Identifying Aging Genes in the Aging Mouse Hypothalamus Using Gateway Node Analysis of Correlation Networks

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Abstract: High-throughput studies continue to produce volumes of data, providing a wealth of information that can be used to better guide biological research. However, models that can readily identify true biological signals from this data have not been developed at the same rate, due in part to a lack of well-developed algorithms that can handle the magnitude, variability and veracity of the data. One promising and effective solution to this complex issue is network modeling, due to its capabilities for representing biological elements and relationships *en masse.* In this research, we use correlation networks for analysis where genes are represented as nodes and indirect relationships (derived from expression patterns) are represented as edges. Here, we define "gateway" nodes as elements representing genes that change in co-expression and possibly co-regulation between states. We use the gateway node approach to identify critical genes in the aging mouse brain and perform a cursory investigation of the robustness of these gateway nodes according to network structure. Our results highlight the power of the gateway nodes approach and show how it can be used to limit search space and determine candidate genes for targeted studies. The novelty of this approach lies in application of the gateway node approach on novel mouse datasets, and the investigation into robustness of network structures.

1 INTRODUCTION

Recently, network analysis methods have been developed to analyze and draw signal from large, high-throughput datasets. These methods include the use of correlation networks, protein-protein interaction networks, genetic interaction networks, metabolic networks, and more. Commonly used to describe networks of co-expression, the correlation network model uses nodes to represent genetic probes and edges to represent a correlated pattern of gene expression between samples, defined by condition, time, or other environmentally quantifiable criterion. This technique has been proposed for identifying differentially expressed genes where traditional methods (such as Gene Set Enrichment Analysis) do not always return desirable results(Benson, Breitling 2006, Reverter, Chan 2008, Horvath, Dong 2008). As such, correlation networks also serve as a valuable supplement to traditional approaches.

While typically used for studying one particular state individually, the correlation network can also be used for comparison of states. A recent study by Dempsey and Ali(Dempsey, Ali 2014) uses clustering in correlation networks, particularly clustering that identifies small, densely coA nnected groups of genes, to compare datasets from the same cell lines under different conditions. This analysis revealed that clusters between states typically do not overlap except for at a limited number of genes. These genes that connect differentially to two different states are termed "gateway nodes." It has been proposed that these gateway nodes, which are thought to represent genes that are co-expressed with two different sets of genes at different states, can reveal a small, finite set of genes related to the phenotype under scrutiny, making this approach appealing when using high-throughput data – typically, in studies comparing 10,000 to 40,000 genes in two or more different states, typically only 20 to 100 gateway nodes result from analysis, depending on parameterization. Further studies on clusters in correlation networks have found that almost all clusters contain predicted and actual transcription factor binding sites for common regulatory elements(Dempsey, Ali 2014). This

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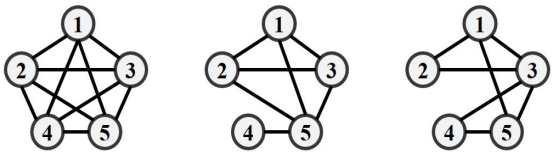
indicates that potentially, gene co-expression and even possibly co-regulated could be mined from this type of network, if such a relationship exists.

Since 1999, the network model that is representative of biological data has found structure and function to be related(Barabasi, Albert 1999), particularly when the network is built using clean data. In the protein-protein interaction networks, high degree or hub nodes typically are more likely to be lethal(Jeong et al. 2001, Barabasi, Oltvai 2004, Albert 2005) clusters in these same networks represent proteins that complex together for functional purpose(Bader, Hogue 2003). In a genetic interaction network, which represents the relationships between genes when both are simultaneously knocked out or down, the relationship represents some measure of how beneficial (or, more likely, detrimental) the duel silencing is on the organism(Michaut et al. 2011). Structures identified in these networks can lead to identifying of genes with common pathways. The correlation network is also known for these structure function relationships – hubs, while not as obviously lethal, can be enhanced to reveal lethal properties(Dempsey et al. 2012), clusters have been found to represent real sets of functionally related genes(Horvath, Dong 2008), and gateway nodes give insights into which genes play a pivotal role in the changes in expressions from one environment to another.

