Supplemental material

Figure S1. Identifying molecular stages of disease in the optic nerve head by hierarchical clustering. (A) Two comparisons were used to identify disease-associated DE genes for clustering ONH samples. The first comparison identified genes that were DE due to glaucoma progression or *Gpnmb* genotype. The second comparison, between 4.5 months old DBA/2J and 10.5 months D2-*Gpnmb*$^{+}$ mice allowed subtraction of genes that differed due to *Gpnmb* genotype. 4.5 months old DBA/2J mice have never demonstrated high IOP or glaucoma in our colony (34). **(B-C)** Distributions of axon numbers. There is no significant axon loss in either eyes with NOE damage (B) or molecular stages 1 and 2 (C), compared to *Gpnmb*$^{+}$ eyes. Half of the eyes in molecular stage 3 have no axon loss indicating that the similar patterns of gene expression that define all eyes in this stage are established prior to axon loss. Significant axon loss is clearly present in all eyes with conventionally defined moderate (MOD) and severe (SEV) glaucoma (B), and the subset of these eyes in stages 4 and 5 (C). The green diamonds indicate the mean and the 95% confidence interval.

Figure S2. Composition of the three groups (dataset 1, 2, and 3)

In this study, 50 eyes were grouped based on three distinct criteria. The middle column shows all 50 eyes used in the gene expression profiling study. Each eye is named based on the conventional morphological criteria (optic nerve damage assessment) followed by a sequential number. The way in which the eyes clustered into molecular groups based on hierarchical clustering of either the ONH (left, Figure 1) or retina (right, Figure 7) gene expression profiles are shown. Blue lines connect the same eye in each column.

Figure S3: ECM-receptor interaction pathway in ONH.

(A) A high scoring network of DE genes in the ONH that was generated using IPA when comparing stage 2 to D2-*Gpnmb*$^{+}$ controls. The network contains genes of the ECM-receptor interaction pathway. Within this network, genes that control the function of other ECM-relevant genes include IL1B (IL1 in network) and ITGB3. Gene names are standard names provided by IPA. Up-regulated genes are shown in red, down-regulated genes in green. The darker the color, the greater the relative fold changes with respect to D2-*Gpnmb*$^{+}$ controls. Genes are classified based on their function. The shape associated with the gene name(s) relates to the known/putative functional class (diamonds = enzymes, ovals = transcription factors, triangles = kinases, and circles = others). Solid lines indicate direct interactions and dotted lines indicate indirect interactions. (**B**) Expression levels for *Itgb3*, *Il1b* and *Casp1* (also known as interleukin converting enzyme, an activator of *Il1b*). All of these genes are differentially expressed compared to D2-*Gpnmb*$^{+}$ controls ($q \leq 0.05$) across all stages of disease.

Figure S4: Figure of merit analysis for k-means clustering. The slope of the line indicates the most ideal number of clusters for k-means clustering. In Figure of merit analysis, most information will be obtained as the line levels out. We assessed k=4, 6, 8, 10 and 12 (arrows). Based on this merit analysis, we present the data for k=8 (red arrow, see Figure 5 and Table 1).

Figure S5. Early DE genes provide molecular markers to assess glaucoma status in individual eyes. An independent set of eyes (not included in the microarray study) were assessed. (**A**) Total number of DE genes (from all genes assessed, Table S2) for each eye. Individual eyes are shown in the same order for ONH and retina. Eyes with NOE glaucoma are ordered based on the increasing number of DE genes in the ONH. Any eye was regarded as different to the controls when its number of DE genes was at least twice as high as for the highest control eye (horizontal dashed line, 19 of 21 (ONH) and 14 of 21 eyes (retina, including eyes 1, 11 and 19). For most NOE eyes, the number of DE genes in the ONH was over half of that in the moderate eyes. This was true for only 6 eyes in the retina. However, in a few eyes the retina appeared more similar to moderate when compared to the ONH. (**B**) The activation status of each tested pathway was determined, based on the number of pathway-specific DE genes. A pathway was considered activated when the number of DE genes for that pathway was twice as high as in the highest control eye (horizontal dashed line). For the ONH, eyes 1-3 are predicted to be at the earliest stages of glaucoma (as maximally only 1 of 3 tested pathways is activated in these eyes). All pathways are activated in eyes 4-16. Interestingly, the complement cascade is activated in the retina in eyes 1-3. The complement cascade is activated in only 10 of the other no or early eyes (including eyes 7, 10 and 20). All pathways were activated in the three moderate eyes.

