High Throughput RNA Sequencing of Mice Retina Reveals Metabolic Pathways Involved in the Gut-Retina Axis

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Abstract

Background and aims:

Connections between the gut microbiome and retinal diseases such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinopathy of prematurity (ROP), and primary open-angle glaucoma (POAG) are recently being established. Communication between the gut microbiome and retina, referred to as the gut-retina axis, has been proposed; however, the biologic pathways and mediators involved in the interactions have not yet been elucidated. Using high-throughput RNA sequencing (RNA-seq) of whole retinas, we compare the retinal transcriptome from germ-free (GF) and specific pathogen-free (SPF) mice to investigate effects of the gut-microbiome on both retinal gene expression and biologic pathways.

Methods:

RNA was extracted from whole retinas of GF and SPF mice (four animals per group) and cDNA libraries were created. RNA-seq was performed on NovaSEQ6000 using the paired-end method. After preprocessing the RNA-seq data, gene expression value was calculated by count per million (CPM). The differentially expressed genes (DEGs) were identified with edgeR Bioconductor analysis of expression data. Functional enrichment and protein-protein interaction (PPI) network analyses were created for the differentially expressed genes (DEGs).

Results:

RNA-sequencing reveals a cohort of 396 DEGs, of which, 173 are upregulated and 223 are downregulated in GF mouse retina. Enrichment analysis reveals that the DEGs are involved in glucocorticoid effects, transcription factor binding, cytoskeletal stability, lipid metabolism, and mitogen-activated protein kinase (MAPK). Multiple biologic pathways, including obesity/metabolic syndrome, longevity, and 5' AMP-activated protein kinase **(**AMPK) signaling pathway are affected in the GF retinas. PPARG1a (PGC1a) gene is involved in 12 of the 16 significantly modulated pathways. Proteins with the most number of interactions in the PPI are E1A binding protein P300(EP300), forkhead box O3(FOXO3) and PGC1a.

Conclusions:

To our knowledge, this is the first study demonstrating the involvement of the gut microbiome in driving the retinal transcriptome, providing evidence for the presence of a gut-retina axis. Future studies are needed to define the precise role of the gut-retina axis in the pathogenesis of retinal diseases.

Keywords (according to MeSH)
Gut-retina axis; transcriptome; high-throughput RNA-sequencing; germ-free mouse; gut microbiome; age-related macular degeneration; diabetic retinopathy; primary open angle glaucoma

Introduction
The gut microbiome is the collective microbiota and their genetic material residing symbiotically in the gut. [1] Effects of the gut microbiome in diseases of anatomically distant sites, for instance, skeletal muscle, lung, and brain, are well-recognized. [2,3]. However, its role in ocular conditions, especially retinal diseases is only recently being recognized. [4,5] Communication between the gut microbiome and retina, also referred to as the gut-retina axis has been proposed; but to date, the biologic mediators and pathways affected in the retina by the gut microbiome are not known.

The concept of the gut microbiome affecting anatomically distant organs, especially the regulation at an immune-privileged site, like the eye, is very perplexing. [6] In the past few years, data for the gut microbiome's involvement in retinal diseases is accumulating. [7,8] Autoimmune uveitis was the first ocular disease linked with an altered gut microbiome. [9] Early microbiome studies show germ-free (GF) mice completely devoid of microbiota, have a modified retinal lipidome. [10] Huihui Chen *et al*. showed that GF mice relative to mice with an intact microbiome are protected from glaucoma by a T-cell mediated effect. [11] Alteration of the microbiome by diet (also known as gut dysbiosis) illustrates that high-sugar and fat diets exacerbate worsened features of dry age-related macular degeneration (dAMD) and neovascular AMD, respectively. [4,12,13] On the other hand, it is shown that restricting the gut microbiome by fasting prevents diabetic retinopathy (DR) and prolongs survival in mice. [14] Human studies have determined that the gut microbiome is compositionally and functionally different in wet AMD, [14] primary open-angle glaucoma (POAG), [15] retinal artery occlusion (RAO) [17] and retinopathy of prematurity (ROP) [18] in contrast to healthy subjects. Gut microbiota modulation may serve as a novel strategy for the prevention and treatment of retinal diseases.

A recent study shows that remodeling the gut-microbiome has a favorable influence on retinal morphology and decreases age-related retinal ganglion cell (RGC) loss. [19] Another investigation suggests that administration of probiotics may modulate clinical signs of autoimmunity in the eye. [20] Andriessen et al show the gut microbiota influences the development of neovascular lesions associated with AMD and modifying microbiota can decrease systemic and local chorioretinal inflammation, subsequently decreasing pathological choroidal neovascularization. [12] Despite this knowledge, the gut microbiome's precise role in retinal disease pathogenesis remains unknown; nonetheless, the functional differences between health and disease states support the presence of a gut-retina axis.

Multiple animal models have been used to study the microbiome, using antibiotics and diet to modulate the microbiome are the most common methods for these studies. [4, 12] Nevertheless, these models do not allow for assessing changes caused by antibiotics and diet, as opposed to the effects caused by the microbiome alone. [21] 16S rRNA gene sequencing can be used to compare taxonomic differences between gut microbes of patients and healthy controls. [22] All these techniques provide significant primary evidence for linking the gut-microbiome to the retinal

diseases; but, to move beyond the correlation phase of experiments; preclinical mouse models are essential. [21,23] The GF mouse model is considered the gold-standard for proof-of-concept microbiome investigations and has been used in milestone discoveries to determine causal links between the microbiome and disease. [24,25,26] Presently, no studies use the GF mouse model to describe the gut microbiome's role in the gut-retina axis.

Here, we profiled the retinal transcriptome to identify differentially expressed genes (DEG) between GF and specific pathogen-free (SPF) mice retina; the latter serving as a control group. Advances in high throughput RNA-sequencing and computational bioinformatics analysis have evolved as powerful methods to yield detailed information on novel genes and pathways involved in different conditions. [27] The study design is illustrated in Figure 1. To our knowledge, no microbiome studies have illustrated alterations in the retinal transcriptome utilizing RNA-seq technology. We identified DEGs between the two groups revealing dysfunctional biological processes (BPs), molecular functions, cell components, and pathways. Furthermore, protein-protein interactions (PPIs) of the DEGs are also explored.

Changes in gene expression by modulating the gut-microbiome have already been established in the GI tract, [28] skeletal muscle, [29] and the liver. [30] We show that similarly the gut microbiome can also modulate gene expression in the retina. Our study provides a global insight into the retinal genes and pathways influenced by the gut microbiome. Future investigations are needed to elucidate the function of the microbiome in specific retinal diseases, which can aid in developments in alternate or adjunct prevention strategies and therapies for medically refractory and incurable retinal diseases.

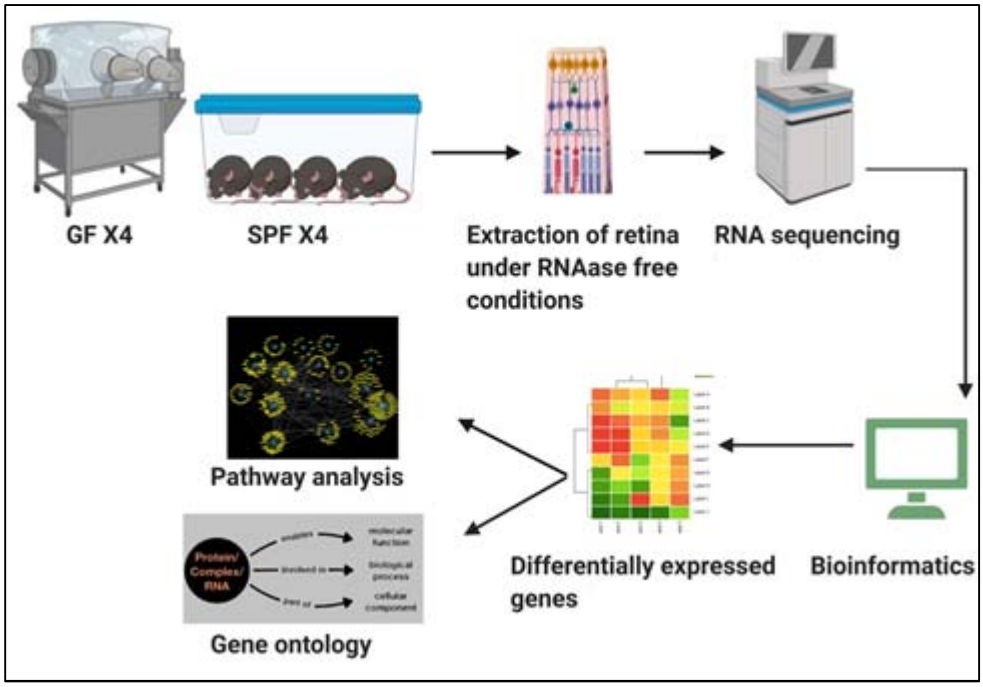


Figure1. Schematic diagram illustrating the experiment methodology

Materials and Methods

Animals

All experiments were performed per-the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in Ophthalmic and Vision Research and were institutionally approved. Adult male mice C57Bl/6 mice were utilized for this study. The GF mice were bred in the Gnotobiotic Research Animal Facility (GRAF) at the University of Chicago (U of C). SPF mice were purchased from the Jackson Laboratories and bred in at the Animal Resources Center (ARC) at the U of C SPF animal vivarium. SPF mice were kept in sealed cages in a ventilated rack system.