To investigate the novelty of gateway nodes in a number of datasets with mediocre differential gene analysis results, three datasets from varying brain tissues of mice at 2 to 3 ages were analyzed using the gateway nodes approach. It can be speculated that cluster density has an impact on biological function in correlation networks. The nature of correlation network construction suggests that in a network where genes are nodes and edges are correlated patterns of expression, a clique (a network where all nodes are connected to all other nodes) is theoretically a more reliable or likely representation of co-expression than a less connected cluster (also known as a semi-clique). Consider two "clusters" of 5 genes each, one where all 5 nodes are completely connected (10 edges) and another where the cluster is only semi-complete (say, having 7 edges or 70% edge density). In the example 1 below, clusters A, B, and C all contain 7 edges – in example B, it seems likely that edge 4—5 is incorrect, and 1-2-3-4 are likely co-expressed. In C, it seems likely by examining K_3s that 1-2-3, 1-3-5, and 3-4-5 are all highly correlated, but if that were truly the case, it would stand to reason that 2-5 and 2-4 should also be connected.



(A) (B) (C)

The best evidence without examining cluster substructure is example A in this case, the densest. To investigate the influence of density related cutoffs on the gateway node, clusters were analyzed using a density filter of 65-100% (65%), 75-100% (75%) and 85-100% (85%). The goal of this study was to analyze aging in the brain and possibly identify the pathway players with roles in neuronal growth and differentiation. Gateway node analysis was again used for its application to aging and for its design for identifying temporal expression changes. The beauty of this and other case studies is that they satisfy a need for application of methods to real world data and testing of hypotheses. The results of this study reveal a number of genes that are known players of change in aging in the mouse brain, and highlights how gateway nodes can be used to identify targets of further study in similar cases.

2 METHODS

The network model used was created using data prepared and analyzed with pairwise Pearson Correlation (see *Network Creation & Enrichment Analysis*) and was then clustered and gateway nodes were identified (see *Gateway Node Analysis*). Targets were then identified via model creation.

2.1 Network Creation and Enrichment Analysis

Data was drawn from three microarray expression datasets for this analysis; three were prepared in total: (1) Cerebellum from Balb/C mice at three time points (Young, Middle-aged, and Aged), (2) Striatum from Balb/C mice at three time points (Young, Middle-aged, and Aged), and (3) Hypothalamus from male C57 mice at two time points (Young and Middle-aged). Correlation networks were generated using probes and expression values from using pairwise computation of the Pearson Correlation Coefficient (ρ) and

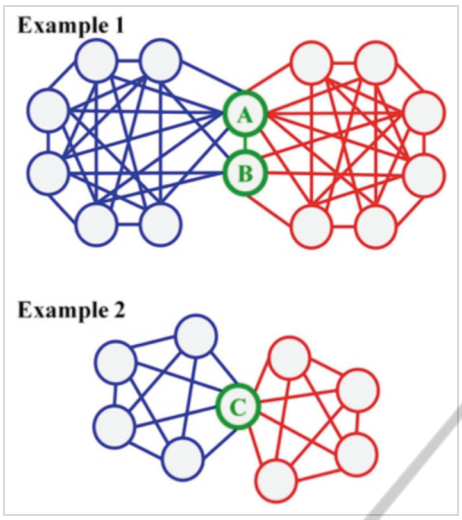


Figure 1: An example of how gateway nodes are scored. In example 1, gateway nodes A and B are identified from the overlap between network 1 (blue) and network 2 (red). Node A has 6 edges in network 1 and 6 edges in network 2 (not including edge A-B). Node B has 3 edges in network 1 and 3 edges in network 2 (not including edge A-B). Therefore, the total edge responsibility (total edges) connecting gateway nodes is 18. A therefore has 12/18 or 66% gatewayness and B has 6/18 or 33% gatewayness. In example 2, gateway node C has 4 edges in both network 1 and network 2, for a total of 8/8 or 100% gatewayness.