Figure S6. Decrease in ratio of lumen to blood vessel area in eyes with glaucoma. (**A**) Each black bar represents the average ± SEM of all measurements for individual eyes (see Methods). (**B**) Ratios at individual locations within each assessed eye. There is a clear decrease at some locations in NOE eyes that are at early stages of glaucoma. 51/56 ratios that were below 0.5 were for vessels in DBA/2J eyes. Only 5/54 of D2-*Gpnmb*$^{+}$ control ratios were below this value.

Supplemental Tables.

Table S1: Probe sets used for k-means clustering (see excel file)

Table S2: Genes assessed by real time PCR in individual eyes

Pathway	**Tissue Assessed**	**Genes**
ECM-receptor interaction	Optic nerve head	*Cd44, Cd47, Col11a2, Col3a1, Col4a2, Col4a6, Col5a1, Col5a2, Col6a1, Dag1, Itga1, Itga6, Itgb3, Itgb4, Itgb5, Lama4, Lamb1-1, Lamb2, Lamc1, Sdc4, Spp1, Sv2a, Thbs3, Tnxb, Vwf*
MAPK signaling	Optic nerve head	*Bdnf, Cacna1b, Cacna1f, Cacna2d3 Cacna2d4, Cacnb2, Dusp8, Fas, Fgf9, Gadd45a, Gng12, Mapkapk3, Mapt, Myc, Nfkb2, Nlk, Ntrk2, Pla2g4a, Relb, Rps6ka1, Rps6ka6, Rras, Tgfbr2, Tnfrsf1a*
Toll-like receptor signaling	Optic nerve head	*Casp8, Ccl3, Cd86, Cxcl10, Ifnar1, Ifnar2, Il6, Irak1, Irak4, Irf5, Irf7, Lbp, Ly96, Myd88, Ripk1, Stat1, Ticam1, Tlr2, Tlr4, Tlr6*
Complement cascade	Retina	*C1qa, C1qb, C1qc, C2, C3, C3ar1, C4b, C5ar1, C6, C8b, Cr2, Crry*
Miscellaneous (DE, stages R1 and/or R2)	Retina	*Ccl12, Chi3l1, Ecel1, Gal, Hrk, Lcn2, Osmr, Stat3, Steap4, Tnfrsf12a*

Table S3: Primer sequences for RTPCR (see excel file)