Mice were fed normal rodent chow. Food and water were provided *ad libitum* to both groups and they are exposed to 12:12 light: dark cycle. The environmental parameters of temperature and humidity were kept within the range required by The Guide for the Care and Use of Laboratory Animals, Eighth Edition. [31] At 15 weeks of age, the mice were sacrificed via CO_2 asphyxiation followed by cervical dislocation. All measures were taken to minimize stress and pain throughout the procedure. Sample processing for this experiment was immediately started on ice as an extra cautionary measure to prevent biologic/ genetic material contamination and degradation.

Sterility Monitoring

To ensure sterility in GF mice, they were housed, bred, and maintained in a sterile flexible film isolator at the U of C GRAF. Diets for GF mice were autoclaved (250 F, 30 minutes). Weekly fecal pellets were collected for sterility monitoring via microbial cultures, as previously described. [32] No positive cultures were identified in any of the GF mice used in this study.

RNA extraction

After the mice were sacrificed, eyes were enucleated and carefully dissected and the retinas were extracted on ice using sterile technique. All surfaces, tools, tubes, and equipment were treated with RNase decontamination solution (RNaseZAP, Thermofisher Scientific Waltham, MA). The retinas were immediately stored in RNA*later* (Qiagen, Germantown, MD) in RNase free Eppendorf tubes and stored at -80C until RNA extraction. For the mRNA extraction, the RNeasy (Qiagen, Germantown, MD) was used. Each sample was analyzed using Nanodrop (NanoDrop 2000cc, Thermo Scientific, Waltham, MA) to determine total RNA concentration before sequencing.

RNA sequencing

Eight samples of purified RNA (4 from each group) were used for this analysis. Before sequencing, the RNA quality was assessed via the Bioanalyzer and the samples were confirmed to have the required RNA integrity numbers (RIN) concentration (both done at U of C Genomics core). The cDNA library of each sample was prepared using Tru-Seq RNA Sample Prep Kits (Illumina, San Diego, CA) for 100 bp paired-end reads, according to the manufacturer's instructions. Each of the eight cDNA libraries was indexed for multiplexing. These indexed

libraries were sequenced on NovaSEQ6000 (Illumina, San Diego, CA) using PE100bp. Data were recorded in the FASTQ format and then imported in R for bioinformatics analysis.

Statistical Analysis

The secondary analysis of sequence data was performed on Globus Genomics,[33] an enhanced, cloud-based analytical platform that provides access to different versions of Next-Generation Sequence analysis tools and workflow capabilities. Tools such as STAR[34], featureCounts,[35]and Limma were run from within the Globus Genomics platform. We used STAR (version 2.4.2a, Stanford University, CA) aligner default parameters to align the RNA-seq reads to the reference mouse genome (GRCm38) for all the eight samples. The raw gene expression count matrix was then generated by featureCounts (version subread-1.4.6-p1). [35] The gene annotation was obtained from the Gencode vM23.[37] STAR default parameter for the maximum mismatches is 10 which is optimized based on mammalian genomes and recent RNA-seq data.

We obtained a matrix with 55213 genes and eight samples as the final output. The data was imported to R for downstream analysis. The raw gene count matrix for all samples was transformed into log-CPM (count per million) values. Genes without sufficiently large counts were filtered using EdgeR Bioconductor. [38] 20,287 genes were selected for the downstream analysis. We used Limma Voom [39] normalization to standardize the gene expression matrix, where the raw library sizes were scaled using TMM (trimmed mean of M values). Multidimensional scale plots were generated on top 6 leading FC dimensions which showed the similarity between samples.

The differential expression analysis was performed on contrast group SPF against GF using Limma. [40] Significant DEGs with p-value < 0.05 were extracted for further downstream analysis. The enrichment analysis in Toppgene suite took both the upregulated and downregulated DEGs in GF and extracted the over-represented gene ontology functional classification (molecular functions, biological processes, and cellular component). [41] The significance of the association between the datasets and bio functions were measured using a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes in that pathway. This enrichment analysis was based on mouse-to-human orthologs. A list of all DEGs and their p-values is available in Supplementary Table 1.

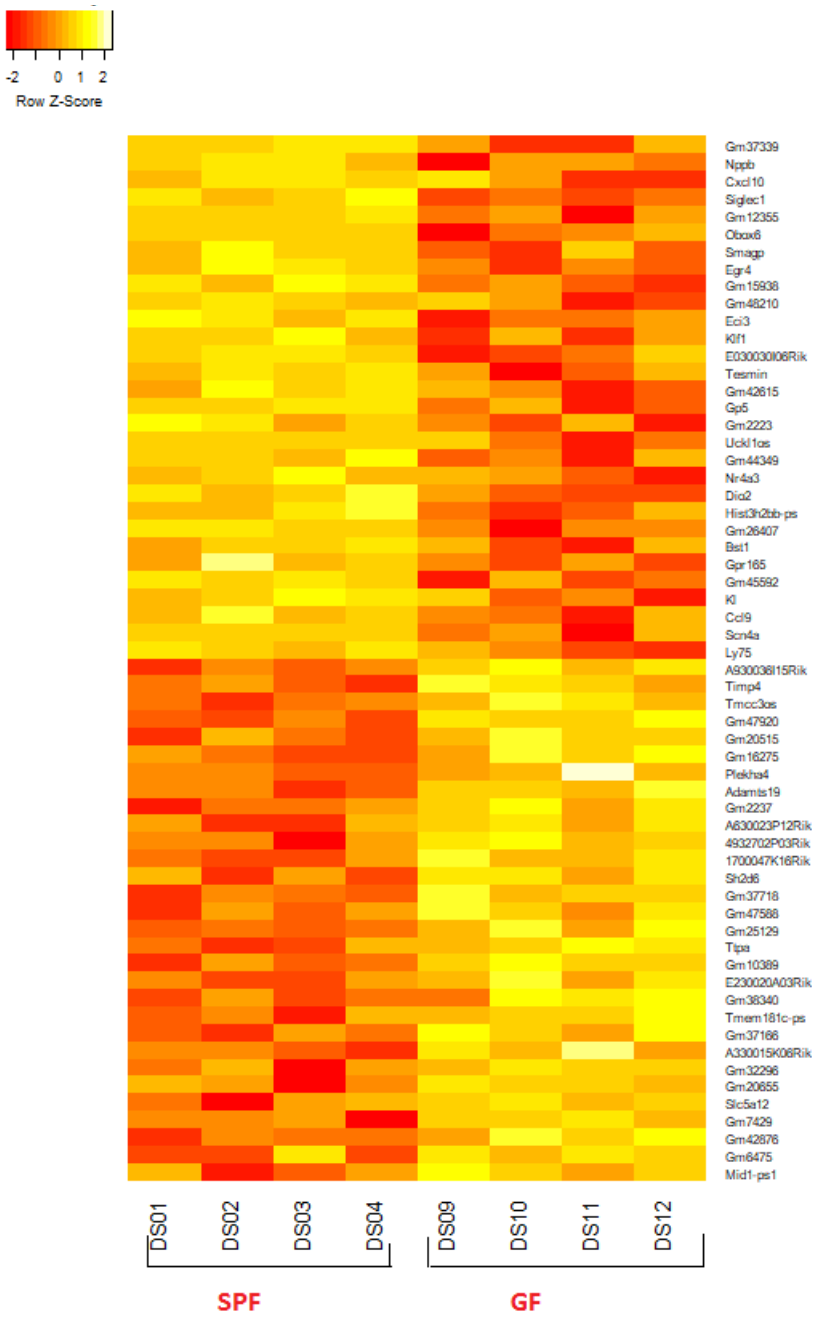
Proteins are known to work together to exert a specific function. Therefore, we mapped the identified DEGs into the STRING (Search Tool for the Retrieval of Interacting Genes) database (http://string-db.org/), which contains a wealth of validated and text mined PPIs among the proteins, to further explore the relationships of these DEGs from protein level.[42] We used the DEGs (p-value < 0.05) to identify PPI for both the upregulated and downregulated genes in GF mice.