correlation threshold of $0.85 \leq \rho \leq 1.00$. For each pairwise correlation computation, hypothesis testing using the Student's T-test was performed; only significant correlations (P-value <0.0005) were kept. Gene Set Enrichment Analysis was performed using the GeneTrail Analysis tool (http://genetrail.bioinf.uni-sb.de/)(Backes et al. 2007). Parameters for each analysis were set as follows:

- Organism: *Mus musculus*
- Analysis Type: KEGG, Gene Ontology (manually curated only)
- P-value adjustment: FDR Adjustment
- P-value threshold: 0.05
- Minimum # categories: 2

2.2 Gateway Node Analysis

In brief, gateway nodes are identified by first clustering networks, then networks are overlapped and nodes that have edges in networks from both conditions are iteratively identified. To perform clustering, AllegroMCODE(Bader, Hogue 2003) was used on each network under the following parameters: Degree cutoff:10, Node Score: 0.2, K-Core:10, MaxDepth: 10. Clustering time ranged from 89.436 seconds (Male C57 young network) to 29,499.495 seconds (Cerebellum Balb/C young network). Clustering correlation networks is known to improve the lethality enrichment of high degree nodes, largely because important hub nodes in correlation networks are understood to be contained within clusters. While the choice of clustering method may vary, the lethality enrichment findings were conducted using AllegroMCODE which identifies many small, dense clusters. As such, this work also includes a cursory review of how clustering density impacts the robustness of the gateway node. After clustering was performed, clusters were filtered to three different arbitrarily chosen density thresholds: clusters at or above 65% density, at or above 75% density, and at or above 85% density. Density is defined as the number of total edges in the network divided by the number of possible edges – in a network with N nodes and no duplicate edges or self-loops, the number of possible edges is equal to $[N*(N-1)]/2$. As the density threshold changes, the number of gateway nodes present within the overlaid network changes, and as such, it is important to consider numerous thresholds to see if a gateway exists as an artifact of clustering or it exists as a true gateway node, or gene that is co-expressed with a unique group of genes in two or more different states.

After the clustering step, networks are overlaid on top of one another to identify gateway nodes. The process used to identify these nodes in an automatic way is extensively described in Dempsey and Ali 2014. Briefly, for each node in the clustered, overlaid network, each node is first classified as having edges in one or both networks. If the node has edges in both networks, it is technically considered a gateway. Scoring is then performed by examining the number of edges per gateway per cluster overlap versus the total number of gateway edges (excluding intra-gateway edges). This ratio is the gatewayness score, and reflects the "responsibility" of each gateway in terms of how many edges pass through that particular node from one stage to another. An example of the difference is shown in Figure 1. Gateway nodes were identified at each density threshold, heretofore referenced as 65% (at or above 65% cluster density), 75% (at or above 75% cluster density), and 85% (at or above 85% cluster density).

After determination of gateway nodes at each density threshold and Gene Ontology (GO) enrichment of each gateway-connected cluster (Ashburner et al. 2000), a model was drawn to connect genes based on shared processes in which