Figure S1

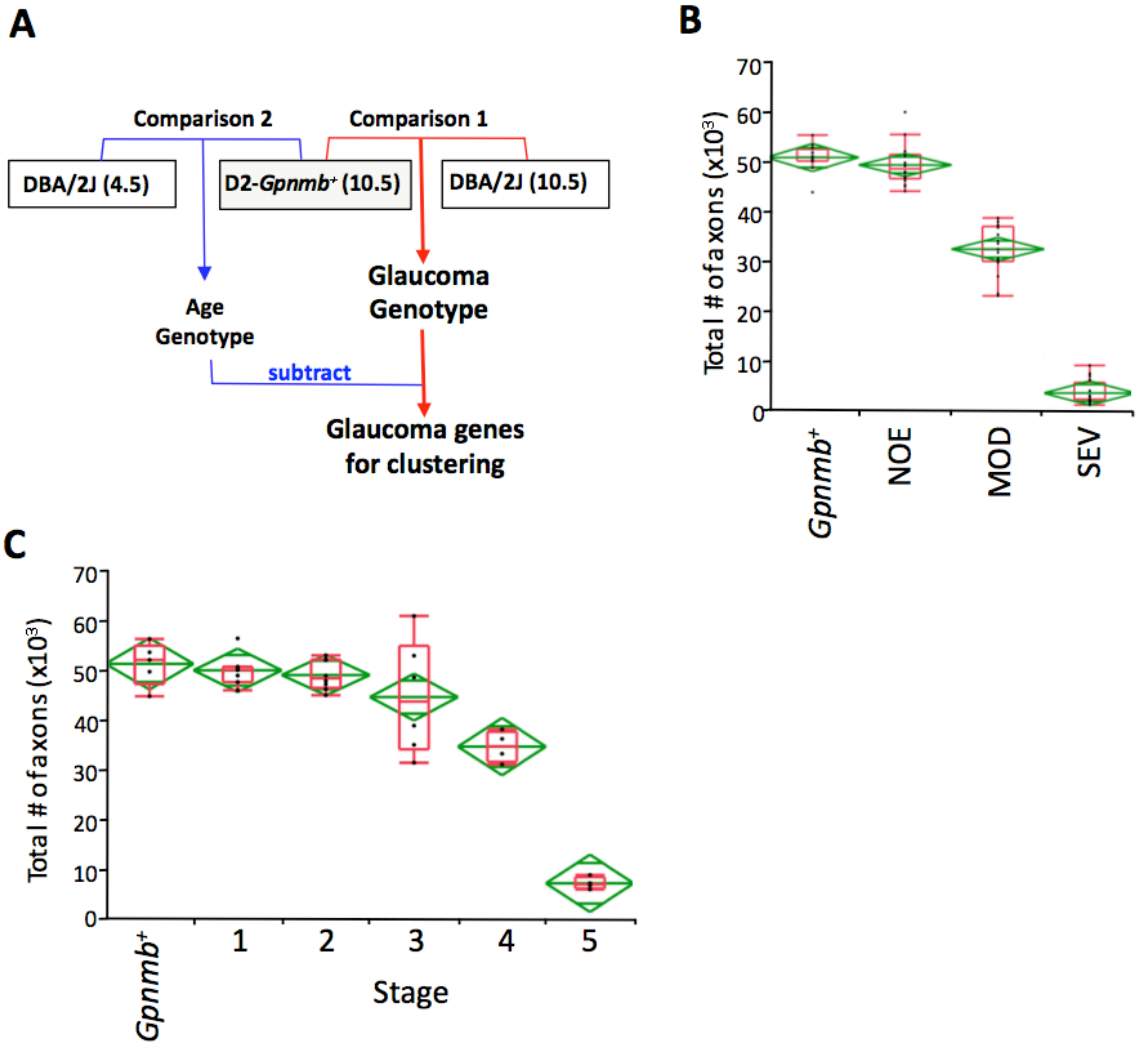

A
Comparison 2
Comparison 1
DBA/2J (4.5)
D2-Gpnmb+ (10.5)
DBA/2J (10.5)
Age Genotype
Glaucoma Genotype
subtract
Glaucoma genes for clustering
B
Total # of axons (x10³)
70
60
50
40
30
20
10
0
Gpnmb+
NOE
MOD
SEV
C
Total # of axons (x10³)
70
60
50
40
30
20
10
0
Gpnmb+
1
2
3
4
5
Stage

