statistical nature of human expression variation has not been reported and the role of gene methylation is just beginning to be understood. Moreover, the contribution that age, sex and tissue-specific factors have on expression variability are not well understood.

Results: Here we used a novel analytic that accounts for sampling error to classify human genes based on their expression variability in normal human breast and brain tissues. We find that genes with high expression variability differ markedly between tissues, indicating that tissue-specific factors govern population expression variance. In addition, high expression variability is almost exclusively unimodal, indicating that variance is not the result of segregation into distinct expression states. Importantly, we find that genes with high population expression variability are likely to have age-, but not sex-dependent expression. Lastly, we find that methylation likely has a key role in controlling expression variability insofar as genes with low expression variability are likely to be non-methylated.

Conclusions: We conclude that gene expression variability in the human population is likely to be important in tissue development and identity, methylation, and in natural biological aging. The expression variability of a gene is an important functional characteristic of the gene itself. Therefore, the classification of a gene as one with Hyper-Variability or Hypo-Variability in a human population or in a specific tissue should be useful in the identification of important genes that functionally regulate development or disease.

Keywords: Expression variability, Tissue Specificity, Essentiality, Methylation, Aging

Background

Within the last decade, many studies have established that gene expression patterns vary between individuals, across tissue types [1], and within isogenic cells in a homogenous environment [2]. These differences in gene expression lead to phenotypic variability across a population. Differential gene expression gene expression is typically detected by analyzing expression data from a population of samples in two or more genetic or phenotypic states, for example a cancerous and non-cancerous sample or between two different individuals. Various differential gene expression algorithms, such as edgeR and DESeq, are then used to identify genes whose expression mean differs significantly between the states. While differential co-expression analyses have successfully been used to identify novel disease-related genes [3], the statistical methods used in these analyses consider gene expression variance within the sample population as a component of the statistical significance estimate. However, expression variability within populations has been emerging as an informative metric of cell state an informative metric of a phenotypic state, particularly as it relates to human disease [4, 5].

There are several sources of expression variability in a population. The first are polymorphisms that contribute, both genetically and epigenetically, to promoter activity, message stability and transcriptional control. Another source of gene expression variability is plasticity, whereby an organism adjusts gene expression to alter its phenotype in response to a changing environment [6]. However, gene expression patterns can also vary among genetically identical cells in a constant environment [7–10]. This is commonly described as “noise”.

Expression variability, whatever its source, is an evolvable trait subject to natural selection, whereby each genes have an optimal expression level and variance required for an organism's fitness and selection minimizes this variability [7, 10–14]. In this case, genes with low variability have been subjected to heavy selection pressure to minimize population expression variance. Conversely, high variability genes have been least selected for. Genes with high expression variability could be drivers of phenotypic diversity, as suggested by position association between expression noise and growth [15–18]. In this interpretation, genes with high variability allow for growth in fluctuating environments. Understanding the role of the gene expression variability patterns across human populations will therefore provide crucial insights into how genetic differences contribute to phenotypic diversity, susceptibility to disease [19, 20], differentiation of disease subtypes [5], embryonic development [21, 22], and alterations in gene network architecture [23].

In this analysis, we used a novel method to analyze global gene expression variability in non-diseased human breast, cerebellum, and frontal cortex tissues. Our method differs from other protocols in that we account for sampling error in our analysis as well as estimate expression variability independent of expression magnitude. In addition, we analyzed gene methylation in conjunction with expression variability. Our work suggests that expression variability is an important part of the development and aging process and that identifying genes with very high or very low expression variability is one way to identify physiologically and important genes.

Results

Estimating expression variability. We measured human gene expression variability (EV) [1] in post-mortem non-diseased cerebellum (n=465) and frontal cortex samples (n=455) and biopsied normal breast

tissues (n=144). We excluded probes corresponding to non-coding transcripts as well as those with missing probe coordinates, resulting in a list of 42,084 probes. To estimate a gene's EV independent of its expression magnitude, we modified the method initially described by Alemu et al [1]. First, we calculated the median absolute deviation (MAD) for each probe. Then we modelled the expected MAD for all genes as a function of median expression using a locally weighted polynomial regression (Fig. 1A, red line). The expected MAD regression curves for each tissue type exhibit a flat, negative parabolic shape where the lowest and highest expression probes represent the troughs of the curve. Variability in gene expression levels has previously been shown to decrease as expression approaches either extrema [7, 9, 24]. The EV for each probe was calculated as the difference between its bootstrapped MAD and the expected MAD at each median expression level (Fig. 1A). Positive EV values indicate that the gene has a greater expression variability than genes with the same expression magnitude mean. Conversely, negative EV values imply reduced population expression variability. We next plotted the probability density function of EV for each tissue (Fig. 1B). The EV distributions in all three tissue types exhibit large peaks around the zero mean and a long tail for positive EV probes. Breast tissue exhibited a larger shoulder of the negative EV probes compared to cerebellum and frontal cortex tissues. This is likely attributable to the lower number of breast samples (144 compared to 456 and 455 samples respectively).

We then confirmed the independence of EV on expression by modelling the relationship between the two variables using a linear regression (Fig 1C). Based on the poor adjusted R^2 values ($2x10^{-4}$, $8x10^{-4}$, and $5x10^{-3}$ for breast, cerebellum, and frontal cortex respectively) and the flat slopes, we conclude that there is no substantial correlation between EV and gene expression magnitude.

Next, we then classified each probe into three categories based on their EV. We used the term "Hyper-Variable" to describe probes whose EV was greater than $\tilde{x}_{EV} + 3 * MAD_{EV}$. Probes with an EV less than $\tilde{x}_{EV} - 3 * MAD_{EV}$ were deemed "Hypo-Variable". The remaining genes that fell within the range of

$\tilde{x}_{EV} \pm 3 * MAD_{EV}$ were considered "Non-Variable". We propose that these three distinct gene groups, categorized based on EV, correspond to distinct functional and phenotypic gene characteristics.

Figure 1. Expression variability (EV) in human breast, cerebellum, and frontal cortex tissue. *(A) Expected expression MAD for curve as a function of median probe expression (solid black line). (B) Probability density function of EV. The vertical black lines represent the EV classification ranges. (C) Expression variability as a function of median gene expression. Adjusted R^2 values for the linear regression model shown in red were 0.0002, 0.0008, and 0.005 for breast, cerebellum, and frontal cortex tissues respectively.*

Accounting for sampling error in EV classification. We were concerned that the classification of a gene into Hyper-, Hypo- and Non-Variable classes might be the result of sampling errors. To minimize this possibility and to increase the accuracy of our EV classification method, we divided each of our tissue samples into two equally sized probe subsets and repeated the EV analysis. This 50-50 split-retest procedure was repeated 100 times. Figure 2 shows the probability distribution of a concordant EV classification for each gene into Hyper-, Hypo- and Non-Variable class across the three subsets in each tissue type. Fig 2 demonstrates that classification of a gene as Hyper or Hypo-variable based on a single analysis of the population is problematic because of sampling bias. We see a substantial decrease in the number of genes in the Hyper- and Hypo-Variable gene sets after conducting our split-retest protocol(Fig. 2B). Thus, our split-retest method likely increases the robustness and accuracy of EV classification

Figure 2. Cross-Validation of EV Classifications. *(A) Probability distribution of gene EV classification accuracy between original distribution and 50-50 split retest replicates (n=100). (B) Number of genes in each EV gene set before and after split-retest protocol.*

Statistical nature of Hyper-variability. A previously unexplored aspect of expression Hyper-variability is the statistical characteristics of expression amongst genes with this wide range of gene expression. Specifically, high EV could be the result of a multimodal distribution of gene expression with two or more distinct expression means or might simply result from a broadening of expression values around a unimodal mean value. In order to distinguish between the two possibilities, we modeled each gene expression as a mixture of two Gaussian distributions (Fig. 3). Next, we identified the peaks of the probability density function for each Gaussian distribution and compared the distance between the peaks as well as the ratio of peak heights. Probes with peaks that were greater than one median absolute deviation apart and displayed a peak ratio greater than 0.1 were classified as having a bimodal expression distribution. Probes that did not satisfy both criteria were considered to have a unimodal distribution. Only a small minority of the Hyper-Variable (high EV) probes (15/3453 breast tissue probes, 6/2980 cerebellum probes, and 6/3487 frontal cortex probes) showed a bimodal distribution of gene expression. The remaining majority of Hyper-Variable probes had a unimodal distribution. This indicates that high expression variability is a result of a widening of possible expression values across a single mean rather than the gene expression existing in two or more discrete states.