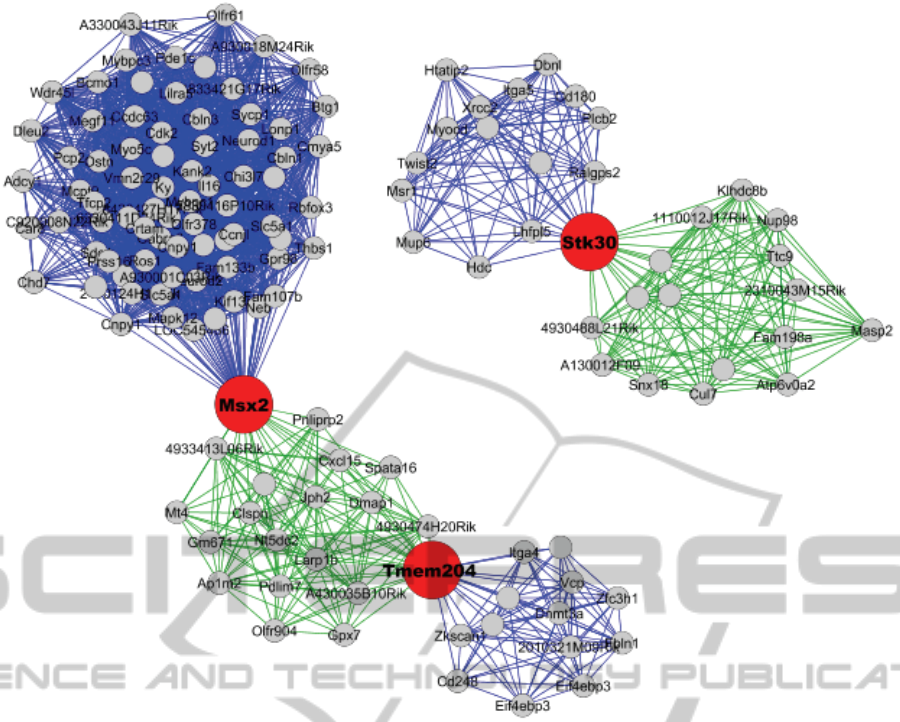


Figure 2: The 65% gateway clusters from Male c57 Hypothalamus networks. Gateway nodes are in red. These are the clusters examined using Gene Ontology in Table 2.

Table 1: Gateways by dataset. Gateways not present at 1 density only not shown. Column 1: Dataset in which gateway was found. Column 2: Array ID for the gateway. Column 3: Gene Symbol for the gateway. Column 4: Edges running through the gateway. Column 5: Total edges running through gateways connecting the two clusters. Column 6: Gatewayness score. Column 7: If the gateway was found using 65% edge density clustering, the box is marked. Column 8: If the gateway was found using 75% edge density clustering, the box is marked. Column 9: If the gateway was found using 85% edge density clustering, the box is marked.

Dataset	Array ID	Gene Symbol	Edges	Total Edges	Gateway Score	65%	75%	85%
Male c57 Hypothalamus	A_51_P493919	Stk30	31	31	100.00%	X	X	X
	A_51_P478132	2210019G11Rik	205	205	100.00%	X	X	
	A_52_P78684	D330040H18Rik	174	174	100.00%	X	X	
Balb/c Cerebellum	A_51_P346893	Extl1	182	182	100.00%	X	X	

the gateway nodes are involved, if known. This model was manually curated using the following resources: Literature via PubMed search and review, KEGG pathway database (Aoki, Kanehisa 2005), and NCBI, and included regulatory relationships, inhibitory relationships, binding relationships, etc. This section must be in one column.

3 RESULTS

Before clustering, network sizes ranged from 38k41k nodes and 312k-7,600k edges. After clustering, node counts ranged from 30-8k and edges from 300-86k. Thus, network sizes changed depending on state and tissue. As described in Table 1, the Male c57 Hypothalamus dataset contained the fewest gateway nodes (3), with only one gene (*Stk30*) found to be robust to changes at 65%, 75%, and 85% cluster densities. The other two gateway nodes were only found at 65% density (*Tmem204*, *Msx2*). All three gateway nodes in this case had scores of 100% gatewayness. The Balb/c cerebellar dataset contained 7 gateway nodes, none of which were robust to all three thresholds. Three were robust to two thresholds, but only one of these are non-RIKEN probes (*Extl1*). Two gateways in this set that did not have 100% gatewayness were found only at 65% cluster density and were shared between