Figure S2

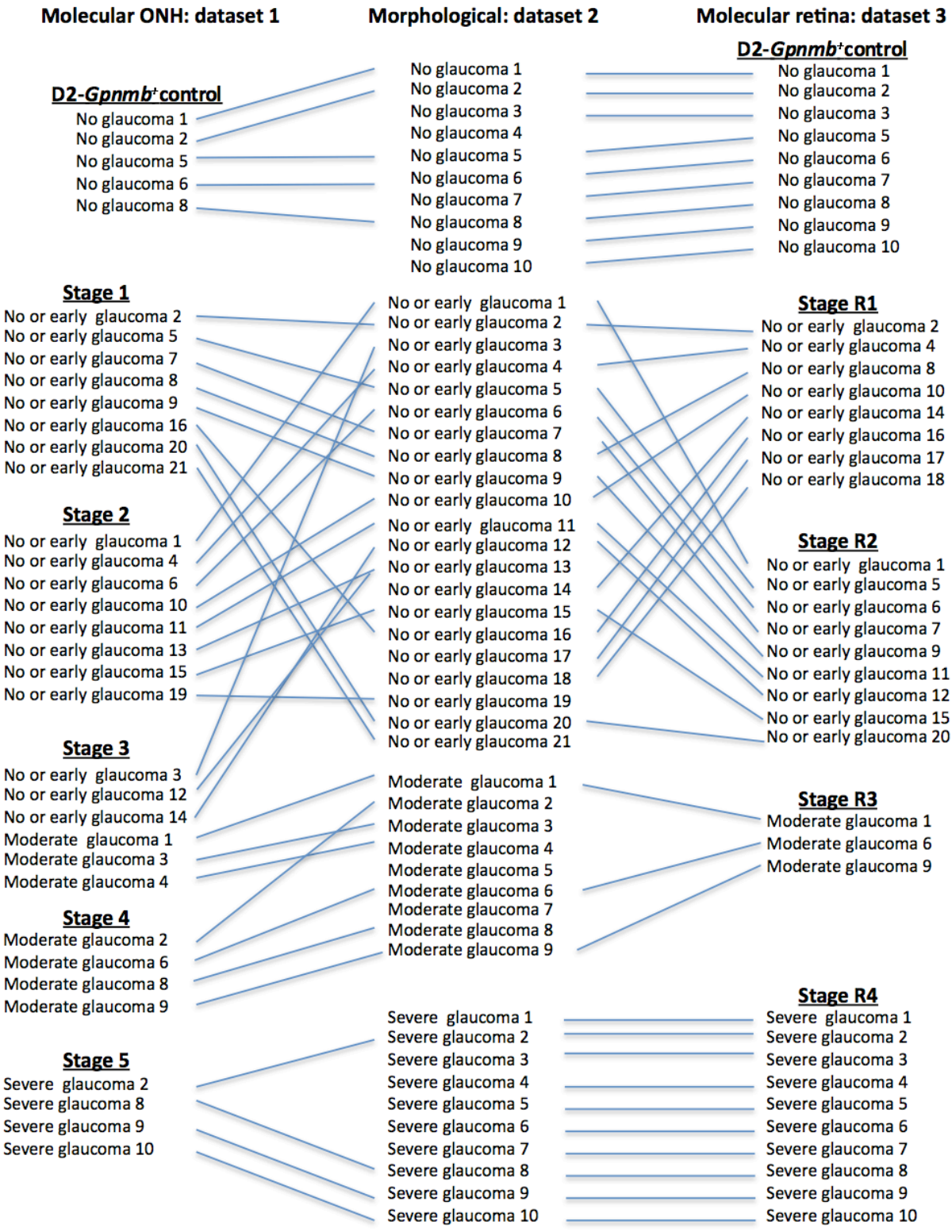
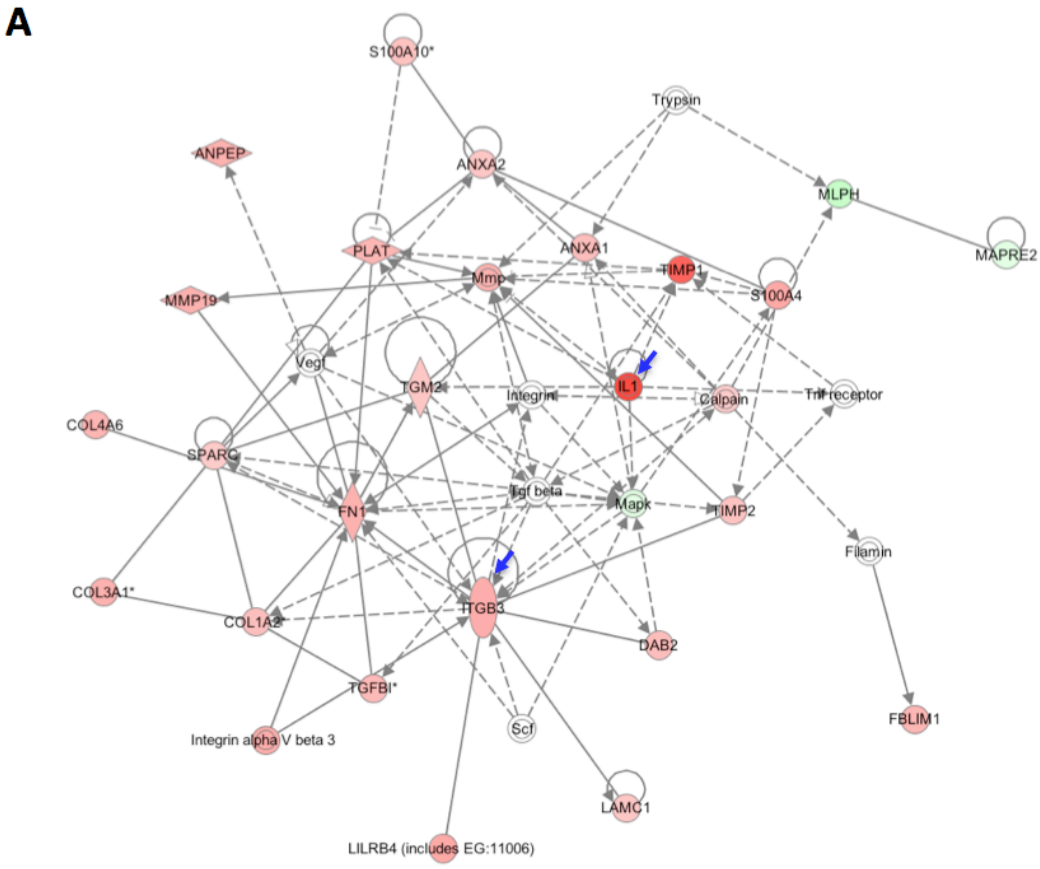