Results

Gut microbiome modifies global retinal gene expression

To compare the retinal transcriptome profile of SPF vs.GF mice, we performed high throughput RNA-seq analysis of mouse retinas from the two groups. To obtain precise results, four separate retinas were obtained from each sample group (biological replicates). After the correction of the

raw data to remove background noise, 20,287 genes were selected for differential gene analysis. DEGs were labeled as the genes with a p-value of <0.05. A comparison between the two groups revealed that 396 DEGs were identified, 173 upregulated and 223 downregulated genes in the GF mice group. Using the criteria of log fold-change ($\log_2 FC$)>1, we performed an unsupervised analysis of these DEGs. This revealed 60 genes; 40 upregulated and 20 downregulated in the GF mice retina. Heatmap are plotted to show the hierarchical clustering of these genes (Figure 2). The volcano plot demonstrates a distinct retinal gene expression in retinas from SPF vs GF mice (Figure 2). Our data support s that there are substantial differences in the global gene expression in GF and SPF retina; hence, supporting the presence of gut-retina axis. Detailed statistics of the DEGs are available in Supplementary Table 1.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2
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Nppb
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Gm2223
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Gpr165
Gm45592
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DS11
DS12
SPF
GF

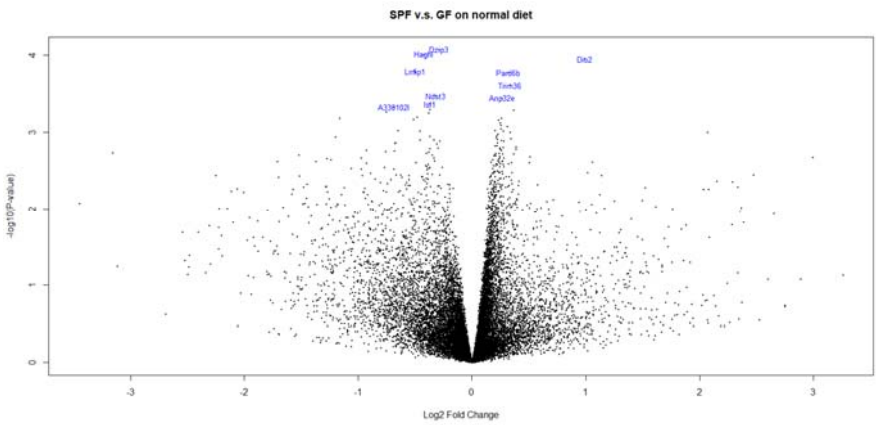


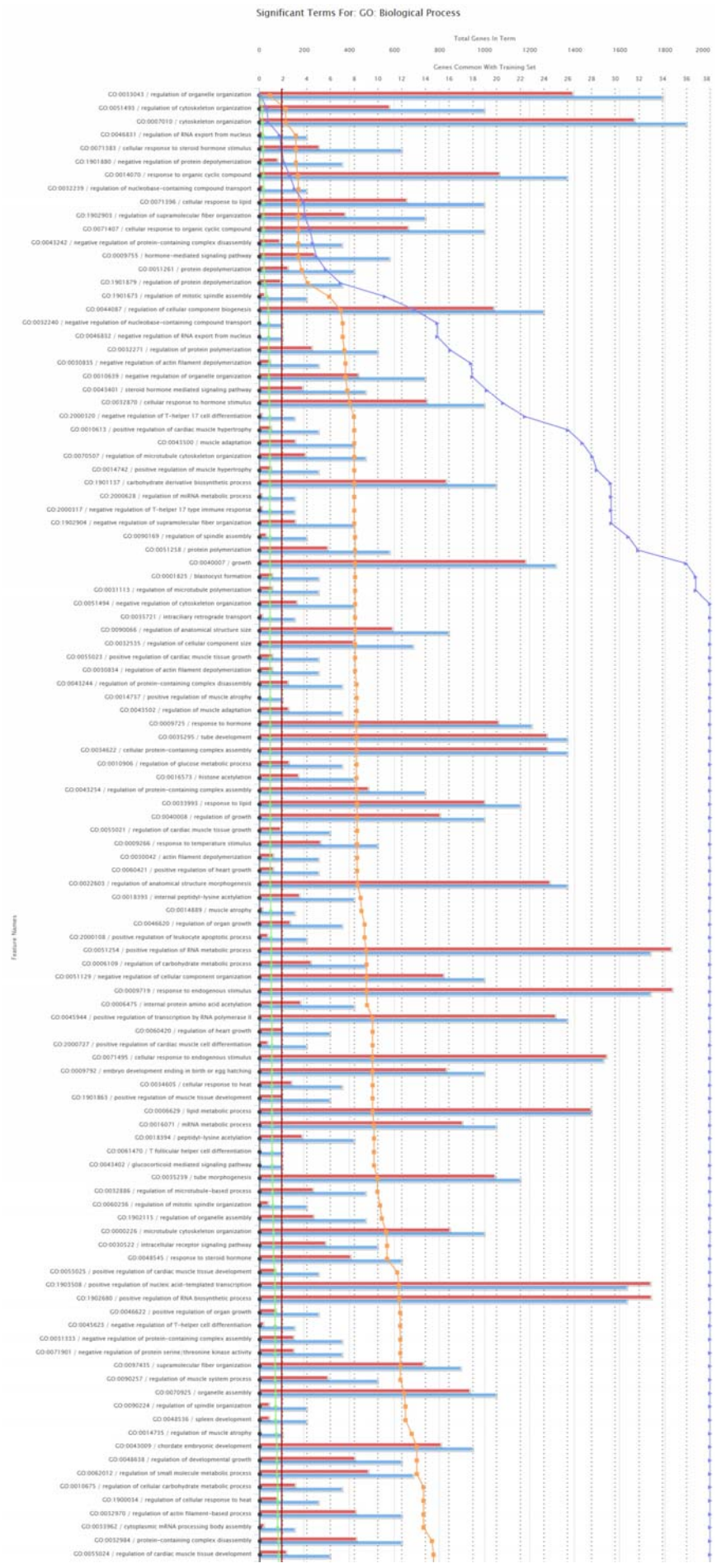
Figure2. Comparing the gene expression profiles of the GF and SPF mice
(A). Hierarchical clustering of retinal genes of GF vs SPF mice demonstrated by the heatmap. Red and yellow indicate upregulated and downregulated genes in the GF mice retina, respectively. DEGs with log2FC > 1 (more than 2-fold difference) are included in this heatmap. (B). Volcano plot showing DEGs in GF vs SPF mice shows a distinctive gene expression profile of two groups with minimal overlap. X-axis represents log2 FC and the Y-axis represents -log10 (P-values). (DS01, DS02, DS03, DS04- SPF and DS09, D10, DS11, DS12- GF)

Enrichment Analysis reveals Gut Microbiome Regulates Multiple Key Biological Pathways in Mouse Retina

The enrichment analysis for both Gene Ontology and pathways was performed using Toppgene [(41).] The genes were mapped from mouse ortholog. The enrichment analysis identifies significantly over-represented gene ontology functional categories (Figure 3) and pathways based on DEG (Figure 4). The analysis revealed that the DEGs are involved in glucocorticoid receptor binding (GO:0035259), steroid hormone effects (GO:0071383), transcription factor binding (GO:0008134), cytoskeletal stability (GO:0007010), lipid metabolism (GO:0071396) and mitogen-activated protein kinase (MAPK) activity (GO:0017017).

	Name	Source	p-Value	Number of Genes affected from dataset
1	GO:0033043	regulation of organelle organization	6.379E-7	34
2	GO:0051493	regulation of cytoskeleton organization	4.160E-6	19
3	GO:0007010	cytoskeleton organization	4.804E-6	36
4	GO:0046831	regulation of RNA export from nucleus	1.112E-5	4

5	GO:0071383	cellular response to steroid hormone stimulus	1.185E-5	12
6	GO:1901880	negative regulation of protein depolymerization	1.335E-5	7
7	GO:0014070	response to organic cyclic compound	1.646E-5	26
8	GO:0032239	regulation of nucleobase-containing compound transport	1.910E-5	4
9	GO:0071396	cellular response to lipid	2.414E-5	19
10	GO:1902903	regulation of supramolecular fiber organization	2.450E-5	14