Table 2: Cluster Gene Ontology Set Enrichment Analysis for Male c57 Hypothalamus dataset. Column 1: The gateway name. Column 2: The cluster connecting that gateway – young or mid (not both combined) and edge color. Gene Ontology enrichment (GO) or KEGG enrichment (KEGG). Column 3: GO/KEGG annotation or pathway name. Column 4: Annotation/pathway ID. Column 5: The number of genes in that annotation/pathway name. Column 6: The p-value associated with that enrichment. Column 7: If "down", the cluster has fewer genes in that annotation/pathway than expected for random. If "up", the cluster has more genes in that annotation/pathway than expected for random. *FDR Adjustment was used, but if a * is included in the P-value column, this indicates the annotation did not survive P-value adjustment and the noted P-value is the unadjusted value.

Gateway	Cluster Description	GO/ KEGG	Category	ID	# Genes	P-value	Enrich.
Tmem204	Aged-Blue	G.O.	Cell	GO:0005623	4	0.04*	down
		G.O.	cell part	GO:0044464	4	0.04*	down
Tmem204	Yng – Green	G.O.	membrane	GO:0016020	2	0.044*	down
		G.O.	multicellular organismal process	GO:0032501	4	0.045*	down
		G.O.	cytoplasm	GO:0005737	5	0.048*	down
Msx2	Yng – Green	G.O.	membrane	GO:0016020	2	0.044*	down
		G.O.	multicellular organismal process	GO:0032501	4	0.045*	down
		G.O.	cytoplasm	GO:0005737	5	0.048*	down
Msx2	Aged-Blue	KEGG	ECM-receptor interaction	4512	2	0.013*	down
		KEGG	Malaria	5144	2	0.013*	down
		KEGG	Olfactory transduction	4740	3	0.015*	down
		G.O.	biological regulation	GO:0065007	22	0.009*	down
		G.O.	cell	GO:0005623	24	0.012*	down
		G.O.	cell part	GO:0044464	24	0.012*	down
		G.O.	regulation of biological quality	GO:0065008	4	0.012*	down
		G.O.	multicellular organismal process	GO:0032501	19	0.012*	down
		G.O.	cellular process	GO:0009987	26	0.017*	Down
		G.O.	membrane part	GO:0044425	7	0.024*	Down
		G.O.	regulation of biological process	GO:0050789	21	0.026*	Down
		G.O.	non-membrane-bounded organelle	GO:0043228	6	0.034*	Down
		G.O.	intracellular non-membrane-bounded organelle	GO:0043232	6	0.034*	Down
		G.O.	regulation of localization	GO:0032879	4	0.035*	Down
		G.O.	cellular component assembly	GO:0022607	7	0.035*	Down
		G.O.	cellular component biogenesis	GO:0044085	7	0.035*	Down
		G.O.	negative regulation of biological process	GO:0048519	7	0.041*	Down
		G.O.	membrane	GO:0016020	10	0.044*	Down
		G.O.	system process	GO:0003008	12	0.045*	Down
		G.O.	regulation of cellular process	GO:0050794	16	0.046*	Down
		G.O.	molecular_function	GO:0003674	51	0.046*	Up
Stk30	Aged-Blue	KEGG	Phagosome	4145	2	0.038*	Up
		G.O.	binding	GO:0005488	8	0.021*	Up
		G.O.	plasma membrane	GO:0005886	4	0.031*	Up
		G.O.	membrane	GO:0016020	4	0.031*	Up
		G.O.	cytosol	GO:0005829	3	0.035*	Up
		G.O.	regulation of localization	GO:0032879	2	0.038*	Up
Stk30	Yng – Green	G.O.	organelle	GO:0043226	2	0.042	Up
		G.O.	membrane-bounded organelle	GO:0043227	2	0.042	Up
		G.O.	intracellular organelle	GO:0043229	2	0.042	Up
		G.O.	intracellular membrane-bounded organelle	GO:0043231	2	0.042	Up
		G.O.	intracellular part	GO:0044424	2	0.042	Up