Molecular ONH: dataset 1
Morphological: dataset 2
Molecular retina: dataset 3
D2-Gpnmb+control
No glaucoma 1
No glaucoma 2
No glaucoma 5
No glaucoma 6
No glaucoma 8
D2-Gpnmb+control
No glaucoma 1
No glaucoma 2
No glaucoma 3
No glaucoma 4
No glaucoma 5
No glaucoma 6
No glaucoma 7
No glaucoma 8
No glaucoma 9
No glaucoma 10
D2-Gpnmb+control
No glaucoma 1
No glaucoma 2
No glaucoma 3
No glaucoma 5
No glaucoma 6
No glaucoma 7
No glaucoma 8
No glaucoma 9
No glaucoma 10
Stage 1
No or early glaucoma 2
No or early glaucoma 5
No or early glaucoma 7
No or early glaucoma 8
No or early glaucoma 9
No or early glaucoma 16
No or early glaucoma 20
No or early glaucoma 21
Stage 2
No or early glaucoma 1
No or early glaucoma 4
No or early glaucoma 6
No or early glaucoma 10
No or early glaucoma 11
No or early glaucoma 13
No or early glaucoma 15
No or early glaucoma 19
Stage 3
No or early glaucoma 3
No or early glaucoma 12
No or early glaucoma 14
Moderate glaucoma 1
Moderate glaucoma 3
Moderate glaucoma 4
Stage 4
Moderate glaucoma 2
Moderate glaucoma 6
Moderate glaucoma 8
Moderate glaucoma 9
Stage 5
Severe glaucoma 2
Severe glaucoma 8
Severe glaucoma 9
Severe glaucoma 10
No or early glaucoma 1
No or early glaucoma 2
No or early glaucoma 3
No or early glaucoma 4
No or early glaucoma 5
No or early glaucoma 6
No or early glaucoma 7
No or early glaucoma 8
No or early glaucoma 9
No or early glaucoma 10
No or early glaucoma 11
No or early glaucoma 12
No or early glaucoma 13
No or early glaucoma 14
No or early glaucoma 15
No or early glaucoma 16
No or early glaucoma 17
No or early glaucoma 18
No or early glaucoma 19
No or early glaucoma 20
No or early glaucoma 21
Moderate glaucoma 1
Moderate glaucoma 2
Moderate glaucoma 3
Moderate glaucoma 4
Moderate glaucoma 5
Moderate glaucoma 6
Moderate glaucoma 7
Moderate glaucoma 8
Moderate glaucoma 9
Severe glaucoma 1
Severe glaucoma 2
Severe glaucoma 3
Severe glaucoma 4
Severe glaucoma 5
Severe glaucoma 6
Severe glaucoma 7
Severe glaucoma 8
Severe glaucoma 9
Severe glaucoma 10
Stage R1
No or early glaucoma 2
No or early glaucoma 4
No or early glaucoma 8
No or early glaucoma 10
No or early glaucoma 14
No or early glaucoma 16
No or early glaucoma 17
No or early glaucoma 18
Stage R2
No or early glaucoma 1
No or early glaucoma 5
No or early glaucoma 6
No or early glaucoma 7
No or early glaucoma 9
No or early glaucoma 11
No or early glaucoma 12
No or early glaucoma 15
No or early glaucoma 20
Stage R3
Moderate glaucoma 1
Moderate glaucoma 6
Moderate glaucoma 9
Stage R4
Severe glaucoma 1
Severe glaucoma 2
Severe glaucoma 3
Severe glaucoma 4
Severe glaucoma 5
Severe glaucoma 6
Severe glaucoma 7
Severe glaucoma 8
Severe glaucoma 9
Severe glaucoma 10

