SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Post-Mortem Donor Eye Collection and Preparation

All study tissue samples were preserved within 6 hours post-mortem or less except one eye at ~8 hours. Ten donor eyes for histology were injected in the vitreous with 100 µl of Modified Davidson's Fixative (MDF, H0290-500ML) and preserved as whole globes in that same fixative (supplemental table 1). Eyes were fixed for 48 hours and transferred to 70% alcohol for an additional 48 hours, all at room temperature. After fixation, globes were sectioned horizontally so that the sections included the optic nerve head and the macula. Five-micron sections were dried at 60°C for 24 hours prior to in situ hybridization (RNAscope), immunohistochemistry (IHC), and hematoxylin and eosin staining (H&E).

Donor eyes for RNA (supplemental table 2) and protein (supplemental table 3) analysis were dissected and isolated by staff at the eye bank LEITR. The macular region was identified and isolated within the boundaries of superior and inferior temporal vascular arcades with a 6-mm biopsy punch centered on the fovea. The following tissues were collected sequentially from each donor eye: macular neural retina, peripheral neural retina, macular RPE/choroid, and peripheral RPE/choroid. Dissected tissue samples were flash frozen in liquid nitrogen and stored at -80°C. High-resolution color digital fundus photographs of the posterior eye were captured under a dissecting microscope before and after neural retina removal.

Preparation of Eye Lysates

Total RNA was isolated from the homogenized tissue samples using RNeasy micro columns (Qiagen, Venlo, Netherlands). RNA concentrations were determined by NanoDrop 800 (Thermo Scientific) and RNA integrity number (RIN) was assessed by Fragment Analyzer (Advanced Analytical Technology). The average RIN in this study was 9.9 for macular neural retina and 8.0 for macular RPE/choroid. Protein lysates were prepared using 200 µL cell lysis buffer (Meso Scale Diagnostics (MSD), Rockville, MD; #R60TX-2) with protease inhibitor (Thermo Fisher, Waltham, MA), PMSF cocktail (Sigma, St Louis, MO) and EDTA (Invitrogen, Carlsbad, CA), and homogenized with a 5-mm stainless steel bead by a Tissue-lyzer (Qiagen, Hilden, Germany) at 30hz for 2min. Samples were centrifuged at 10,000 rpm for 2 min, and the sample supernatant was collected. Vitreous samples were homogenized similarly with protease inhibitors, but no cell lysis buffer was added. The bicinchoninic acid (BCA) protein assay (ThermoFisher, Waltham, MA) was used to determine total protein concentrations.

Materials for TaqMan

Reagents for TaqMan: Primers for real-time PCR were from Invitrogen/Thermo Fisher (Waltham MA) for GAPDH Hs99999905_m1 (endogenous control), C1Q Hs00706358_s1, C3 Hs00163811_m1, CFB Hs00156060_m1, CFH Hs00962373_m1, CFI Hs00989715_m1, C9 Hs01036216_g1, CFD Hs00157263_m1, CFP Hs01106969_g1, MASP1 Hs00373559_m1.

Reagents for western blot: Primary antibodies were anti-mouse C3d (R & D Systems, Minneapolis, MN) and anti-human FB, C1Q, FH, or FI antibodies (Quidel, San Diego, CA). Anti-mouse C3d antibody recognizes all C3d containing proteins including full-length C3alpha, C3 breakdown products C3b, iC3b, and C3d. Anti-human tubulin antibody (Proteintech, Rosemont, IL) was the loading control for protein level normalization.

MSD assay reagents: C3b (in-house C3b-neo; Quidel #A205), C3 (in-house C3-neo; Abcam #ab13329-1), sC5b-9 (Abcam #ab66768; Biomeda #G36), C1Q (Quidel #A201and #A301), and Factor H (Quidel #A254; in-house FH). The C3b MSD assay recognizes multiple C3 breakdown products including C3b, iC3b, and C3c. Calibrators were proteins purchased from Complement Technology (Tyler, TX).

RNAscope of *C1QA, C3, C5, C9, CFB, CFD, CFH, CFI, MASP1* and Immunohistochemistry of IBA1

The RNAscope ® (ACD, Advanced Cell Diagnostics, and Newark, CA, USA) assay on donor eyes and positive control human liver sections was conducted in Automated Leica Bond RX according to the manufacturer's instructions for RNAscope® 2.5 LS with RNAscope® 2.5 LS Reagent Kit-RED (Cat # 322150) for visualization of the following complement pathway components: C1QA (Cat # 485458), C3 (Cat # 430708), C5 (Cat # 459498), C9 (Cat # 420828), CFB (Cat # 402108), CFD (Cat # 420838), CFH (ACat # 428738), CFI (Cat # 421928), and MASP1 (Cat # 545538). The positive control probes consisted of a probe set for POLR2A (Cat # 310458) or a probe set for PPIB (Cat # 313908), while the negative control probe targeted dapB of Bacillus subtilis (Cat # 312038). Microglia (macrophages) were detected with rabbit anti-IBA1 (Wako, Cat # 019-19741) at 1:1500 dilution in green with Vina Green™ Chromogen (Biocare Medical, Cat # BRR807A) in Automated Leica Bond RX or TEAL chromogen (Roche Diagnostics, Discovery TEAL HRP Cat# 760-247) from Ventana.

Normal human serum collection and preparation

Human blood was obtained from the Novartis Cambridge Research Blood Donor program, in compliance with all federal, state and local laws pertaining to human research. We acquired blood samples from healthy volunteers aged 18-65 under patient informed consent, in

accordance with the Declaration of Helsinki ethical principles for medical research involving human subjects. Blood was collected into vacutainer tubes with a straight needle, immediately placed on ice, and centrifuged (4000rpm, 30min at 4C) to collect serum. Serum was stored frozen at -80C.