Significant Terms For: GO: Biological Process
Total Genes In Term
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200
400
600
800
1000
1200
1400
1600
1800
2000
Genes Common With Training Set
0
2
4
6
8
10
12
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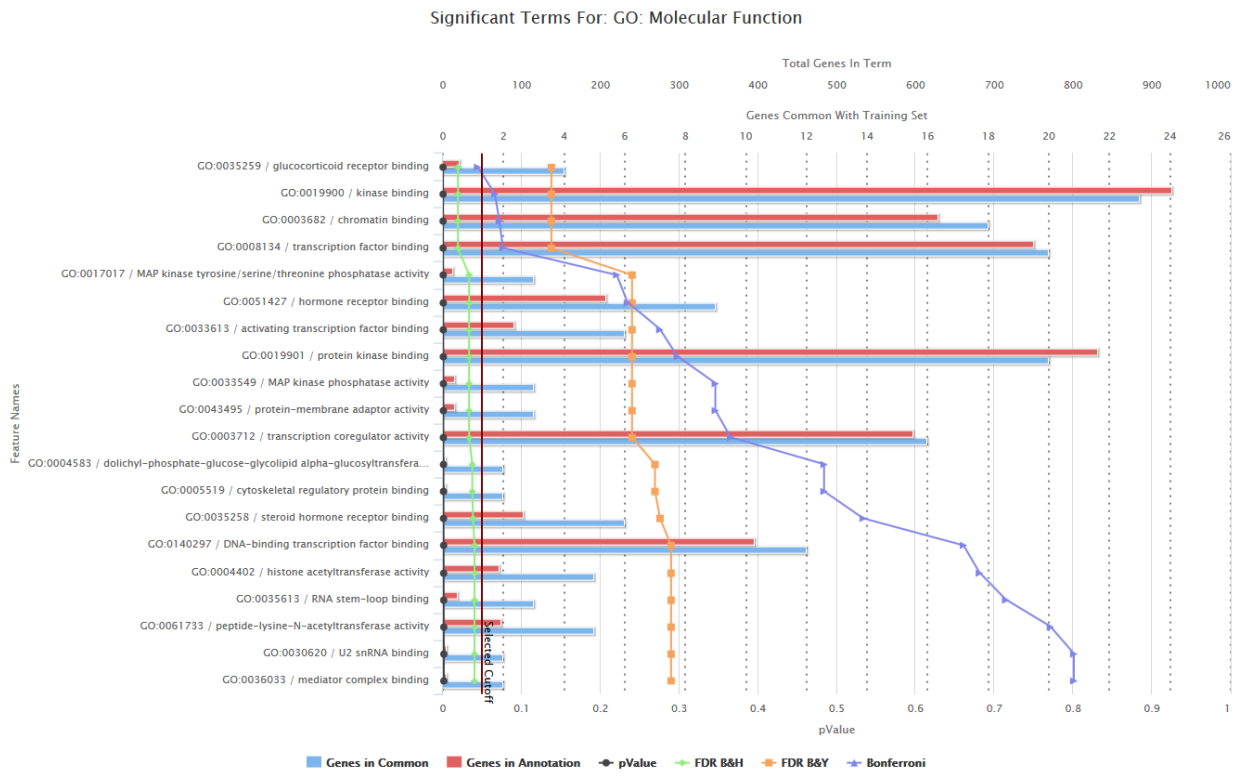
34
36
38
Feature Names
GO:0033043 / regulation of organelle organization
GO:0051493 / regulation of cytoskeleton organization
GO:0007010 / cytoskeleton organization
GO:0046831 / regulation of RNA export from nucleus
GO:0071383 / cellular response to steroid hormone stimulus
GO:1901880 / negative regulation of protein depolymerization
GO:0014070 / response to organic cyclic compound
GO:0032239 / regulation of nucleobase-containing compound transport
GO:0071396 / cellular response to lipid
GO:1902903 / regulation of supramolecular fiber organization
GO:0071407 / cellular response to organic cyclic compound
GO:0043242 / negative regulation of protein-containing complex disassembly
GO:0009755 / hormone-mediated signaling pathway
GO:0051261 / protein depolymerization
GO:1901879 / regulation of protein depolymerization
GO:1901673 / regulation of mitotic spindle assembly
GO:0044087 / regulation of cellular component biogenesis
GO:0032240 / negative regulation of nucleobase-containing compound transport
GO:0046832 / negative regulation of RNA export from nucleus
GO:0032271 / regulation of protein polymerization
GO:0030835 / negative regulation of actin filament depolymerization
GO:0010639 / negative regulation of organelle organization
GO:0043401 / steroid hormone mediated signaling pathway
GO:0032870 / cellular response to hormone stimulus
GO:2000320 / negative regulation of T-helper 17 cell differentiation
GO:0010613 / positive regulation of cardiac muscle hypertrophy
GO:0043500 / muscle adaptation
GO:0070507 / regulation of microtubule cytoskeleton organization
GO:0014742 / positive regulation of muscle hypertrophy
GO:1901137 / carbohydrate derivative biosynthetic process
GO:2000628 / regulation of miRNA metabolic process
GO:2000317 / negative regulation of T-helper 17 type immune response
GO:1902904 / negative regulation of supramolecular fiber organization
GO:0090169 / regulation of spindle assembly
GO:0051258 / protein polymerization
GO:0040007 / growth
GO:0001825 / blastocyst formation
GO:0031113 / regulation of microtubule polymerization
GO:0051494 / negative regulation of cytoskeleton organization
GO:0035721 / intraciliary retrograde transport
GO:0090066 / regulation of anatomical structure size
GO:0032535 / regulation of cellular component size
GO:0055023 / positive regulation of cardiac muscle tissue growth
GO:0030834 / regulation of actin filament depolymerization
GO:0043244 / regulation of protein-containing complex disassembly
GO:0014737 / positive regulation of muscle atrophy
GO:0043502 / regulation of muscle adaptation
GO:0009725 / response to hormone
GO:0035295 / tube development
GO:0034622 / cellular protein-containing complex assembly
GO:0010906 / regulation of glucose metabolic process
GO:0016573 / histone acetylation
GO:0043254 / regulation of protein-containing complex assembly
GO:0033993 / response to lipid
GO:0040008 / regulation of growth
GO:0055021 / regulation of cardiac muscle tissue growth
GO:0009266 / response to temperature stimulus
GO:0030042 / actin filament depolymerization
GO:0060421 / positive regulation of heart growth
GO:0022603 / regulation of anatomical structure morphogenesis
GO:0018393 / internal peptidyl-lysine acetylation
GO:0014889 / muscle atrophy
GO:0046620 / regulation of organ growth
GO:2000108 / positive regulation of leukocyte apoptotic process
GO:0051254 / positive regulation of RNA metabolic process
GO:0006109 / regulation of carbohydrate metabolic process
GO:0051129 / negative regulation of cellular component organization
GO:0009719 / response to endogenous stimulus
GO:0006475 / internal protein amino acid acetylation
GO:0045944 / positive regulation of transcription by RNA polymerase II
GO:0060420 / regulation of heart growth
GO:2000727 / positive regulation of cardiac muscle cell differentiation
GO:0071495 / cellular response to endogenous stimulus
GO:0009792 / embryo development ending in birth or egg hatching
GO:0034605 / cellular response to heat
GO:1901863 / positive regulation of muscle tissue development
GO:0006629 / lipid metabolic process
GO:0016071 / mRNA metabolic process
GO:0018394 / peptidyl-lysine acetylation
GO:0061470 / T follicular helper cell differentiation
GO:0043402 / glucocorticoid mediated signaling pathway
GO:0035239 / tube morphogenesis
GO:0032886 / regulation of microtubule-based process
GO:0060236 / regulation of mitotic spindle organization
GO:1902115 / regulation of organelle assembly
GO:0000226 / microtubule cytoskeleton organization
GO:0030522 / intracellular receptor signaling pathway
GO:0048545 / response to steroid hormone
GO:0055025 / positive regulation of cardiac muscle tissue development
GO:1903508 / positive regulation of nucleic acid-templated transcription
GO:1902680 / positive regulation of RNA biosynthetic process
GO:0046622 / positive regulation of organ growth
GO:0045623 / negative regulation of T-helper cell differentiation
GO:0051333 / negative regulation of protein-containing complex assembly
GO:0071901 / negative regulation of protein serine/threonine kinase activity
GO:0097435 / supramolecular fiber organization
GO:0090257 / regulation of muscle system process
GO:0070925 / organelle assembly
GO:0090224 / regulation of spindle organization
GO:0048536 / spleen development
GO:0014735 / regulation of muscle atrophy
GO:0043009 / chordate embryonic development
GO:0048638 / regulation of developmental growth
GO:0062012 / regulation of small molecule metabolic process
GO:0010675 / regulation of cellular carbohydrate metabolic process
GO:1900034 / regulation of cellular response to heat
GO:0032970 / regulation of actin filament-based process
GO:0033962 / cytoplasmic mRNA processing body assembly
GO:0032984 / protein-containing complex disassembly
GO:0055024 / regulation of cardiac muscle tissue development

	Name	Source	p-Value	Number of Genes affected from dataset
1	GO:0035259	glucocorticoid receptor binding	5.673E-5	4
2	GO:0019900	kinase binding	8.485E-5	23
3	GO:0003682	chromatin binding	9.231E-5	18
4	GO:0008134	transcription factor binding	9.808E-5	20
5	GO:0017017	MAP kinase tyrosine/serine/threonine phosphatase activity	2.844E-4	3
6	GO:0051427	hormone receptor binding	3.017E-4	9
7	GO:0033613	activating transcription factor binding	3.548E-4	6
8	GO:0019901	protein kinase binding	3.832E-4	20
9	GO:0033549	MAP kinase phosphatase activity	4.456E-4	3
10	GO:0043495	protein-membrane adaptor activity	4.456E-4	3



	Name	Source	p-Value	Number of Genes affected from dataset
1	GO:0016604	nuclear body	8.093E-7	24
2	GO:0015630	microtubule cytoskeleton	5.018E-5	29
3	GO:0036477	somatodendritic compartment	1.459E-4	24