Figure S3


A
S100A10*
Trypsin
ANPEP
ANXA2
MLPH
MAPRE2
PLAT
ANXA1
TIMP1
Mmp
S100A4
MMP19
Vegf
TGM2
Integrin
IL1
Calpain
Tnf receptor
COL4A6
SPARC
Tgf beta
Mapk
TIMP2
FN1
Filamin
COL3A1*
ITGB3
COL1A2
DAB2
TGFBI*
FBLIM1
Integrin alpha V beta 3
Scf
LAMC1
LILRB4 (includes EG:11006)

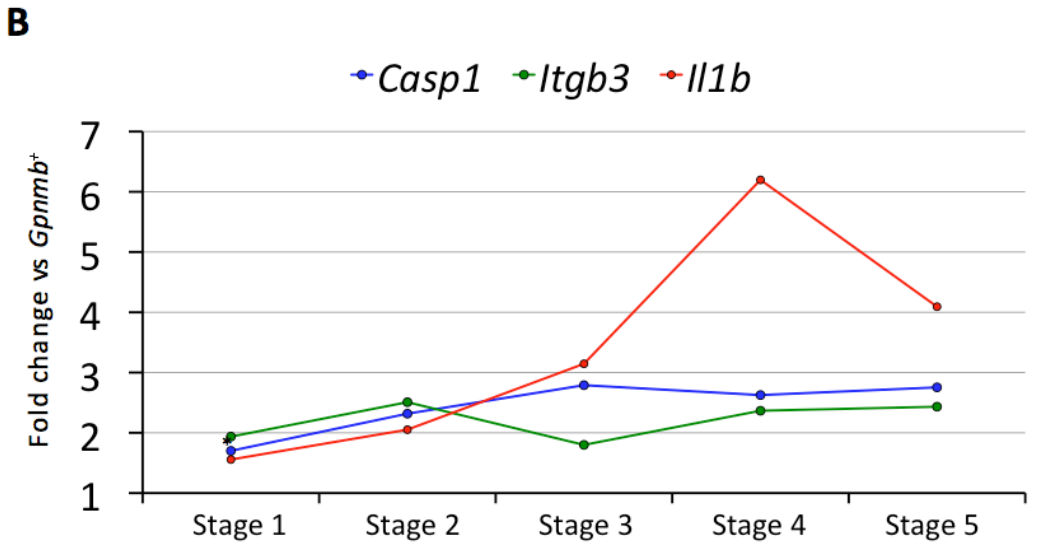

B
Casp1
Itgb3
Il1b
Fold change vs Gpnmb+
7
6
5
4
3
2
1
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5