Figure 3. Bimodal Hyper-Variable gene expression detection*. Gaussian mixture modelling method of detecting bimodal probes. The dashed lines represent the overall gene probability density function of gene expression. The two Gaussian models are shown in dark grey and light grey, and the dotted vertical lines represent the distribution means.*

Tissue-specificity of EV. Because we have calculated EV from different tissues, we were able to determine the extent to which tissue-specific factors might contribute to EV. This is an important question because expression variability exists not only between individuals but between different tissues in the same

organism. Characterizing tissue-specific EV will therefore shed light on tissue specification and identity processes. As shown in Fig. 4A, there was limited overlap between the Hyper-Variable, Hypo-Variable, and Non-Variable genes between the three different tissues. 13-16% of the Hyper-Variable genes were classified as such in the three tissues and 18-26% of the Hypo-Variable were so classified. The Non-Variable probe sets contained over 82% of genes in each tissue type, with over 71% of the measured genes commonly classified as NV in all three tissue types. The poorly overlapping nature of each EV classification suggests that population expression variability is largely determined by tissue-specific pathways.

Figure 4. Tissue Specificity of EV. *(A) Venn diagrams comparing EV classifications between breast, cerebellum, and frontal cortex tissues. (B) Effect of genomic position on EV. Each chromosome is divided into 100 bins (x-axis) based on the maximum gene coordinate annotation, and the average EV in each bin is measured (y-axis).*

EV and gene structural characteristics. To understand possible genomic mechanisms by which population expression variability occurs, we first explored the relationship between EV and various structural features of the genes. Expression variability has previously been reported to be associated with gene size, gene structure, and surrounding regulatory elements [1]. However, we found no significant linear correlation between EV and a gene's exon count, sequence length, transcript size, or number of isoforms (Additional file 1). While certain linear models exhibited statistical significance ($p < 0.05$), the fit of the model and subsequent comparison of the linear model against a local polynomial regression curve showed that the correlation was either not correctly defined by a linear model or simply too small to draw a conclusion.

While we did not find that the physical gene characteristics were correlated to EV, previous studies have shown that the position of a gene on a chromosome has considerable effects on stochastic gene expression variability, independent of gene- and promoter-specific variables [25]. We next tested if

there is a relationship between expression variability and chromosomal position (Fig. 4B). To this end, each chromosome was divided into 100 bins and the average EV all the genes within each bin was determined. If there was no relationship between the two, we would expect to see EV to be uniformly distributed throughout the genome. We found that EV is not uniformly distributed across the genome, and individual regions of chromosomes exhibited peaks of high expression variability or troughs of low expression variability. To further confirm our conclusion, we tested the cosine similarities of the chromosomes within and across the tissue types (Additional file 2). The EV distributions across chromosomes exhibited low similarities within each tissue type, further establishing that EV is not randomly distributed throughout the genome.

Essentiality enrichment in variable genes. Previous studies in yeast have shown that gene expression variability is reduced in genes that are essential for survival. It is believed that evolution has selected for transcriptional networks that limit stochastic expression variation of essential genes [13]. If this were true for humans, we would expect a significant number of essential genes to exhibit Hypo-Variable expression. Conversely, we expect a depletion of essential genes within the Hyper-Variable probe sets.

Table 1. Pearson's Chi-squared test for Essentiality in Hyper-Variable, Hypo-Variable, and Non-Variable probe sets.

Tissue	Probe Set	Total Gene Count	Essential Gene Counts	Standardized Residuals	P-Value
Breast	Hyper	1448	180	12.22	1.50×10^{-34}
	Hypo	957	108	8.27	
	NV	33957	1657	-14.94	
Cerebellum	Hyper	1640	170	8.66	8.27×10^{-63}
	Hypo	837	83	5.54	
	NV	35257	1849	-10.42	
Frontal Cortex	Hyper	1760	196	10.6	1.52×10^{-92}
	Hypo	1254	125	7.04	
	NV	34831	1764	-12.89	

In order to examine a potential correlation between expression variability and essentiality in human tissues, we first tested the independence between EV classification and annotation of human essentiality (Table 2). Essentiality annotations were obtained from the CCDS [26] and MGD [27] databases. Here, direct human orthologs of genes essential for prenatal, perinatal, or postnatal survival of mice were classified as essential. Using the Pearson's chi-square test for the number of essential genes in each probe set, we find that that the Hypo-Variable gene set in breast, cerebellum, and frontal cortex tissues were significantly enriched for genes with essentiality annotation (p-value = 1.50×10^{-34}, 8.27×10^{-63}, and 1.52×10^{-92}, respectively). Thus, expression variability for many essential genes is constrained in humans, likely reflecting a similar biology to essential yeast genes. However, we also observe a significant enrichment of essential genes within the Hyper-Variable gene sets. This was a surprise to us since essential genes are thought of as being dose-sensitive and changes in the level of gene expression would be predicted to be deleterious or lethal.

To better understand the implications of high variability in essential genes, we examined the functional annotations associated with Hyper-Variable essential genes (Table 3 and Additional file 4). The breast essential Hyper-Variable gene set was enriched for embryonic development, responses to growth factors, cell-substrate junction assembly, regulation of epithelial cell proliferation, and positive regulation of cellular component movement. The cerebellum essential Hyper-Variable gene set was enriched for cell differentiation, anion transport, trans-synaptic signaling, response to growth factors, and cell projection organization. Lastly, the frontal cortex essential Hyper-Variable gene set was enriched for cell differentiation, secretion, tyrosine kinase signaling, cell projection organization, and heart contractions. Overall, the Hyper-Variable essential gene sets tended to be enriched for morphogenic, tissue, and organ system development. This suggests that tight regulation of some essential genes may only be required for embryonic and morphogenic development and dose-sensitivity is lost in adults, allowing for high expression variability.

Table 2. Top 5 common and unique REVIGO GO annotation subsets of Hyper-Variable and Hypo-Variable essential genes in breast, cerebellum, and frontal cortex tissues.

	Unique Breast Annotations	Unique Cerebellum Annotations	Unique Frontal Cortex Annotations
Hyper-Variable Essential Genes	Embryo development ending in birth or egg hatching	Positive regulation of cell differentiation	Positive regulation of cell differentiation
	Cellular response to growth factor stimulus	Anion transport	Regulation of secretion
	Cell-substrate junction assembly	Trans-synaptic signalling	Transmembrane receptor protein tyrosine kinase signaling pathway
	Regulation of epithelial cell proliferation	Cellular response to growth factor stimulus	Regulation of cell projection organization
	Positive regulation of cellular component movement	Regulation of cell projection organization	Heart contraction
Hypo-Variable Essential Genes	Negative regulation of cellular component organization	DNA repair	DNA repair
	DNA repair	Negative regulation of cellular component organization	Covalent chromatic modification
	Regulation of cellular protein localization	Positive regulation of viral release from host cell	mRNA transport
	Embyro development ending in birth or egg hatching	Regulation of cellular protein localization	Progesterone receptor signaling pathway
	Apoptotic process	Regulation of cell cycle process	Regulation of cell cycle process

Functional analysis of Hyper-, Hypo- and Non-Variable genes. In order to understand the overall biological significance of gene EV, we examined the functional aspects that are enriched in the Hyper-Variable, Hypo-Variable, and Non-Variable probe sets by conducting a gene set enrichment analysis for each probe set group. We determined the over-represented Gene Ontology (GO) terms that were unique in each tissue type, as well as GO terms that were common in all three tissue types. The resulting GO annotations were simplified and visualized using a REVIGO treemap. The top five terms for each tissue type can be found in Table 1, while the complete list of GO term treemaps can be found in Additional file 3.

Table 3. Top 5 common and unique REVIGO GO annotations in the Hyper-Variable and Hypo-Variable gene sets of breast, cerebellum, and frontal cortex tissues.

	Common Annotations	Unique Breast Annotations	Unique Cerebellum Annotations	Unique Frontal Cortex Annotations
Hyper-Variable	Regulation of bone remodeling	Epithelial cell differentiation	Regulation of nervous system development	Histamine secretion
	Regulation of inflammatory response	Primary alcohol metabolism	Regulation of transmembrane transport	Regulation of cell morphogenesis
	Response to zinc ion	Positive regulation of cellular component movement	Regulation of neuron death	Trans-synaptic signaling
	Carboxylic acid biosynthesis	Response to corticosteroid	Negative regulation of response to external stimulus	Regulation of neurological system process
	Regulation of ion transport	Transmembrane receptor protein tyrosine kinase signaling pathway	Response to calcium ion	Dephosphorylation
Hypo-Variable	Proteolysis involved in cellular protein catabolism	Golgi vesicle transport	DNA conformation change	ncRNA metabolism
	Ribonucleoprotein complex assembly	Nucleoside monophosphate metabolism	Modification-dependent macromolecule catabolism	Response to interleukin-1
	Regulation of cellular amino acid metabolism	Proteolysis involved in cellular protein catabolism	Response to camptothecin	Regulation of enter of bacterium into host cell
	Innate immune response activating cell surface receptor signaling pathway	Cellular response to nitrogen starvation	Retrograde transport, endosome to Golgi	
	Negative regulation of autophagy	Mitochondrial respiratory chain complex I assembly	Regulation of ubiquitin-protein transferase activity	

The breast Hyper-Variable gene set was uniquely enriched for epithelial cell differentiation, primary alcohol metabolism, and positive regulation of cellular component movement. The cerebellum Hyper-Variable gene set was uniquely enriched for regulation of nervous system development, transmembrane transport, and neuron death. The frontal cortex Hyper-Variable gene set was enriched for histamine secretion, regulation of cell morphogenesis, and trans-synaptic signalling. The breast, cerebellum, and frontal cortex Hyper-Variable gene sets were commonly enriched for regulation of tissue remodeling, inflammatory responses, and responses to inorganic substances. Of note, many of the enriched GO annotations of the Hyper-Variable genes are involved in signalling pathways. These pathways

require dynamic control based on internal and external stimuli and their high EV likely represents differing environmental or hormonal conditions amongst the individuals.