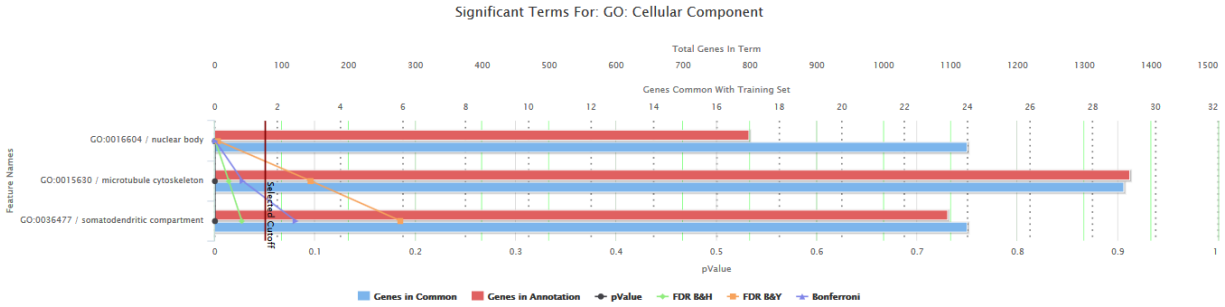
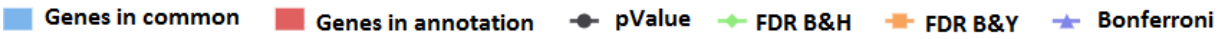


Figure 3A, B, C. Table for top 10 biologic processes, molecular function and cell component revealed by enrichment analysis in the DEGs. Corresponding bar-charts show the genes affected

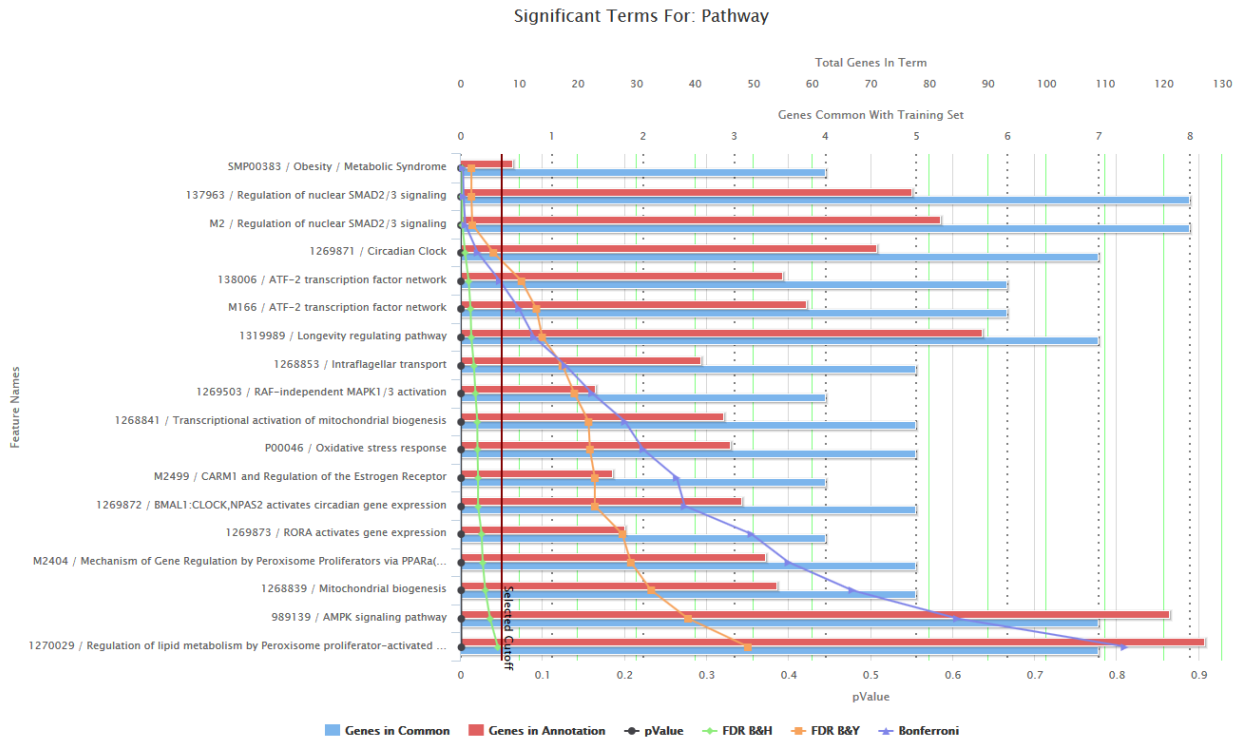
in our dataset compared to the total number of genes involved in the condition. The figure is made using Toppgene.



The absence of the gut microbiome influenced sixteen biologic pathways in the GF retina (Figure 4). All these pathways are downregulated in the GF mice retina compared to the SPF group. These pathways were involved in obesity/metabolic syndrome, adenosine monophosphate-activated protein kinase (AMPK), nuclear SMAD 2/3 signaling, mitochondrial biogenesis, lipid metabolism, longevity, and oxidative stress response, which are already known to be crucial in the pathogenesis of numerous retinal diseases. However, novel pathways with no known function in retinal diseases, but demonstrated to be regulated by the gut-microbiome, for example, intraflagellar transport, and the circadian clock, are also shown to be affected by the gut microbiome. Our data shows that the DEGs in the modified pathways have considerable overlap (Figure 4). PPARGC1A (now referred to as PGC-1α) is involved in twelve of the sixteen pathways and seems to be especially important.

Pathway	P-value	Number of Genes involved from the dataset
Obesity / Metabolic Syndrome	1.90E-06	PPARGC1A,NCOA2,NR3C1,EP300
Regulation of nuclear SMAD2/3 signaling	2.42E-06	MEF2C,NCOA2,FOXO3,SIN3A,NR3C1,ATF2,EP300,SMAD7
Circadian Clock	1.50E-05	NRIP1,MEF2C,PPARGC1A,NCOA2,NR3C1,ATF2,EP300
ATF-2 transcription factor network	3.48E-05	PPARGC1A,CUL3,DUSP5,DUSP8,ATF2,EP300
Longevity regulating pathway	6.54E-05	PPARGC1A,KL,PIK3CB,RPTOR,FOXO3,ATF2,ADIPOR1
Intraflagellar transport	9.37E-05	TTC26,WDR35,TNPO1,WDR19,DYNC2LI1
RAF-independent MAPK1/3 activation	1.18E-04	JAK1,DUSP5,DUSP7,DUSP8
Transcriptional activation of mitochondrial biogenesis	1.47E-04	MEF2C,PPARGC1A,NCOA2,ATF2,ATP5F1B
Oxidative stress response	1.64E-04	MEF2C,DUSP5,DUSP7,DUSP8,ATF2
BMAL1:CLOCK,NPAS2 activates circadian gene expression	2.01E-04	NRIP1,PPARGC1A,NCOA2,NR3C1,EP300
Mitochondrial biogenesis	3.51E-04	MEF2C,PPARGC1A,NCOA2,ATF2,ATP5F1B
AMPK signaling pathway	4.44E-04	ACACA,PPARGC1A,PIK3CB,RPTOR,FOXO3,PPP2R3A,ADIPOR1
RORA activates gene expression	2.61E-04	NRIP1,PPARGC1A,NCOA2,EP300
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	4.91E-04	NRIP1,ME1,CITED2,PPARGC1A,EP300

Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	5.94E-04	ME1,PPARGC1A,NCOA2,SIN3A,MED13L,MED19,EP300
CARM1 and Regulation of the Estrogen Receptor	6.28E-04	NRIP1,MEF2C,PPARGC1A,EP300