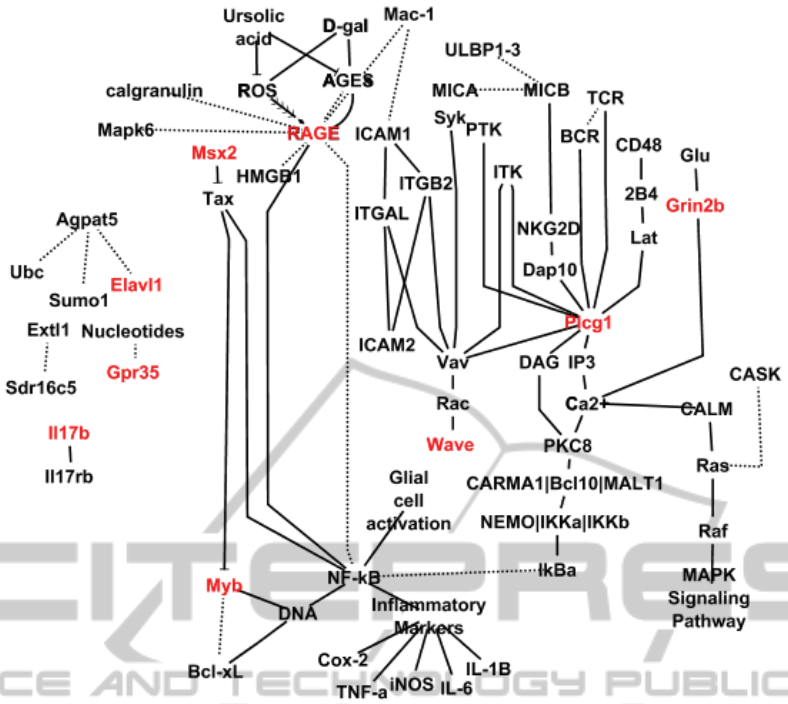


Figure 3: The curated gateway nodes model. Genes/proteins in red are gateway nodes as listed above. Not all nodes listed above are in the model if they do not fit or pathway information is not available. This model is not comprehensive.

two clusters – *Gm8221* at 48.63% gatewayness and *Apol7c* at 51.37% gatewayness. The Balb/c striatum dataset contained the most gateway nodes at 67; however, 19 of these were RIKEN or unknown/unnamed genes. All the gateway nodes in this set were not robust past 65% cluster density.

The top gateway nodes identified from Table 1 that were robust to density changes were *Stk30*, two RiKEN hypothetical genes, and *Extl11*. Gene Ontology enrichment analysis was performed on all three datasets; the enrichment data was not used particularly for gateway analysis but for consistency and integrity of analysis to ensure biological functions were found, indicating cluster relevance. Results for GO enrichment on Male C57/Bl/6 mice clusters are shown in Table 2 (results for Balb/c datasets not shown). Based on the gateway node analysis, *Stk30* (coding for the RAGE protein) and *Extl1* are the only gateways that are non-RIKEN genes that are robust to multi-clustering thresholds. Based on literature collection and model curation, *Stk30* (aka RAGE) is the most upstream target that interacts with *reactive oxygen species* and is also upstream of *NF-kB*. Gateway node *Msx2* is also upstream of the NF-kB pathway acting as an inhibitor of *Tax* gene which induces NF-kB enhancing transcription factors. *Myb* is a downstream target gateway that has ties to the apoptotic pathway and the NF-kB pathway. *Plcg1* is acted upon by multiple proteins and goes on to influence DAG and IP3, both (way) upstream of NF-kB. Upstream of the same route to NF-kB as *Plcg1,* the gateway node *Grin2b* is influenced by glucose.