In the case of the Hypo-Variable gene sets, all three tissue types were enriched for protein catabolism and metabolism, ribonucleoprotein complexes, and negative regulation of autophagy. The breast Hypo-Variable gene set was enriched for Golgi vesicle transport, nucleoside metabolism, and protein catabolism. The cerebellum Hypo-Variable gene set was enriched for DNA conformation change, modification-dependent macromolecule catabolism, and retrograde transport.

Genes with higher expression variability have previously been shown to be functionally and physically involved with the physical cell periphery, localizing in the membrane, transmembrane, or extracellular matrix regions [23]. Our results corroborated these findings as the Hyper-Variable gene sets from breast, cerebellum, and frontal cortex were enriched for GO annotations associated with cell surface signalling pathways, as well as cellular component ontologies enriched at the plasma membrane. In contrast, genes with low variability genes tended to regulate nucleic acid and metabolic pathways, localizing in the cell interior. These Hypo-Variable genes are likely involved in complex, dose-sensitive gene networks and require tight regulation of their expression to function correctly.

DNA methylation and expression variability. One factor that has been postulated to regulate EV is DNA methylation. While the relationship between methylation and gene expression is complex, low promoter methylation is associated with high levels of gene expression [28–31]. Like gene expression, DNA methylation is highly variable at the cell, tissue, and individual level [32], suggesting that EV could result from variations in gene methylation. To explore this idea, we used DNA methylation annotations that were available in 724 out of 911 brain tissue samples.

DNA methylation in CpG sites is thought to be bimodal, meaning that the gene is either hypomethylated or hypermethylated [31]. In order to differentiate between low, medium, and high

methylation states in our samples, we modelled gene methylation using Gaussian mixture models for the mean methylation for each gene. The distribution of gene methylation in both cerebellum and frontal cortex tissue was best modelled as a three-component system. The first component was a sub-population Gaussian mixture while the second and third components were modelled as single Gaussian distributions. Genes whose methylation fell within the first component were classified as Non-Methylated genes. Genes were classified as Medium Methylated for those in the second component and Highly Methylated if they were in third. The distribution of methylation amongst the genes is predominantly bimodal with only a minority of genes being Medium Methylated (Fig. 4A). In contrast, over 62% of cerebellum genes are non-methylated and 23% highly methylated. Similarly, 58% of frontal cortex genes are non-methylated and 22% are highly methylated).

Next, we explored the correlation between methylation and expression based on the EV. When we subset the methylation distribution by EV classification (Fig. 4B), we observe that Hypo-Variable genes have a visibly different methylation pattern than Hyper- or Non-Variable genes insofar as Hypo-Variable genes are visibly overrepresented in the Non-Methylated gene group compared to both the Hyper-Variable and Non-Variable genes.

Figure 5. Methylation in human cerebellum and frontal cortex tissue. *(A) Probability density function of average gene methylation. Gaussian mixture models were used to classify the genes into Non-, Medium- and Highly- methylated clusters. (B) Probability density function of average gene methylation by EV classification. The dashed vertical lines represent the methylation state cluster cut-offs generated by the Gaussian mixture modelling. The y-axis is scaled by the square root of the methylation density.*

To further quantify the overrepresentation of Hypo-Variable genes in the Non-Methylated gene group, we conducted a chi-squared test of independence between the methylation state clusters and the EV classifications (Table 4). Both the cerebellum and frontal cortex tissues exhibited a significant relationship between the methylations clusters and EV classifications ($p = 7.57 \times 10^{-36}$ and $p = 1.58 \times 10^{-59}$, respectively). By examining the standardized residuals of the chi-square test of independence, we quantitatively confirmed the enrichment of Non-Methylated genes within the Hypo-Variable gene set. We also observe a significant enrichment of Highly Methylated genes in the Non-Variable gene set as well as an enrichment of Medium Methylated genes in the Hyper-Variable gene set. The high significance and non-overlapping enrichments across each of the three groups suggests that there is a strong relationship between methylation and EV classifications: low gene methylation is important for the tight expression constraint in Hypo-Variable genes while high gene methylation contributes to Non-variable expression.

Table 4. Chi-Squared Test Standardized Residuals*. We tested the independence between the methylation state clusters and the EV classifications in cerebellum and frontal cortex tissues and found a significant relationship between the two variables ($p = 7.57 \times 10^{-36}$ and $p = 1.58 \times 10^{-59}$, respectively).*

	Cerebellum Tissue			Frontal Cortex Tissue		
	Non-Methylated	Medium Methylated	Highly Methylated	Non-Methylated	Medium Methylated	Highly Methylated
Hypo-Variable	11.98	-5.69	-9.04	14.84	-7.11	-10.79
Non-Variable	-7.52	0.06	8.59	-10.00	-0.04	11.73
Hyper-Variable	0.07	4.21	-3.58	-0.23	6.23	-5.47

Effects of age, sex, and PMI on variability. To further understand the biological relevance of EV, we focused on the Hyper-Variable genes to identify potential mechanisms of decreased constraint on gene expression across the samples. We systematically analyzed EV as a function of sex, age, and post-mortem interval (PMI). The breast tissue lacked these clinical annotations and were excluded from this analysis. We employed a probe-wise linear regression analysis to model the relationship between Hyper-Variable

gene expression and age, sex, and PMI (Table 5). The resulting p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure and considered significant when the adjusted p-value was less than 0.01.

Table 5. Probe-Wise Multiple Linear Regression of PMI, Sex, and Age. Probes that exhibit an FDR < 0.01 are considered significant for the specific coefficient.

	PMI			Sex			Age		
	Up	Down	Total	Up	Down	Total	Up	Down	Total
Cerebellum	12	10	22	2	0	2	247	267	514
Frontal	8	15	23	7	9	16	373	354	727

PMI might be a source of apparent expression variability because an extended PMI might compromise sample RNA integrity and lead to degradation of labile RNA [33]. Brain samples had PMI times ranging from 1 hour to 94 hours (mean = 36.14 hr), but we observe a negligible number of probes that are correlated with PMI. This suggests that sample integrity is unlikely to be a source of EV changes. Somewhat more surprisingly, however, is the low number of probes that are correlated with sex. Only 2 out of 1640 Hyper-Variable cerebellum genes and 16 out of 1760 Hyper-Variable frontal cortex genes show sex-dependent differences in EV. While other studies have shown widespread sex differences in post-mortem adult brain gene expression [34], we conclude that in our analysis, EV is overwhelmingly sex-independent.

However, we observe that age has a substantial effect on gene expression variability. Age is correlated with over 31% of Hyper-Variable cerebellum probes and over 41% of Hyper-Variable frontal cortex probes. This means that the expression of these genes becomes either more or less constrained during aging. In the cerebellum, there were 247 Hyper-Variable genes whose expression increased as a function of age and 267 genes with decreased expression. Similarly, the frontal cortex contained 373 genes with increased expression and 354 genes with reduced expression. Given that age is correlated with

a considerable number of Hyper-Variable genes, we classified the age of the samples in the cerebellum and frontal cortex tissues into three age clusters according to BIC for expectation-maximization (EM) initialized by hierarchical clustering for parameterized Gaussian mixture models. The oldest cluster contained samples whose ages were between 58 and 98 ($\bar{x}_1 = 79$). The second cluster ranged between 32 and 57 years ($\bar{x}_2 = 45$), while the youngest age cluster contained samples aged 1 through 31 ($\bar{x}_3 = 17$).

To further explore this effect, we examined the age-dependent changes in expression of the Hyper-Variable probes across the three clusters. In each tissue type, we labeled genes whose expression was positively correlated with age as "Upregulated", while the negatively correlated genes were termed "Downregulated". Then, we used a hierarchical clustering method with an expression heatmap to visualize how these upregulated and downregulated genes are expressed throughout the age clusters (Fig. 6). The resulting gene hierarchical trees were clustered into groups via manual tree cutting. The complete list of GO term treemaps for significant gene clusters can be found in Additional file 5.