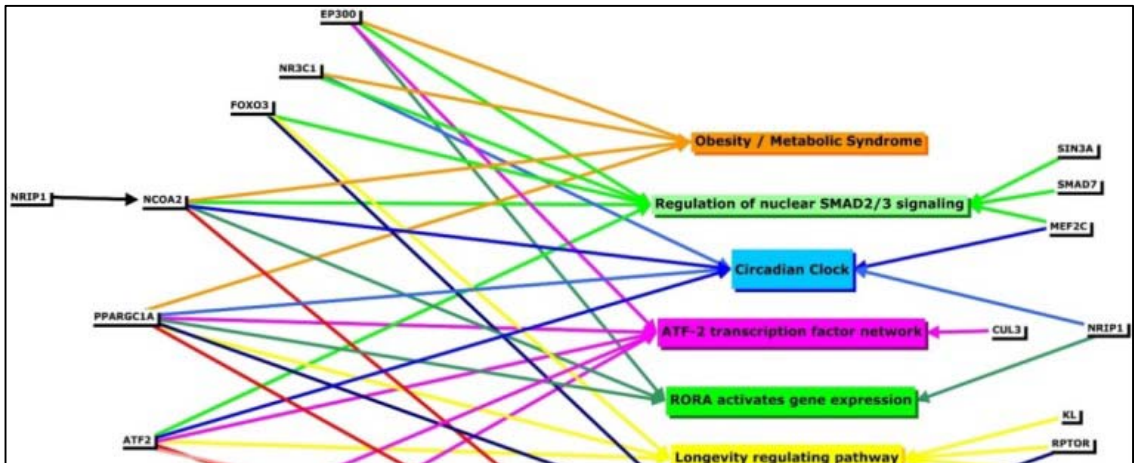
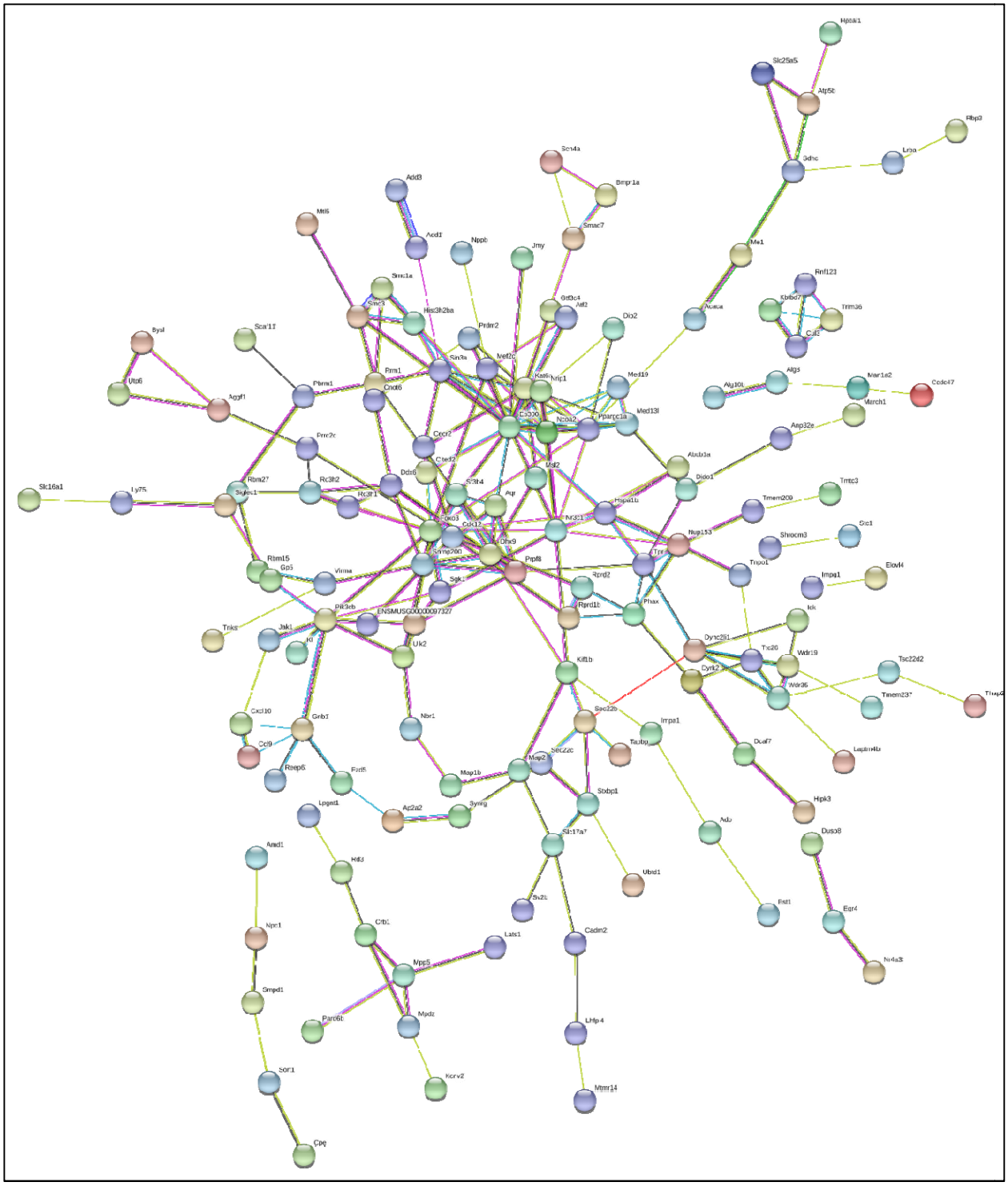


Figure 4: The pathways downregulated by the DEGs revealed by enrichment analysis. Corresponding bar-charts showing the genes affected in our dataset compared to the total number of genes involved in the GF mice. The figures are made using Toppgene.
Figure 4b. Select pathways and their corresponding involved genes revealed by enrichment analysis. The downregulated pathway is in the middle of the figure. Genes involved in multiple pathways are represented on both the left and right of the figure.

Protein-Protein interactions

Using the STRING database with both the upregulated and downregulated DEGs in the GF retina groups, PPI analysis was done. In the PPI network, a node represents a protein and the degree of a node represents the number of the interplayed pairs with other proteins of this specific node. The proteins which are the predominant nodes with a high degree of connections to other proteins (hub nodes) include histone acetyltransferase p300 (EP300), PGC1a, and forkhead box O3 (FOXO3) in the analysis conducted on the DEGs downregulated in the GF mice. The PPI constructed from the DEGs upregulated genes is sparser and more disconnected, as would be expected from the earlier enrichment analysis (Fig. 5). The pathway analysis of the downregulated proteins GF retina shows that they are involved in are AMPK and longevity (Supplementary table 2).


Hpsa1
Slc25a5
Atp5b
Rbp3
Lrba
3dhc
Sch4a
Bmpr1a
Add3
Mtl6
Acd1
Nppb
Jmy
Smac7
Me1
Rnf123
Kbtbd7
Trim36
Cul3
Acaca
Smc1a
Smc3
Hist3h2ba
Gtf3c4
Atf2
Dio2
Prdm2
Bysl
Sca11
Sin3a
Mef2c
Prm1
Utp6
Aggf1
Pbrm1
Cnot6
Kat6
Nrip1
Med19
Alg3
Alg10b
Mar1e2
Cede47
March1
Ppargc1a
Med13l
Ccdc2
Ccdc2
Ccdc2
Prrc2c
Msl2
Aap32e
Abdb1a
Dido1
Slc16a1
Ly75
Rbm27
Rc3h2
Rc3h1
Ddx6
Sf3b4
Aqr
Hspa1b
Tmtc3
Tmem209
Foxo3
Cdk12
Nr3c1
Nup153
Shroom3
Sce1
Dhx9
Snrnp200
Prpf8
Tpr
Tnpo1
Rbm15
Gp5
Virma
Sgk1
Rprd2
Elovl4
Impg1
Rprd1b
Phax
Ikk
Pik3cb
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Tnks
Jak1
Kl
Ulk2
Dync2li1
Tsc20
Wdr19
Tsc22d2
Kif1b
Cyrk2
Wdr36
Tmem237
Thap2
Cxcl10
Grb1
Nbr1
Sec22b
Impa1
Ccl9
Tacbp
Dcaf7
Rbep6
Fzd5
Map1b
Map2
Sec22c
Lapbm4b
Sbbp1
Hpk3
Lpgat1
Ap2a2
Synrg
Ado
Duso8
Amd1
Rd3
Slc17a7
Ubid1
Sv2b
Est1
Egr4
Npc1
Crb1
Lats1
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Kcnv2
Mtmr14
Cpq

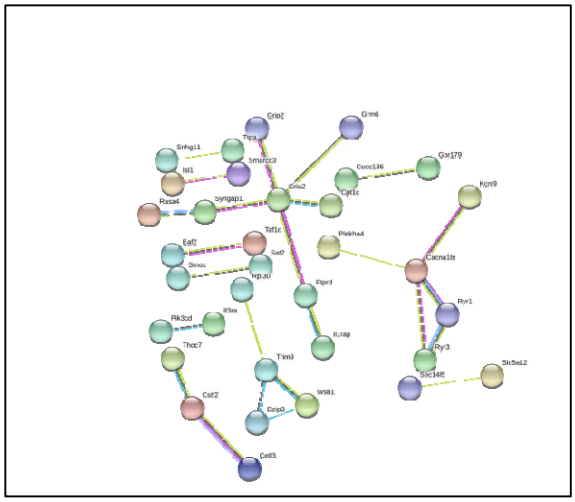

Figure 5. String networks of protein-protein interaction (PPI) generated using DEGs (p-value < 0.05). A. Shows the proteins that were involved in the downregulated genes in the GF mice B. Figure shows the network of the proteins downregulated in GF mice

Discussion

In this study, we used high throughput RNA sequencing to compare the entire retinal transcriptome of whole retinas from GF and SPF mice. Our results show that retinal gene expression is altered by the gut-microbiome the presence of gut microbiotas. 396 DEGs (p-value <0.05) are identified between the two groups. Functional categories the DEGs belong to include glucocorticoid receptor binding, steroid hormone response, lipid metabolism, and transcription factor binding. Additional genes involved in cytoskeletal stability and regulation of T-helper-17 (Th17) differentiation are also affected. Analysis of the DEGs shows pathways affected by the gut-microbiome are obesity/metabolic syndrome, MAPK, mitochondrial biogenesis, longevity, AMPK, and oxidative stress (Figure 4). Molecules proved shown to be regulated by the gut-microbiome in the gastrointestinal tract like tissue metallopeptidase inhibitor 4 [43] and mucin 2 [44] are also affected in the GF mice retina. Hub nodes in the downregulated PPI network include PGC1a, EP300, and FOXO3; all of which are involved in the pathogenesis of retinal diseases. Notably, the pathways downregulated in GF retina protein analysis are AMPK and longevity pathways, further confirming that these pathways are indeed influenced in the retina by gut microbial status.