4 CONCLUSIONS

Based on the gateway node analysis, *Stk30* (coding for the RAGE protein) and *Extl1* are the only gateways that are non-RIKEN genes that are also robust to multi-clustering thresholds. The literature collected and resulting model reveal that *Stk30* aka RAGE is the most upstream target that interacts with *reactive oxygen species* and is also upstream of *NF-kB*. In entirety, the model proposed above points to activation of inflammation via NF-kB and RAGE as a map for aging in normal Balb/C and C57 mouse brain. A 2009 review by Kriete and Mayo confirms a link between NF-kB activation and aging, but calls for further investigation of the role of NF-kB outside its well-studied role in the innate immune system (Kriete, Mayo 2009). In our model, gateway node *Msx2* is also upstream of the NF-kB pathway acting as an inhibitor of *Tax* gene which induces NF-kB enhancing transcription factors. *Myb* is a downstream target gateway that has ties to the

apoptotic pathway and the NF-kB pathway. *Plcg1* is acted upon by multiple proteins and goes on to influence DAG and IP3, both (way) upstream of NF-kB. Upstream of the same route to NF-kB as *Plcg1* is *Grin2b*, influenced by glucose. All of these genes have potential as effectors for change in the NF-kB pathway, either upstream or downstream, but perhaps the most important element in the model due to gateway robustness is the RAGE protein, encoded by gateway node *Stk30.* A 2003 study by Deane *et al.* revealed that RAGE is a mediator of disease-causing amyloid-beta proteins into the central nervous system, and even suggests it as a target for potential future therapies for Alzheimer's disease (Deane et al. 2003). RAGE has been found to be up-regulated in Alzheimer's patients (Leclerc et al. 2009).

A 2004 study of transgenic mice with manipulated RAGE (mAPP^{-}/RAGE^{-}) expression by Arancio *et al.* found that pups displayed issues with spatial memory and the NF-kB pathway is activated, and again find it a potential target for Alzheimer's intervention (Arancio et al. 2004). Multiple other evidences exist to substantiate the speculation that RAGE plays a role in normal aging; an October 2013 PubMed search of "RAGE" + "Aging" reveals over 100 articles relating RAGE and aging dating back to 1999.

Application of the gateway nodes approach allows for the utilization of the gateway nodes approach to determine better targets for study in the aging mouse brain. The accompanying model provides a roadmap that points us toward RAGE, Msx2, and Plcg1 as upstream targets for manipulation for manipulation of expression in the mouse brain. These genes all have indirect roles in the NF-kB pathway; it has recently been shown that inhibition of NF-kB in the mouse hypothalamus resulted in a 20% increase in lifespan, improved cognition, and levels of muscle, bone, and skin tissue typically observed in younger mice. This suggests that the gateway nodes approach is able to identify genes with major roles in aging, particularly using a robust approach. This method is able to take sets of 30,000+ genes or gene probes and narrow it down to only a few targets of interest, and their potential relationships based on network modeling of expression correlation and integration of publicly available databases. Particularly in areas of research where little is understood, funding is not readily available, or resources are tight, the gateway nodes approach can provide a robust, reproducible, and reliable way to identify targets of interest in further research.

Certainly, current methods for analyzing gene expression capture just a snapshot of cellular activity at a given time, not a dynamic process. However, the minimal overlap of co-expression relationships in the network form confirm that the cellular environment is dynamic and spontaneous. This begs the question – does a snapshot of the cell, even in multiple replicates – accurately capture the goings-on of cellular activity? If we were able to understand how we got from point A to point B, we would better understand how these gateway nodes came about. Surely on a short-term basis gateway nodes could arise form differential regulation of expression, but in the long term, the question is whether the clusters captured are a result of a short-term cellular change or a compensatory effect of loss of previous gene function. To improve the dimensionality of these analyses without vastly increasing the data load, one might consider modifying their gene expression research design to include 3 or more time points and to include a high number of replicates for each time point (ideally, 5 or more). While this is certainly not always feasible due to cost, labor, or difficulty in sample preparation, it could be considered to help understanding of cellular dynamics using a network model.

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