While the cerebellum is generally considered a regulator of motor processes, it is also implicated in cognitive and non-motor functions [35]. Many of these age-dependent upregulated Hyper-Variable genes corroborate previous studies exploring the relationship between brain aging and changes in gene expression, including cellular responses to chemical stimuli (gold cluster). In particular, reactive oxygen and nitrogen species have been shown to change ion transport channel activity, and serve as an important mechanism in brain aging [36]. While all the genes selected were age-regulated, some genes exhibit outlier samples whose expression remains high across all genes in the dark orange cluster, regardless of age. These genes are more likely to be overexpressed in the samples as age increases and are enriched for peripheral nervous system neuron development and neuron apoptotic pathways. Similar enrichments of neurogenic and chemical stimuli response pathways are seen in the upregulated frontal cortex genes (gold cluster). The dark orange cluster in the upregulated frontal cortex age-dependent genes exhibits an

entirely sample-specific over- or under-expression of genes. These bimodally expressed genes are enriched for glial cell differentiation, adenosine receptor signaling pathways, and antigen processing. Lastly, we see a random scattering of expression in the yellow cluster of the frontal cortex heatmap that steadily increases with age. These genes are enriched for glial cell differentiation, cellular response to alcohol, and defense responses to fungus.

Figure 6. Hierarchical clustering of Hyper-Variable genes by age in (A) cerebellum tissue, and (B) frontal cortex tissue. *The vertical axis represents the age-regulated Hyper-Variable genes while the samples were clustered by age and plotted on the horizontal axis. The top heatmaps represent the positively correlated age-regulated genes while the bottom heatmaps represent the negatively correlated age-regulated genes. The age clusters decrease in age from left to right in both heatmaps and correspond to the following age ranges:* $\bar{x}_1 = 79\ [58,98]$*,* $\bar{x}_2 = 45\ [32,57]$*, and* $\bar{x}_3 = 17\ [1,31]$*.*

Most of the downregulated age-dependent Hyper-variable genes in the cerebellum fall into the green cluster where expression of the genes in the cluster increases with age. These genes are involved in leukocyte-mediated immunity and defense responses to other organisms, which is supported by previous studies [37]. Interestingly, the yellow cluster exhibits U-shaped expression levels, whereby the lowest expression is seen in the middle age cluster. These genes are enriched for optic nerve development, response to interferon-gamma, and synaptic signalling. In the frontal cortex, the majority of downregulated age-dependent genes fall in the red cluster, and are enriched for ion transport, cell morphogenesis, and trans-synaptic signalling. Overall, the functional annotations of the age-regulated

Hyper-Variable gene clusters suggest that population EV is one outcome of age-dependent gene expression changes.

We next investigated a possible impact of methylation changes on gene expression in the Up- and Down-regulated Hyper-Variable genes. Fig. 7 shows the histogram distribution of correlation between the sample-specific gene expression and gene methylation. We observe no strong correlation between expression and methylation, suggesting age-dependent changes in expression of the age-regulated Hyper-Variable genes are not the result of methylation changes.

Figure 7. Expression and methylation correlation plot. *Histogram of Pearson correlation coefficient between paired gene expression and gene methylation levels in Hyper-Variable and Hypo-Variable genes.*

Discussion

Gene expression variability in a population is the cumulative result of intrinsic genetic factors, extrinsic environmental factors, and stochastic noise. A fundamental issue in biology is understanding the cause of expression variability within an individual organism and between isogenic and genetically dissimilar individuals of a population. In this report, we study population gene expression variability in human breast, cerebellum, and frontal cortex tissues.

Our investigation into human gene expression variability yielded several main findings. First, we find that Hyper-Variability in gene expression is fundamentally unimodal and does not represent population switching between two or more discrete expression stages. In addition, both Hypo-Variable (highly constrained expression) and Hyper-Variable (lowly constrained expression) gene sets are enriched for essential genes and that both Hyper- and Hypo-variability are largely regulated by tissue specific

factors. We also find that gene methylation has an important role in expression variability. Lastly, we find that Hypervariability is primarily associated with age and not sex.

Our observation that Hyper-Variable genes have a unimodal expression pattern is significant because population gene expression diversity could manifest itself in multimodal or unimodal distributions. Unimodal distributions represent a continuum of expression levels in a population while multimodal distributions represent two or more distinct expression states between which cells might switch between [39]. Since Hyper-Variability is almost exclusively unimodal, high EV in both brain and breast is the result of a wide range of permissible expression levels in a population rather than the existence of discrete expression subpopulations. In the case of organismal phenotypes, the benefits of a unimodal phenotype distribution is highest in environments with very rapid or very noisy changes [40]. The unimodal distribution of Hyper-Variability indicates that similar evolutionary constraints apply to gene expression.

The enrichment of essential genes in the Hypo-Variable gene sets is in agreement with previous findings in yeast showing that essential yeast genes are likely to have low expression variability. However, we detected a significant number of essential genes amongst the Hyper-Variable genes in breast, cerebellum, and frontal cortex tissue. Inactivation of these essential genes leads to pre- or neonatal fatality in mice and humans [41] and functional enrichment analysis indicates that the essential genes are indeed involved in developmental pathways. This was a surprise to us since we expected that expression of developmental genes should be tightly regulated, yet we observe highly variable expression being tolerated in obligate developmental pathways. It is possible that these “essential” genes are required for embryonic development but have different post-embryonic roles and may not be essential post-natally. Alternatively, it is possible that these essential genes are not dose-sensitive in humans, meaning that only a certain level of baseline expression is required and expression above this baseline might be well tolerated. One additional possibility is that their protein abundance could be regulated translationally

rather than transcriptionally. Inefficient translation of certain genes may have been selected for during evolution to prevent fluctuations in protein concentrations[32]. Perhaps a combination of these factors is at play. Regardless, tight regulation of some embryonically essential pathways is not required in fully developed adults, allowing for a wider range of expression values within the human population, resulting in high expression variability.

The non-random distribution of Hyper-Variable and Hypo-Variable genes across the genome suggests that EV is dependent on epigenetic factors. Examining the methylation status of the genes allowed us to determine the relationship between gene methylation and expression variability. Firstly, we find that Non-Variable genes in the cerebellum and frontal cortex are likely to have high gene methylation. Secondly, we find that Hypo-Variable genes are likely to be non-methylated. We propose a model for methylation-dependent expression variability where the highly constrained levels of Hypo-Variable gene expression require non-methylated genes. We speculate that the lack of methylation allows transcriptional regulators requiring non-methylated DNA for binding to tightly control gene expression. On the other hand, high gene methylation reduces transcription noise and epigenetically inhibits promoter variability in human populations. Future studies should investigate the role that these putative regulators of expression play on EV, including cis-regulatory elements and transcription factors (TF).

We find that there is limited overlap in gene identity between Hyper- and Hypo-Variable genes in breast and brain tissue. This suggests that expression variability is controlled by tissue-specific factors. We propose that tissue identity is created and preserved, at least in part, by changes in gene expression control pathways. Thus, genes that are Hypo-Variable in any given tissue have a constrained expression pattern because they are likely to be important in the tissue-specific function and physiology of that organelle. While there is limited overlap of genes within the corresponding EV gene sets of different tissues, the Hyper-Variable gene sets of the different tissues have similar functional enrichments and cellular protein localizations. Specifically, proteins encoded by Hyper-Variable genes tend to localize at

the cell periphery and are enriched for cell surface signalling pathways and tissue development, including tissue remodeling and ion transport. We therefore propose that high expression variability in genes associated with tissue development pathways is an important component of tissue identity.

We did not observe any substantial sex dependent effects in expression variability. However, an important conclusion of our study is that many Hyper-Variable genes have age-dependent expression variability: that is, their expression increases or decreases during aging. One main cause of accelerated brain aging and a causal factor of neurodegeneration is a reduction in immunological functions [42, 43]. We see evidence of downregulated immune responses in the cerebellum, specifically leukocyte mediated immunity, defense responses to other organisms, and interferon-gamma response pathways. Many studies also suggest that aging is associated with the upregulation of inflammatory responses [44], which is a pathogenic mechanism implicated in many age-related diseases, including cardiovascular disease, Alzheimer's disease, and Parkinson's disease [45]. Consistent with this idea, we see an enrichment of acute inflammatory response in the cerebellum gold cluster. Another mechanism that has been implicated with age-related diseases, such as Alzheimer's disease and Parkinson's disease, is synaptic dysfunction that can affect neuroendocrine signaling [46–48]. We see a downregulation of ion transport and trans-synaptic signaling in the frontal cortex, which are key components of neurotransmission and membrane excitability, and whose downregulation likely causes deficiencies in these complex processes. Furthermore, we see an upregulation of genes associated with glial cell differentiation in the frontal cortex across multiple gene clusters. Initially thought of as cells that merely support neurons, emerging research shows that neuron-astrocyte-microglia interactions are crucial for the functional organization of the brain [49]. In addition, genes specific to astrocytes and oligodendrocytes, two different types of glial cells, have been shown to shift regional expression patterns upon aging, and are better predictors of biological age than neuronal-specific genes [50]. This suggests that the Hyper-Variability and age-dependent

upregulation of genes associated with glial cell differentiation is caused by differences in normal brain aging between the samples.