We hypothesized that the gut microbiome regulates retinal function by modifying retinal gene expression. To test the hypothesis, we use GF mice which are considered the gold standard for proof-of-concept questions for microbiome studies. As discussed above, the GF mice are important for demonstrating correlative relationships. [21,22,25] They have proved invaluable for establishing causal links between the microbiome and diverse diseases such as inflammatory bowel disease, cancers, autoimmune conditions, and neurologic conditions. [45,46] Recent evidence highlights the importance of the gut microbiota in modulating response to different cancer immunotherapy drugs. [47] Methods for studying the microbiome include microbiota inhibition using antibiotics, diet alteration to cause gut dysbiosis (changes microbial

composition), and the introduction of microbial strains directly into animals that have conventional microbiota. [48] Modulating the microbiome through diet and/or antibiotics is relatively easier, cheaper, and does not require specialized facilities compared to the GF mice. Nonetheless, using antibiotics has limitations, including off-target drug effects, only partial ablation of microbes, possible selection of resistant strains, and direct influence of the drug on the genetic and metabolic machinery of host tissue [48]. Dietary manipulation results in alteration of the microbiota; however, a high-fat diet may cause obesity regardless of the composition of gut microbiota in mice. [49] Therefore, in addition to the indirect dietary influence caused by dysbiosis, it is likely that there is a direct impact of the dietary constituents on the host tissue. Introducing microbial strains by fecal transplant has revealed to be beneficial in ocular surface conditions, e.g. it shows an improved goblet cell density, decreased corneal barrier disruption, and dacryoadenitis in animal models of Sjogren's syndrome. However, only very limited studies comparable investigations have been attempted for retinal diseases. Host-microbe interactions can be studied more accurately and precisely without the background noise from a preexisting microbiome in the GF mice. [21,22] Hence, we chose GF mice to identify genes and pathways involved in the gut-retina axis. To our knowledge, this is the first report using GF mice to outline the transcriptomic changes in the retina caused by changes in the gut microbiome.

As mentioned earlier, there are compositional and functional differences in the enteric microbiome in AMD, POAG, RAO, and ROP patients. Investigators are trying to comprehend how different species of microbiota contribute to genomic changes in the host. Numerous theories have been proposed; the most accepted are the downstream impact from the metabolites produced from the microbiota. Taylor *et al*. show that modifying the gut-microbiome by a high-glycemic-index diet leads to retinal damage; they use metabolomics to compare the severity of the retinal damage with multiple microbial metabolites.[4] Other studies show the protective nature of the gut microbiota metabolites in the retina; exogenous short-chain fatty acids (SCFA) decreases autoimmune uveitis,[50] ursodeoxycholic acid (UDCA) ameliorates DR [51] and tauroursodeoxycholic acid (TUDCA) protects against cell death in retinal degeneration, [52,53]retinal detachment,[54] and protects neural retina in the diabetic mouse model.[55] Further credence is provided to this theory by the presence of SCFA transporters, sodium-coupled monocarboxylate transporter 1 and 2 (SMCT1 and SMCT2), and receptor G protein-coupled receptor 109A (GPR109A) in the retina.[56] Our data shows that SMCT2 (SLC5A12) gene is upregulated in GF mice (Supplementary Table 1). Another recent study shows that modifying the gut microbiome by feeding mice *L.paracasei KW3110* demonstrates favorable changes in retinal function and morphology on the aging retina.[19] These studies lay the groundwork for the connection between the enteric microbiome and the retina; however, these investigations did not reveal if the microbiota or its their metabolites may alter the retinal gene expression. RNA-seq is a revolutionary technique that can generate very precise results to study the entire transcriptome of the tissue. In our study, we used high throughput RNA-seq to evaluate the retinal transcriptome of GF mice retina. We generated a detailed view of the genes and pathways affected in the retina by the gut microbiome but further studies are required regarding the mechanism by which gut microbiota regulates the retinal gene expression as seen in our studies.

The importance of DEGs in retinal diseases including AMD, POAG, and DR (Figure 6) is well-recognized. The most influenced gene in our data is the transcriptional coactivator PGC-1α, which is affected in 12 of the 16 altered pathways (figure 4B). This gene is involved in numerous

retinal diseases. Zhang *et. al* show that AMPK/PGC-1α pathway is dysfunctional in AMD patients. They show that alteration of this pathway leads to increased intracellular accumulation of lipids and cellular waste, decreased mitochondrial biogenesis and turnover, and subsequent mitochondrial dysfunction.[57] Our data shows both PGC-1α and AMPK pathways are differentially expressed in the GF mice retina, indicating that the gut microbiome is involved in the regulation of this pathway in the retina. Additional enrichment analysis reveals genes involved in downstream activities of AMPK/PGC-1α including lipid metabolism and mitochondrial biogenesis are also influenced (Figure 5). AMD is a heterogeneous disease with a very unpredictable course; if the differences in gut-microbiome cause AMPK/PGC-1α dysregulation and explain this heterogeneity in different patients remains to be seen. PGC-1α is also the major transcriptional coactivator for steroid hormone receptors and its nuclear receptors. Expectedly, glucocorticoid receptor binding and cellular effects of steroids are among the top ten dysfunctional molecular functions and biologic processes affected in GF mice (Figure 3). We show for the first time that and PGC-1α- steroid pathways are both modified in the retina through the gut-microbiome. However, further studies of protein expression and hormone activity are needed to determine the microbiome's influence on PGC-1α protein in models of specific retinal diseases.

AMPK can function through genes other than the PGC-1A. One of these principal pathways is the mammalian target of rapamycin (mTOR); RPTOR (Regulatory Associated Protein of MTOR Complex 1), the central gene of the mTOR pathway is downregulated in GF mice retina. mTOR activates protein and lipid anabolism and inhibits autophagy. Overactivity of mTOR has been shown in AMD,[57] glaucoma [58], and diabetic retinopathy. In DR, VEGF synthesis is stimulated via the mTOR pathway which leads to activation of downstream signaling cascade including AMPK and increased angiogenesis and blood-retinal barrier (BRB) breakdown. [59] The activation of AMPK leads to further activation of mTOR forming an activation loop. The bidirectional cross-talk between the microbiome and mTOR is through microbial metabolites, SCFA, and (trans-10, cis-12 conjugated linoleic acid (t10, c12 CLA). Reciprocally, the mTOR pathway influences the composition of gut microbiota by controlling gut barrier function. Interestingly, genes maintaining the retinal-blood barrier, like Elovl and Slc16a1 are also downregulated in GF mice. The concept of a leaky gut syndrome or increased intestinal permeability based on variations in the microbiome has been accepted. [60] This concept has also been extrapolated to the blood-brain barrier; based on our data, we propose that the enteric microbiome may regulate the permeability of the retinal blood-brain barrier.

The most impacted pathway in the GF retina is the obesity/metabolic syndrome (Figure 4). The genes involved are PGC1α, nuclear receptor coactivator 2(NCOA2), nuclear receptor subfamily 3 group C member 1(NR3C1), and EP300. The proteins for these genes also appear to have a prominent position in the PPI, and both PGC1α and EP300 are major hub nodes in the downregulated genes in GF mice (Figure 5). All these genes and their proteins have a prominent function in the steroid hormone metabolism. EP300 is crucial for retinal biology; it is a co-activator of hypoxia-inducible factor 1 alpha (HIF-1A)and stimulates the hypoxia-induced genes such as vascular endothelial growth factor (VEGF).[61] VEGF is the major angiogenic factor involved in AMD. [62,63,64,65,66] Anti-VEGF injections inhibit angiogenesis in neovascular AMD, DR, and retinal vein occlusion (RVO). Our data shows that the EP300-HIF-1-VEGF pathway is downregulated in the GF mice retina which suggests that microbiome modulates the

expression of angiogenesis-related genes and pathways in the retina. [67] Hence, the gut-microbiome may be of worthy consideration in pathogenesis of retinal diseases with pathologic neovascularization. These findings support the involvement of the gut microbiome in disease development and progression; but, precise future studies are needed to determine the exact gut microbiome-retinal angiogenesis pathway.

Another downregulated pathway in the GF retina is the Smad2/3 signaling pathway. The Smad signaling pathway is synergistic with the MAPK. MAPK phosphorylates multiple transcription factors, including c-Jun and ATF-2 and Smad proteins. Phosphorylation by MAPK pathways results in subsequent activation of their transcriptional activity. Predictably, each of these transcription factor genes and MAPK are downregulated in GF retina. There is crosstalk between MAPK signaling and several different pathways, such as VEGF and oxidative stress. [68] Oxidative stress via reactive oxygen species (ROS) serves as an important signal that activates MAPK signaling, which is also dysregulated in the GF eyes. MAPK activation has been implicated in the pathogenesis of several retinal diseases including AMD and DR. However, despite this, several ocular MAPK inhibitors have failed to produce desired outcomes in retinal diseases; in fact, MAPK drugs for tumors may have serious ocular side effects. [69] Given our data, the regulation of MAPK via the gut-microbiome may serve as a probable target for intervention in retinal diseases.