Figure S4

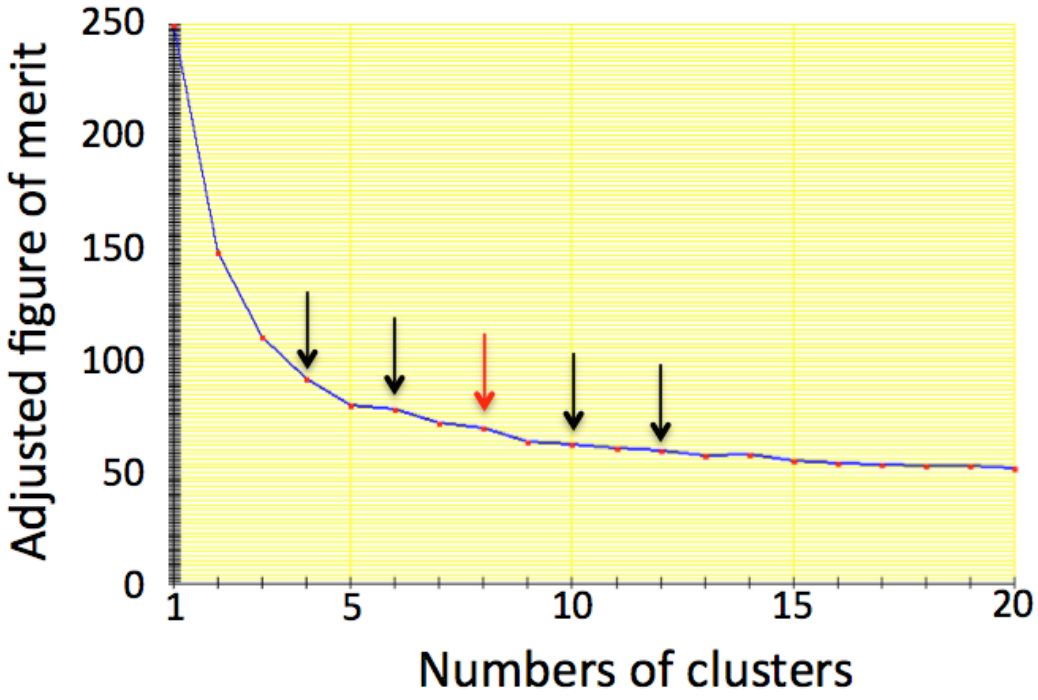

Adjusted figure of merit
250
200
150
100
50
0
1
5
10
15
20
Numbers of clusters

Figure S5

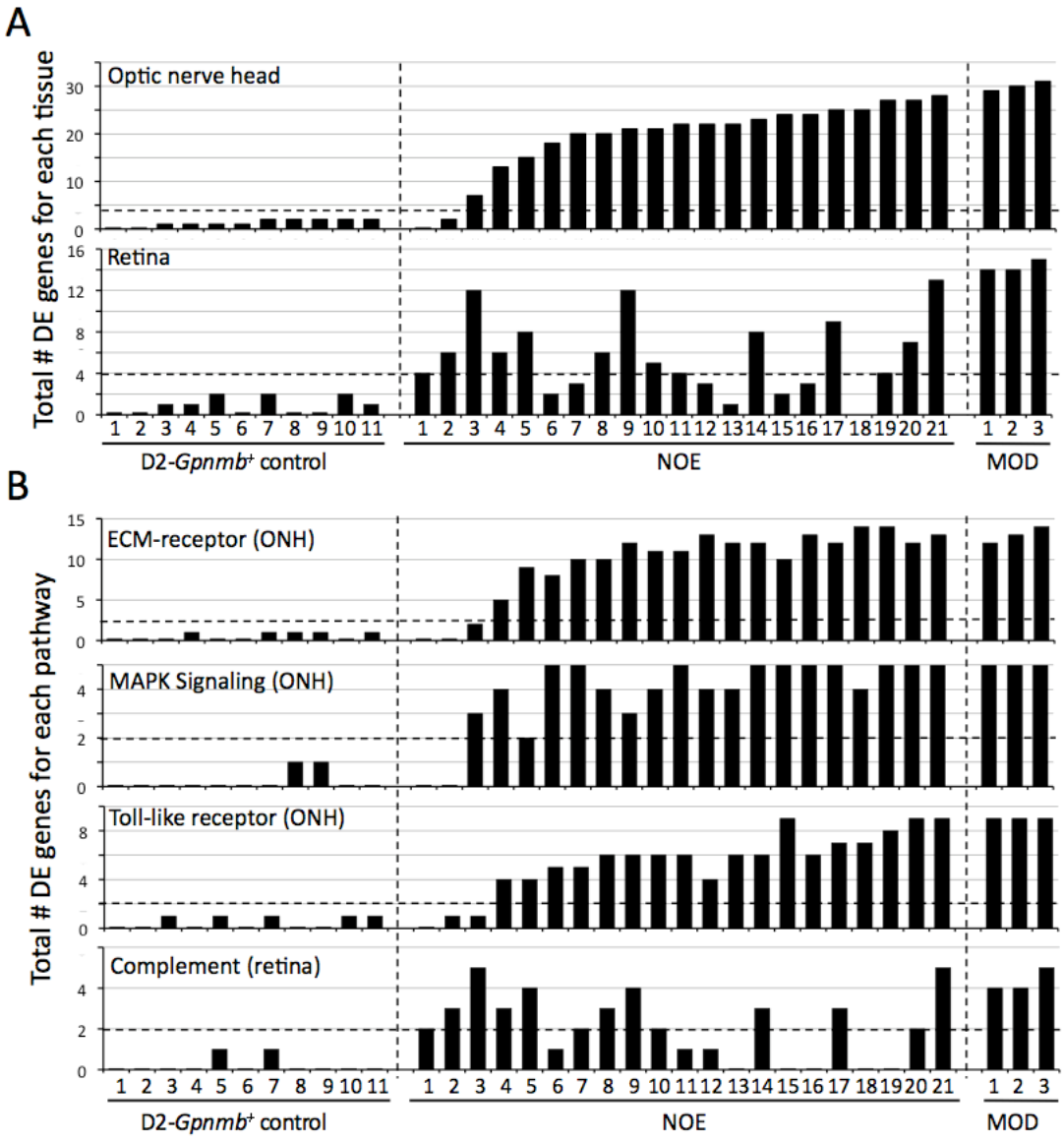
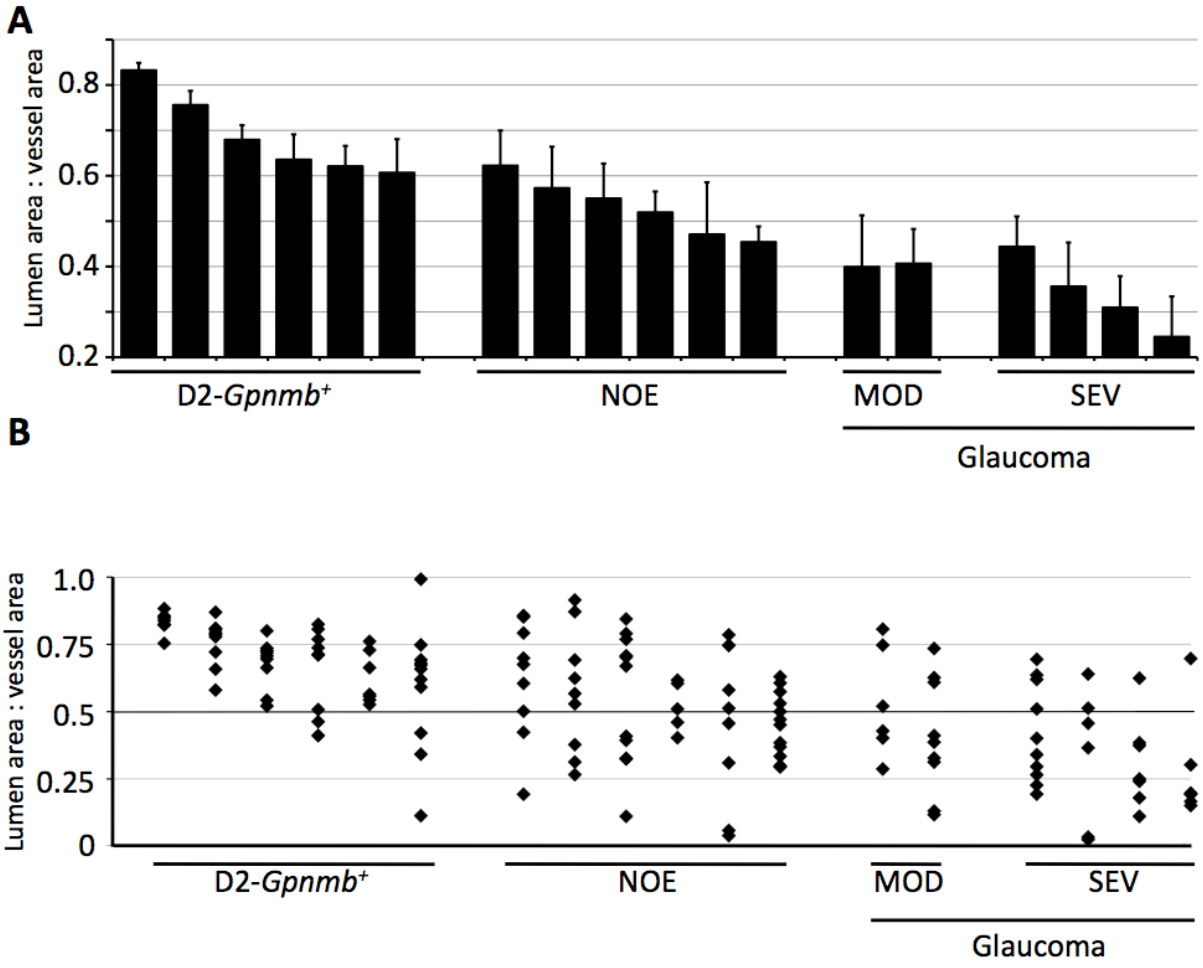

A
Total # DE genes for each tissue
Optic nerve head
Retina
B
Total # DE genes for each pathway
ECM-receptor (ONH)
MAPK Signaling (ONH)
Toll-like receptor (ONH)
Complement (retina)
D2-Gpnmb+ control
NOE
MOD

Figure S6


A
Lumen area : vessel area
0.8
0.6
0.4
0.2
D2-Gpnmb+
NOE
MOD
SEV
Glaucoma
B
Lumen area : vessel area
1.0
0.75
0.5
0.25
0
D2-Gpnmb+
NOE
MOD
SEV
Glaucoma