Without examining the mechanistic control of individuals genes, it is difficult to determine if changes in gene expression result in repression or activation of their associated pathways. For example, we see an upregulation in neurogenesis associated genes during aging in both the cerebellum and the frontal cortex, despite the common theory that neurodegeneration is a ubiquitous effect of normal brain aging. An emerging concept in neuroscience is that homeostatic plasticity of neurons is maintained through local adjustments of neural activities [51]. This overexpression of genes in pathways whose function is known to decline over time may be a compensatory mechanism for an inefficient, aging system. Within the cerebellum, a decline in neuronal function that occurs with aging may cause an upregulation of genes associated with neurogenesis pathways. In addition to mitigating neuronal dysfunction, localized increases in neurogenesis may be induced in response to cerebral diseases or acute injuries for self-repair [52]. Lastly, chronic antidepressant usage has also been shown to result in an increase in neurogenesis [53], suggesting that psychopharmaceuticals can alter neurochemistry and mimic compensatory anti-aging responses. Overall, EV plays an important role in aging, specifically in immune responses and inflammation, neurotransmission, and neurogenesis. Age-dependent gene expression could reflect a loss of regulatory control or be a part of a regulated pathway of development.

In summary, our work shows that gene expression variability in the human population is likely to be important in development, methylation, and in aging. As such, the EV of a gene is an important functional characteristic of the gene itself. Therefore, the classification of a gene as one with Hypervariability or Hypovariability in a human population or in a specific tissue should be useful in the identification of important genes that functionally regulate development or disease.

Methods

Illumina gene expression and methylation microarray data. The analysis was conducted on two separate datasets, both utilizing the Illumina HumanHT-12 V3.0 expression BeadChip. The first dataset provides high quality RNA-derived transcriptional profiling of breast-adjacent tissue from 144 samples***. The associated genotype and expression data have been deposited at the European Genome-Phenome Archive (EGA, http://www.ebi.ac.uk/ega/), which is hosted by the European Bioinformatics Institute, under accession number EGAS00000000083. The microarray readings were preprocessed using the author's own custom script based on existing functionality within the beadarray package in R and were reported as a log2 intensity. This dataset is referred to as breast tissue.

The second gene expression and the methylation datasets were catalogued by the North American Brain Expression Consortium and UK Human Brain Expression Database (UKBEC) [34, 54]. The expression data was obtained from the Gene Expression Omnibus (GEO) database [55] under accession number GSE36192. A total of 911 tissue samples were analyzed from frozen brain tissue from the cerebellum and frontal cortex from 396 subjects. The microarray readings were processed using a cubic spline normalization method in Illumina Genome Studio Gene Expression Module v3.2.7. The expression levels were log2 transformed before any analysis. The methylation data was also obtained from GEO under accession number GSE36194. A total of 724 tissue samples were analyzed from frozen brain tissue from the cerebellum and frontal cortex from 318 subjects. The methylation microarray readings were processed using BeadStudio Methylation Module v3.2.0 with no normalization.

Preprocessing the datasets. Since the brain expression and methylation datasets were individually processed by different tissue banks and in several batches, we corrected for the batch effect using the limma package in R. The breast tissue dataset was previously batch corrected by the authors. Next, we

subset the data into 16 groups based on the clinical annotations provided by the UKBEC database. These annotations included tissue type (Cerebellum and Frontal Cortex), sex (Male and Female), and age group (0-25 years, 25-50 years, 50-75 years, or 75+ years). For each of the 16 groups, we calculated the median expression for each probe and performed a hierarchical clustering via multiscale bootstrap resampling using the pvclust package in R. Using a p-value threshold of 0.01, we see that the ideal clustering method is to subset the data by tissue type, dividing the expression and methylation brain datasets into cerebellum and frontal cortex tissue datasets.

Estimating expression variability. To calculate a magnitude-independent measure of variability for expression and methylation, we used a modified method described in Alemu et al [1]. Briefly, we first calculated a bootstrapped estimate of the median absolute deviation of each gene using 1000 bootstrap replicates. Next, a local polynomial regression curve (loess function with default parameters on R version 3.4.2) was used to determine the expected gene expression MAD as a function of the median value. No additional smoothing was used for the regression curve. We calculated gene EV as the difference between the bootstrapped MAD and the expected MAD at each gene's median expression level.

Identification and removal of bimodal expression probes. Probes expressions that exhibited a bimodal distribution were thought of as having two exclusive phenotypic states. However, our focus in this analysis was to examine the factors affecting the tightly regulated expression of Hypo-Variable probes or the highly variable gene expression of Hyper-Variable probes. In order to identify if a gene's expression was unimodal or bimodal, we modeled each gene expression as a mixture of two gaussian distributions using the mixtools package in R. Next, we identified the peaks of the probability density functions for each gaussian distribution and compared the distance between the peaks as well as the ratio of peak heights. Probes with peaks that were greater than one MAD apart and displayed a peak ratio greater than 0.1 were

treated as having a bimodal expression and subsequently removed from the analysis. Probes that did not satisfy these criteria were considered to have a unimodal distribution and were kept for further analysis.

EV gene set classification. We classified the probes into three distinct probes sets based on their expression variability:

$$\tilde{x}_{EV} \pm 3 * MAD_{Boot} \quad (1)$$

where $\tilde{x}_{EV}$ is the EV median for each dataset, and MAD_{Boot} is the bootstrapped estimate of the median absolute deviation using 1000 replicates. Probes whose EV fell within the range were considered Non-Variable, those above this range termed Hyper-Variable, and the remaining were considered Hypo-Variable.

Bootstrapping EV gene set classifications. To statistically validate our EV classifications, we split our data into two equally sized subsets and repeated the previously explained EV method. This 50-50 split-retest procedure was repeated 100 times per tissue. Next, we determined the accuracy our of original classifications by comparing original classification of each gene with the 50-50 split classifications using a binomial test with a probability of success greater than 0.5. In this hypothesis, a "success" is defined as consistent EV classification across all three subsets, and gene classifications were considered significant with a p-value < 0.05.

Structural analysis of EV genes. Data regarding the structural features of the genes was obtained from the GRCh38/hg38 assembly of UCSC Table Browser [56]. Linear regression analyses were conducted to find any correlation between gene EV and their structural features. For the linear regression analysis of

transcript size, we individually examined the largest and smallest transcripts separately. The sequence lengths excluded introns, 3' and 5' UTR exons, and any upstream or downstream regions.

Gene cluster analysis. The GO term enrichment analyses were conducted using ConsensusPathDB gene set over-representation analysis [26]. The complete list of unique Illumina HumanHT-12 V3.0 expression BeadChip genes was used as a background list of genes. The resulting GO terms were then filtered manually using a q-value cutoff of 0.05. Common and unique GO terms were summarized using REVIGO [57] and visualized through treemaps by the provided R scripts. The parameters used were a medium allowed similarity (0.7) using Homo sapiens database of GO terms.

Enrichment analyses. Using the Pearson's chi-square test, we tested for enrichment of essential genes in each gene set relative to the total number of essential genes in the Illumina HumanHT-12 V3.0 expression BeadChip. A list of 20,029 protein coding genes from the CCDS database was used to test for essentiality enrichment [26]. Only genes that are solely classified as essential are considered in the analysis, resulting in a list of 2377 essential genes present in the dataset. Once the number of annotated genes and gene sets were deemed dependent variables, we determine the enrichment of annotated genes using the Pearson residuals.

The Pearson's chi-square test was also used to test the enrichment of methylation clusters across the Hyper-Variable, Hypo-Variable, and Non-Variable probe sets.

Hierarchical clustering of age-dependent Hyper-Variable genes. With the exception of a few groups, the hierarchical clustering groups with the opposite sex and the same age groups tended to cluster together.

While the p-values of the sex and age groupings during the hierarchical clustering were too high to warrant further subsetting of the brain dataset samples into distinct groups, they were significant enough to inspect on a gene-by-gene basis.

We used a multiple linear regression model to measure the changes in expression of the Hyper-Variable probes as a function of age, sex, and post-mortem interval (PMI):

$$Y_i = \beta + \beta_1 Age + \beta_2 Sex + \beta_3 PMI \quad (2)$$

where Y_i is the expression level of a probe and β_n is the coefficient for each term. The p-values were calculated using a type III sum of squares regression and adjusted for multiple comparisons using the Benjamini-Hochberg method. Probes that exhibit an FDR < 0.01 were considered significant for the specific coefficient, and the sign of the coefficient determines if the probe is positively or negatively correlated with the factor.

The choice to use three age clusters as the optimal number of clusters to examine changes of EV across age samples was determined using an expectation-maximization (EM) algorithm initialized by hierarchical clustering for parameterized Gaussian mixture models in the mclust package of R. The Bayesian information criterion for each hierarchical clustering model was determined, and both the cerebellum and frontal cortex displayed identical optimal numbers of age clusters. Once the samples were correctly clustered by age, the gene clusters were selected by cutting the gene dendrograms manually. The gene expressions were then visualized as heatmaps using the gplots package in R.