A recent study shows melatonin inhibits inflammation and apoptosis in animals with DR via the MAPK pathway. [70] A separate study shows that the gut microbiota is sensitive to melatonin and expresses endogenous circadian rhythmicity. [71] Our study supports that several retinal genes of the circadian rhythm clock are affected by the gut microbiome. The mammalian retina contains a circadian clock system independent from the central clock located in the suprachiasmatic nucleus (SCN) of the brain. [72] Studies show that circadian clock dysregulation has been linked to obesity. Dysregulation of the circadian clock affects the WNT/β-catenin pathway, malfunction of which is also connected to exudative wet AMD. [73] The gut microbiome regulates circadian rhythm in the brain;[74] but, we provide the first evidence that gut microbiome can also affect the circadian circuit in the retina.

Another important set of genes that are affected in GF mice retina is involved in longevity. Currently, only two genetic factors have been consistently found to contribute to longevity; apolipoprotein E (Apo-E) and FOXO3. [75] Predictably, the analysis of PPI of the downregulated genes in the GF mice retina also highlights FOXO3 as a hub node involved with several other proteins. Aging is the most significant factor contributing to the pathogenesis of retinal degenerative diseases, like AMD. FOXO3 transcription factor is involved in cell survival and apoptosis. PI3K/Akt inhibitors via FOXO3 have demonstrated some efficacy in ameliorating neovascularization in mice models. Gut dysbiosis induced by high-fat diet leads to the inactivation of FOXO3 in the intestine. Our data indicate that the gut microbiome can affect the gene expression of FOXO3 in the mouse retina, but more studies are needed to determine the protein expression and activity of these genes in the retina.

Gut-microbiome is well-known to cause downstream sequelae by mediating both innate and adaptive elements of the immune system.[76] GF mice demonstrate an increase in T helper cell type 2 (Th2) compared to T helper cell type 1 (Th-1) cells and a decrease in T helper 17 cells

(Th-17), T regulatory cells (Tregs), and IL-12 formation. [77] Similarly, the Th17 differentiation pathway in GF mice retina is affected through RC3H1, RC3H2, SMAD7 genes. Both AMD and POAG patients have increased Th1/ Th17 response. [78] Several genes of the cytokine-cytokine receptor interaction are also affected by the gut- microbiome in the retina. Membrane cofactor protein (CD46), a regulator of the alternative pathway of the complement system, is also downregulated in the GF mice retina. CD46 decrease can lead to hyperactivation of the complement system and it has been established that there is a central role of inflammatory pathogenesis in AMD. Retinal pigment epithelial (RPE) cells, the main cell implicated in the AMD pathogenesis, lose their CD46 expression in geographic atrophy even before the appearance of morphologic changes. [79] $Cd46^{-/-}$ knockout mice are used as an experimental animal model who spontaneously develop a dry-type AMD-like phenotype.[80] Our experiment reveals that both innate and adaptive immunity pathways are disrupted in the GF mice retina. Can the microbiome be the unknown link between the sterile inflammation at an immune protected site and AMD is yet to be determined.

Toppgene analysis also provides analysis for the number of diseases related to a particular cohort. The retinal diseases that are involved in the DEGs (p-value-<0.0.5) are shown below (Figure 7).

Several long non-coding RNAs (lncRNA) are differentially expressed in the GF and SPF mice retina (Supplementary Table 1). Long non-coding RNAs (lncRNAs) are increasingly recognized to control cellular functions and regulate transcriptional and translational processes of the neighboring genes. Gut microbiota critically regulates the expression of lncRNAs not only locally in the intestine but also remotely in other metabolic organs, like brown adipose tissue and muscle. [80] The exact role of lncRNA in either the retinal disease is unclear at the moment; however, they have an important function in the retinal ganglion cell differentiation. Our study indicate that gut microbiome could affect retinal functions via regulation of retinal lnc-RNAs expression and possible downstream effects on neighboring genes as seen in other organs.

Nonetheless, the GF mouse studies have some limitations since as host physiological parameters are altered in these mice. The lack of microbiota produces systemic changes in these animals, ranging from an inability to efficiently digest food to an under-developed immune system. Other variations include lengthier intestinal epithelial turnover, lower nutritional requirements, and less body fat despite increased consumption. Moreover, our data is based on RNA-seq alone and no studies of protein expression or activity are performed. RNA-seq studies typically provide the basis for further experiments using specific disease animal models and subsequent human diseases.

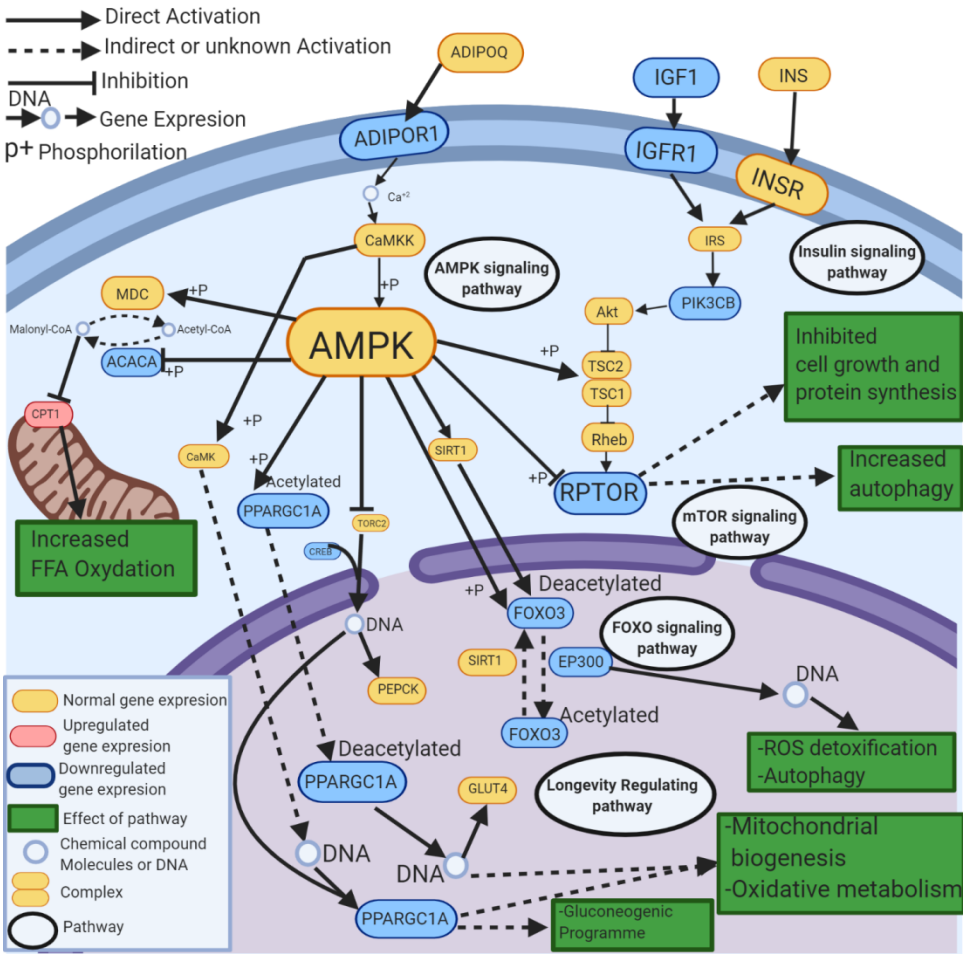

Figure 6. AMPK and the genes and pathways perturbed by the gut-microbiome in GF mice. Image made using Biorender.

Gene Symbol	Gene Name
IMPG1	Interphotoreceptor matrix proteoglycan 1
KCNV2	potassium voltage-gated channel modifier subfamily V member 2
MPDZ	multiple PDZ domain crumbs cell polarity complex component
MEF2C	myocyte enhancer factor 2C
PPARGC1A	PPARG coactivator 1 alpha
KL	klotho
AMD1	adenosylmethionine decarboxylase 1
RBP3	retinol binding protein 3
NR4A3	nuclear receptor subfamily 4 group A member 3
CRB1	crumbs cell polarity complex component 1

Gene Symbol	Gene Name
SLC6A6	solute carrier family 6 member 6
SNRNP200	small nuclear ribonucleoprotein U5 subunit 200
RD3	retinal degeneration 3, GUCY2D regulator

Figure 7. DEGs involved in retinal diseases revealed by bioinformatics analysis (Toppgene)

Conclusion

In this study, we explored the retinal transcriptome using high-throughput RNA-seq to investigate the effect of the gut microbiome on whole retinal transcriptome using GF mice to investigate the genes and pathways involved in gut-retina axis.
Our data provide strong evidence for the regulation of important pathways including AMPK, longevity, oxidative stress, MAPK and mitochondrial biogenesis by the gut-microbiome in the retina. Hence, we propose that regulation of biologic retinal pathway via the gut microbiome may be important in retinal diseases. The microbiome can cause variance in gene expression has been demonstrated in other organs; but, this is the first report providing solid evidence of the existence of the gut-retina axis. Even though our data indicates that gut microbiome is a powerful regulator of retinal gene expression, further analyses are needed to elucidate how retina protein expression and cellular functions are modified by the gut-microbiome and their correlation with retinal disease pathogenesis.

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