List of Abbreviations

EGA European Genome-Phenome Archive

EM	Expectation-Maximization
EV	Expression Variability
GEO	Gene Expression Omnibus
GO	Gene Ontology
MAD	Median Absolute Deviation
PMI	Post-Mortem Interval
TF	Transcription Factors
UKBEC	UK Human Brain Expression Database

Declarations

Ethics approval and consent to participate. Not applicable

Consent for publication. Not applicable

Availability of data and material.

The datasets analyzed in this study are available in the GEO repository under the accession number GSE36192 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36192) and GSE36194 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36194). The remaining dataset analyzed this study is available from European Genome-phenome Archive but restrictions apply to the availability of these data and are not publicly available. Data are however available from the authors upon reasonable request and with permission of EGA.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions. All authors contributed to the analysis of the results. NB performed the computational data analysis, prepared figures, and was a major contributor in writing the manuscript. JL conceived, coordinated, and supervised the work. All authors read and approved the final manuscript.

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Additional Files

Additional file 1: Structural analysis of genes as a function of EV

Additional file 2: EV correlation between different tissue types

Additional file 3: Complete list of GO term treemaps for all genes

Additional file 4: Complete list of GO term treemaps for essential genes

Additional file 5: Complete list of GO term treemaps for age-regulated Hyper-Variable genes

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Figure 1

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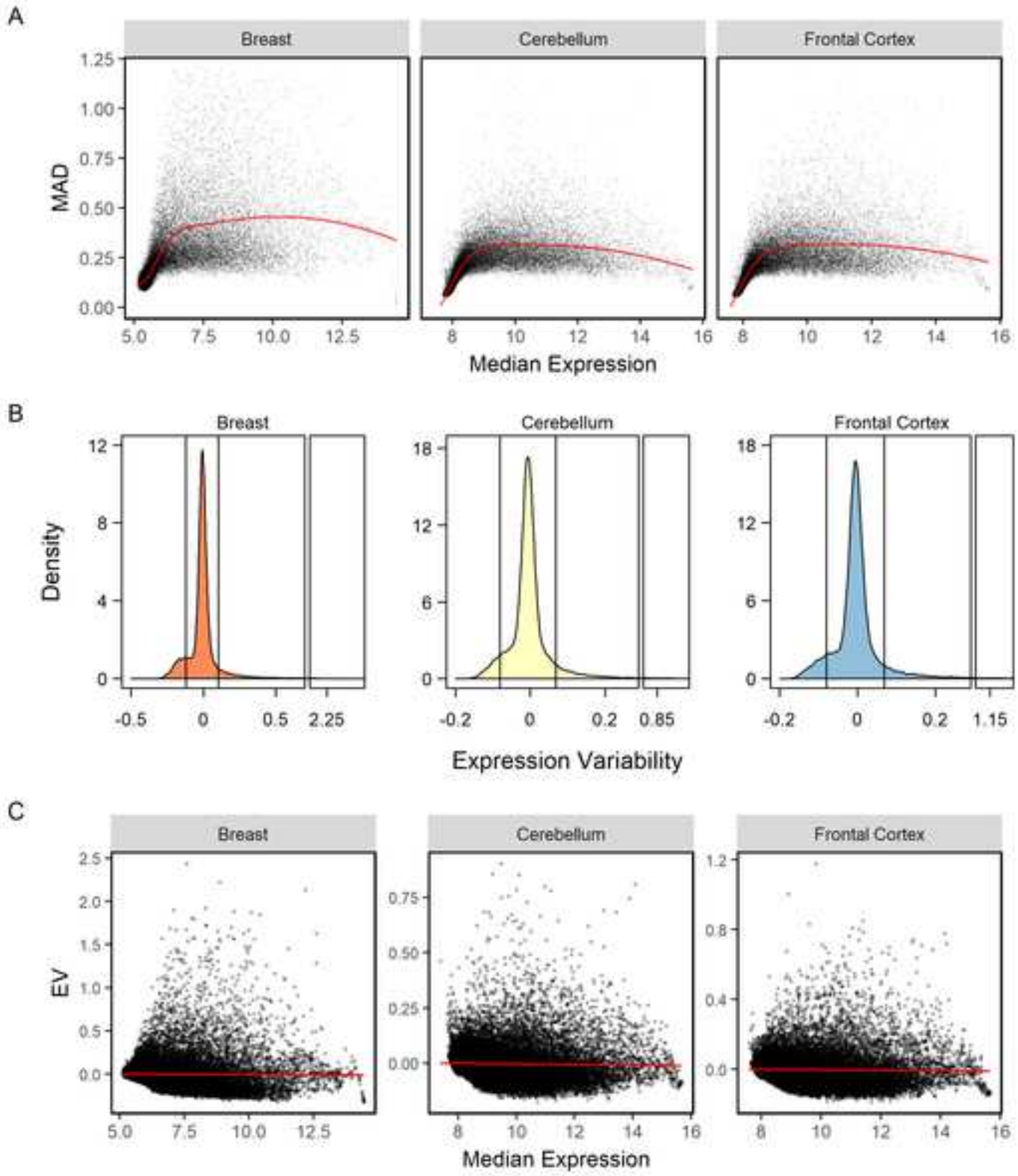

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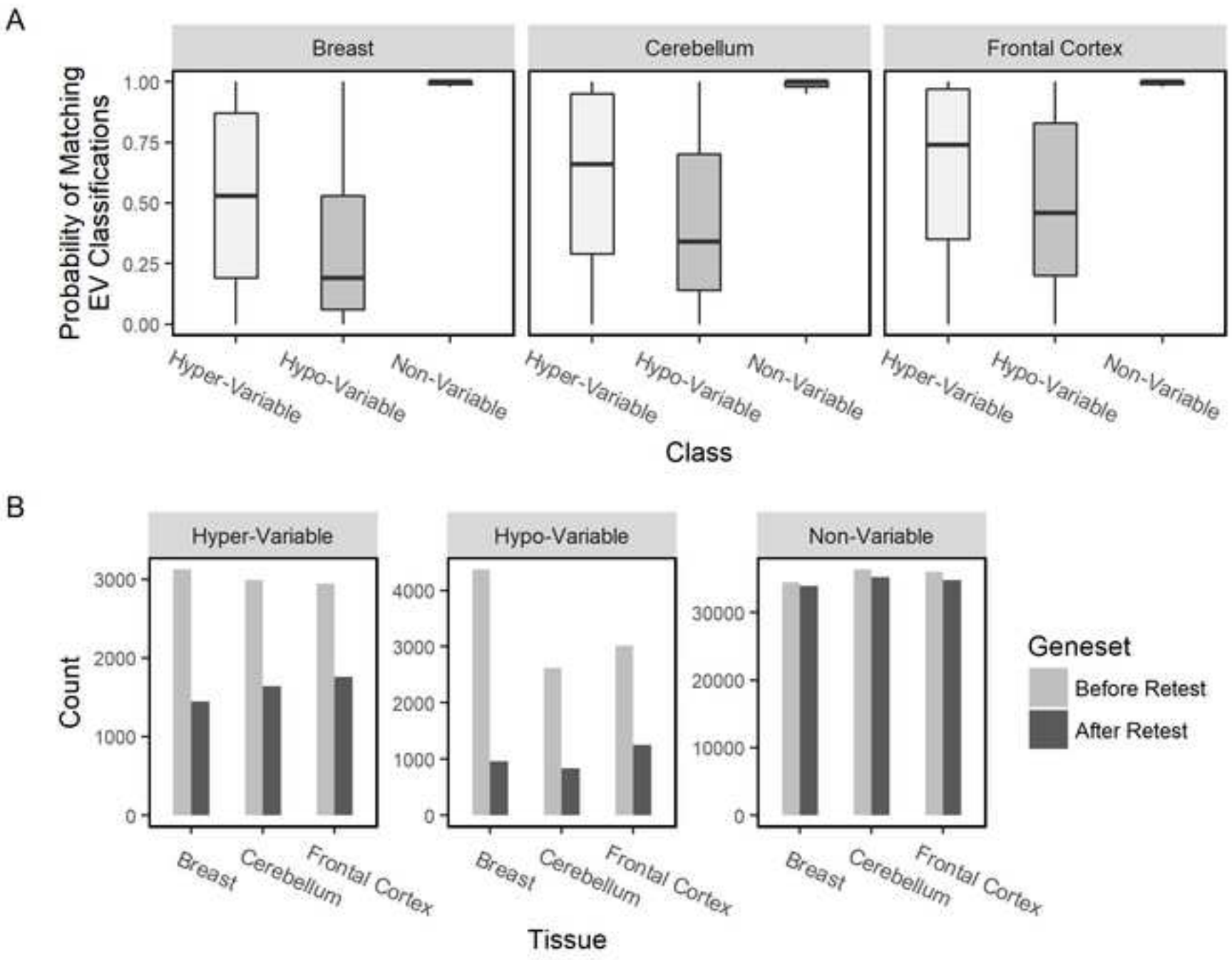

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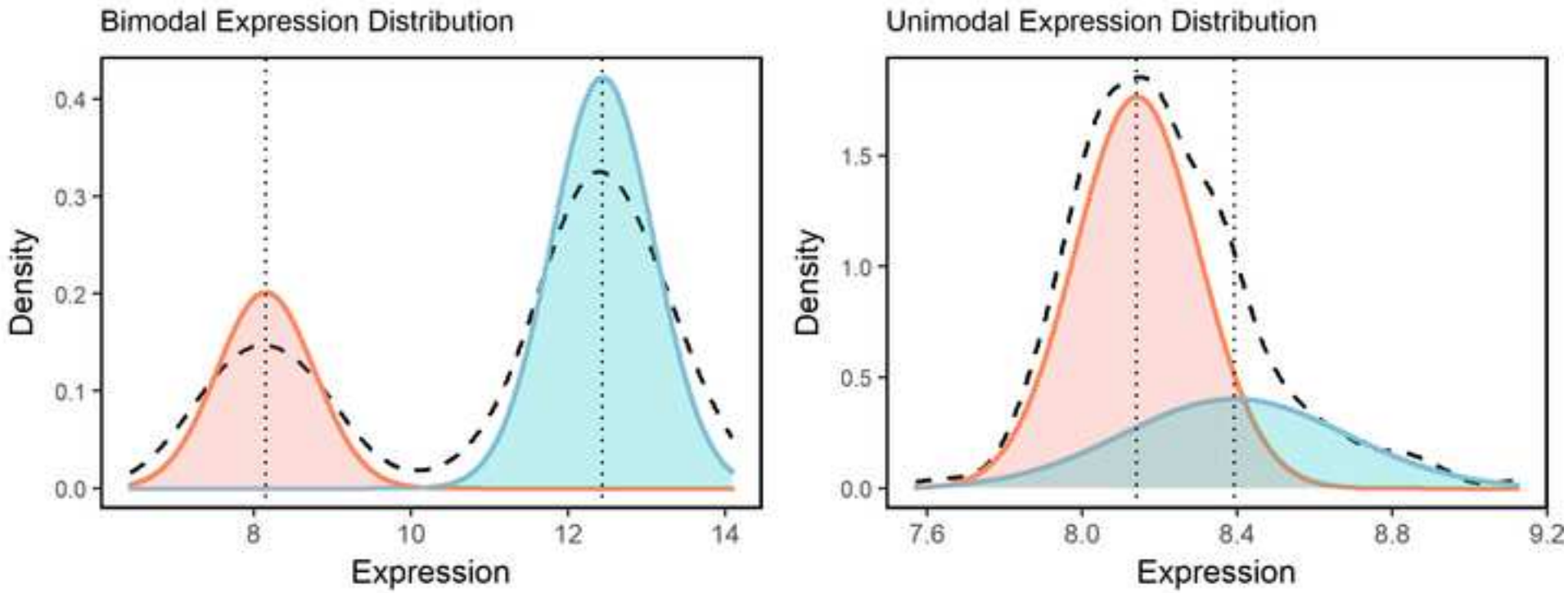

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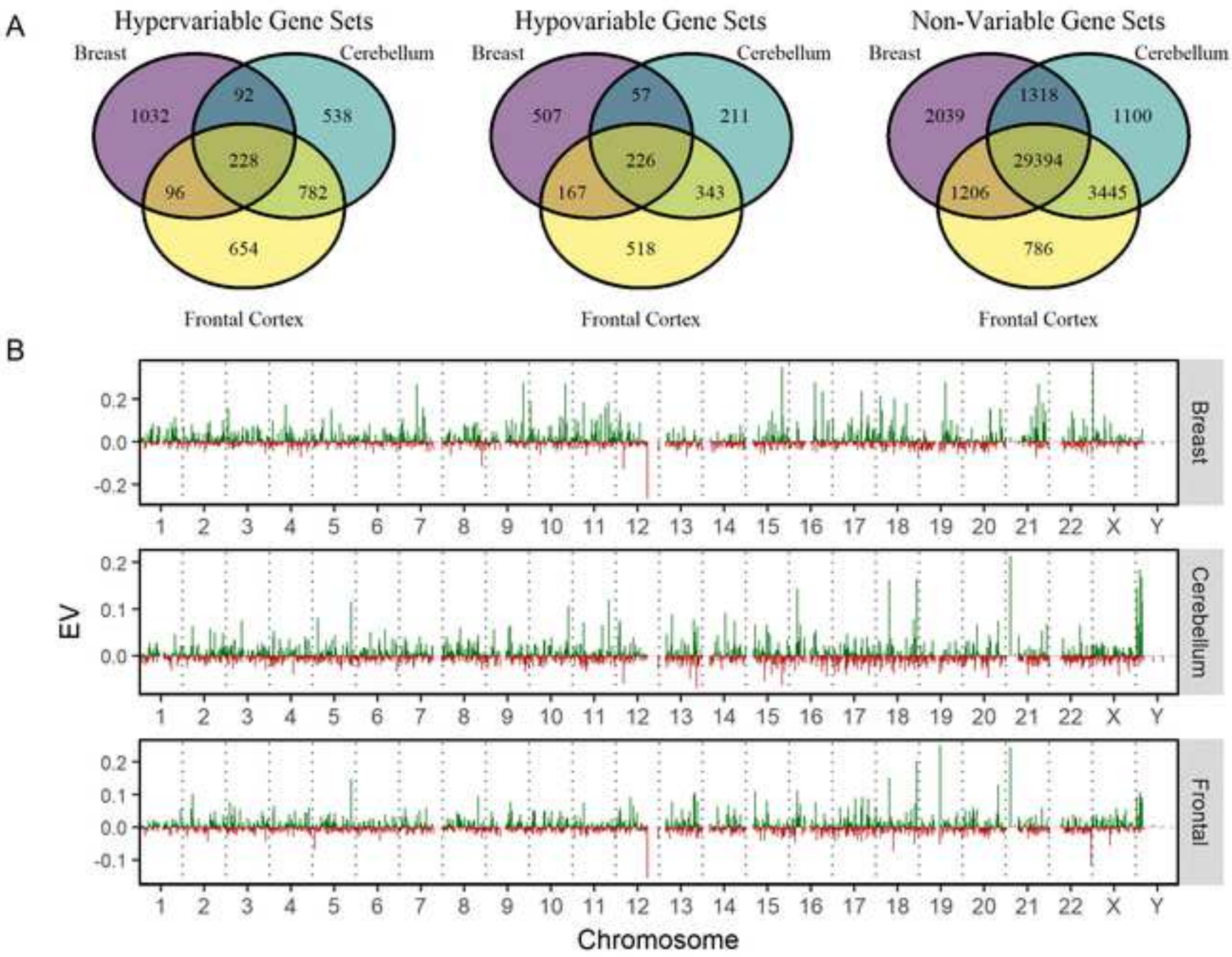

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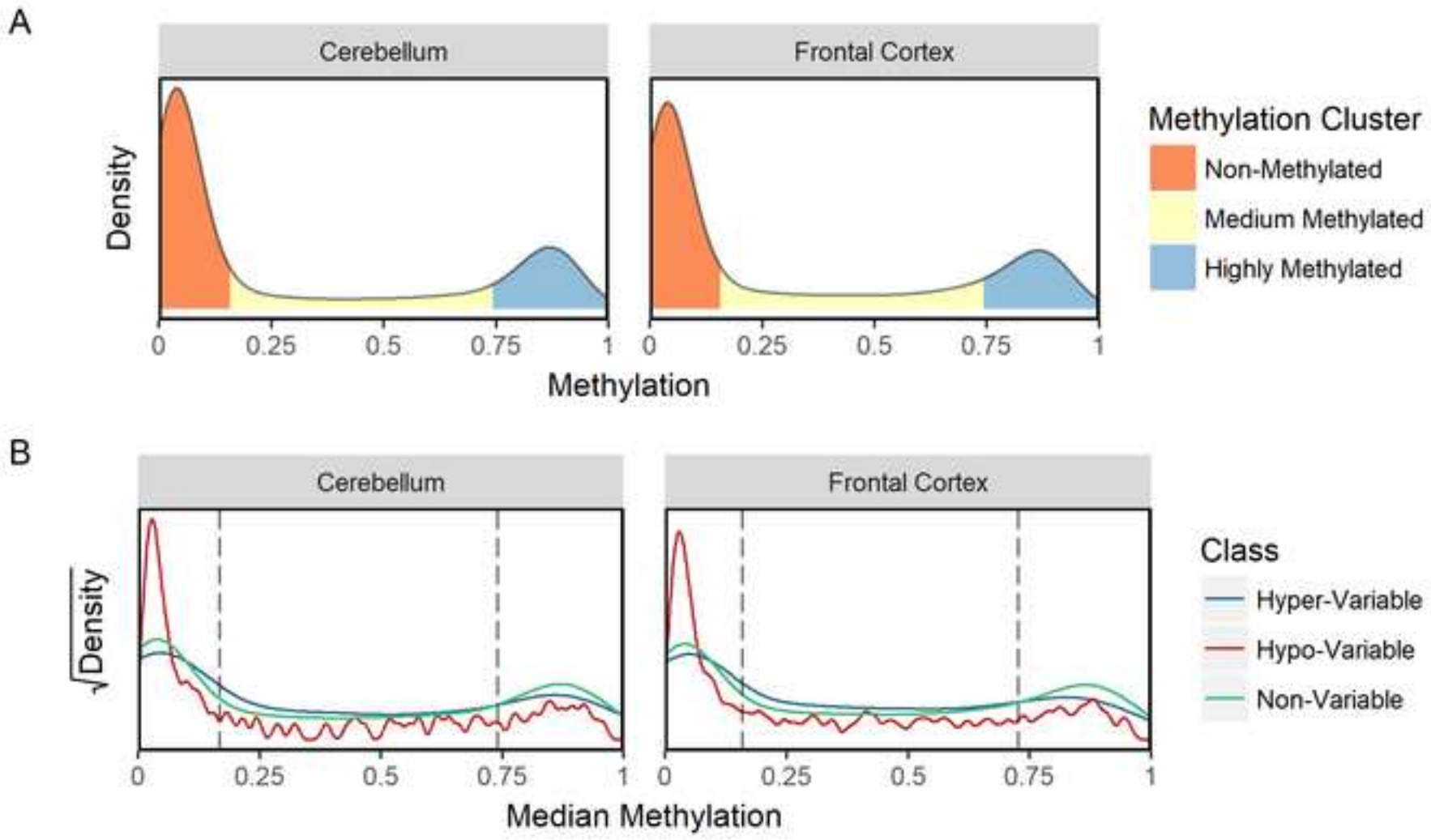

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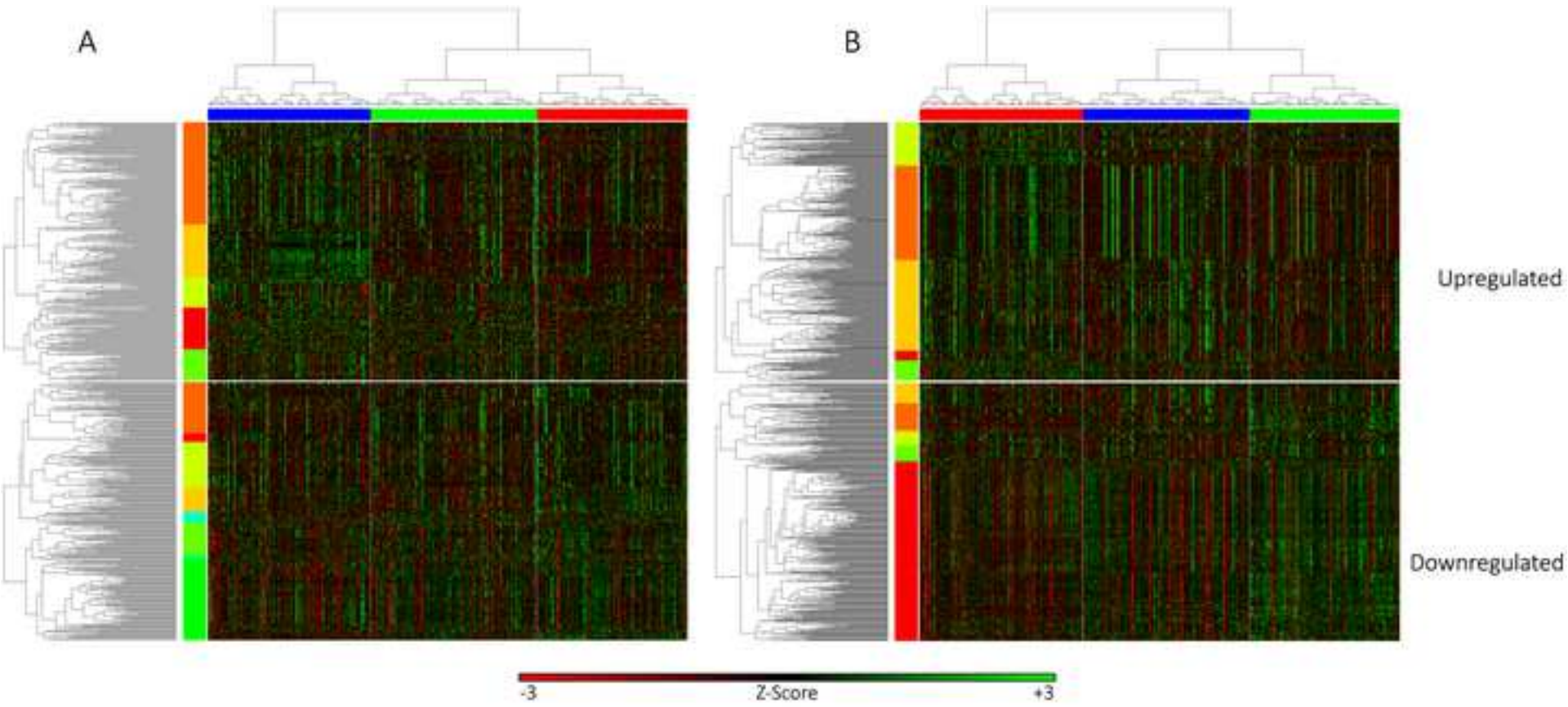

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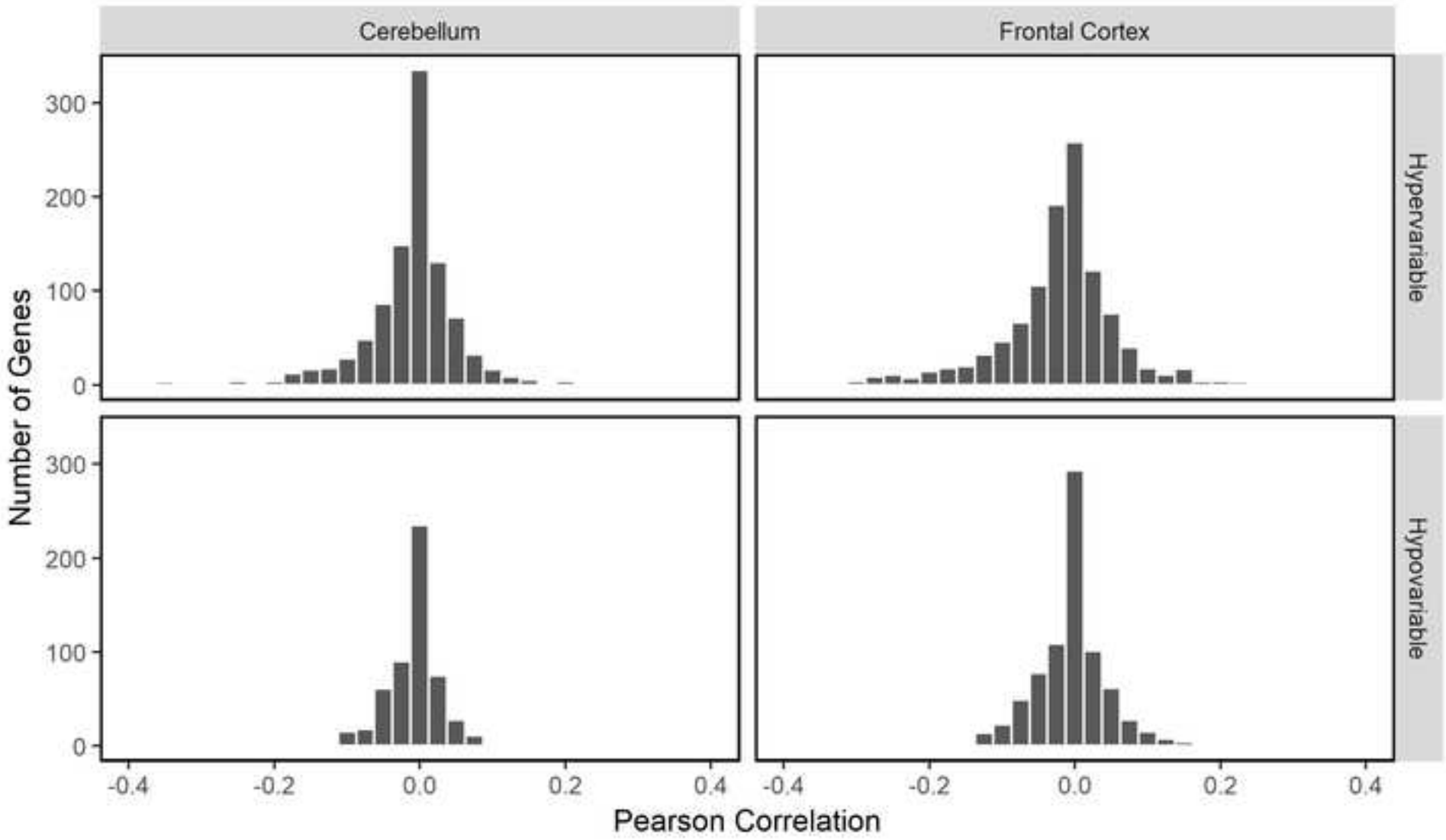
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