Supplementary Table S1 Post-Mortem Eyes for Histology and RNAscope Analysis

AMD grade (histological)	Gender	Age	Race	Death-preservation time (hours:minutes)	Cause of Death
AMD 1	M	61	Caucasian	3:53	Lung cancer
AMD 1	F	83	Caucasian	2:38	Myocardial infarction
AMD 1	M	85	Caucasian	4:46	Cardiopulmonary arrest
AMD 2	F	79	Caucasian	2:24	Lung cancer
AMD 3	M	79	Caucasian	3:50	Chronic obstructive pulmonary disease
AMD 3	F	81	Caucasian	4:43	Breast cancer
AMD 4	F	68	Caucasian	4:21	Cardiopulmonary arrest
AMD 4	M	80	Caucasian	2:24	Pneumonia
AMD 4	M	81	Caucasian	4:09	Metastatic cancer
AMD 4	F	93	Caucasian	3:15	Coronary artery disease

Supplementary Table S2 Post-Mortem Eyes for TaqMan Analysis

Biosample Diagnosis	Gender	Age	Race	Death-preservation time (hours:minutes)	Cause of Death
AMD 1	F	65	Caucasian	3:48	Congestive heart failure
AMD 1	F	66	Caucasian	5:51	Pneumonia
AMD 1	M	68	Caucasian	5:31	Myocardial infarction
AMD 1	M	69	Caucasian	3:18	Sepsis
AMD 1	M	73	Caucasian	5:37	Prostate cancer
AMD 1	M	78	Caucasian	3:35	Cardiopulmonary arrest
AMD 1	M	81	Caucasian	4:56	Coronary artery disease
AMD 1	F	82	Caucasian	3:30	Pneumonia
AMD 1	F	91	Caucasian	4:22	Renal failure
AMD 1	M	93	Caucasian	5:12	Pancreatic cancer
AMD 2	F	67	Caucasian	6:11	acute cardiac crisis
AMD 2	M	68	Caucasian	3:51	Cerebrovascular accident
AMD 2	F	75	Caucasian	3:35	Chronic obstructive pulmonary disease
AMD 2	M	75	Caucasian	4:30	Chronic obstructive pulmonary disease
AMD 2	M	75	Caucasian	4:57	Pancreatic cancer
AMD 2	F	76	Caucasian	4:50	Sepsis
AMD 2	M	78	Caucasian	3:15	Chronic obstructive pulmonary disease
AMD 2	F	78	Caucasian	4:01	Acute cardiac crisis
AMD 2	F	84	Caucasian	2:44	Respiratory failure
AMD 2	M	86	Caucasian	2:16	Acute cardiac crisis
AMD 2	M	87	Caucasian	4:14	Respiratory arrest
AMD 3	M	78	Caucasian	6:02	Chronic obstructive pulmonary disease
AMD 3	M	79	Caucasian	3:50	Chronic obstructive pulmonary disease
AMD 3	F	79	Caucasian	4:24	Acute cardiac crisis
AMD 3	F	79	Caucasian	5:39	Pneumonia
AMD 3	F	84	Caucasian	3:32	Diabetic
AMD 3	M	85	Caucasian	4:59	Acute cardiac crisis
AMD 3	M	85	Caucasian	3:47	Acute cardiac crisis
AMD 3	M	86	Caucasian	2:32	Acute cardiac crisis
AMD 3	M	87	Caucasian	2:15	Sepsis
AMD 3	M	89	Caucasian	5:10	Lung cancer
AMD 3	F	90	Caucasian	4:50	Congestive heart failure
AMD 3	F	92	Caucasian	3:25	Acute cardiac crisis
AMD 3	M	94	Caucasian	6:00	Respiratory failure
AMD 4	F	74	Caucasian	2:00	Acute cardiac crisis
AMD 4	M	76	Caucasian	4:21	Cerebrovascular accident
AMD 4	F	84	Caucasian	5:09	Cerebrovascular accident
AMD 4	M	84	Caucasian	4:23	Chronic obstructive pulmonary disease
AMD 4	M	88	Caucasian	3:06	Renal failure
AMD 4	F	92	Caucasian	5:13	Cerebrovascular accident
AMD 4	F	94	Caucasian	3:05	Chronic obstructive pulmonary disease
AMD 4	M	95	Caucasian	3:13	Sepsis
AMD 4	M	97	Caucasian	5:30	Acute cardiac crisis

Supplementary Table S3 Post-Mortem Eyes for Protein Assays

Biosample Diagnosis	Gender	Age	Race	Death-preservation time (hours:minutes)	Cause of Death
AMD 1	F	61	Caucasian	5:55	Peripheral vascular disease
AMD 1	F	65	Caucasian	6:59	Pneumonia
AMD 1	F	73	Caucasian	6:04	Bowel perforation
AMD 1	M	73	Caucasian	6:06	Leukemia
AMD 1	M	76	Caucasian	3:46	Liver cancer
AMD 1	M	76	Caucasian	4:31	Lung cancer
AMD 1	F	78	Caucasian	8:03	Subarachnoid hemorrhage
AMD 1	F	93	Caucasian	5:50	Chronic obstructive pulmonary disease
AMD 2	M	69	Caucasian	3:27	Multiple Myeloma
AMD 2	F	79	Caucasian	3:55	Intracerebral brain hemorrhage
AMD 2	M	80	Caucasian	3:53	Sepsis
AMD 2	M	81	Caucasian	4:20	Acute cardiac crisis
AMD 2	M	81	Caucasian	3:28	Acute cardiac crisis
AMD 2	M	84	Caucasian	5:16	Acute cardiac crisis
AMD 2	M	85	Caucasian	4:46	Cardiopulmonary arrest
AMD 2	M	88	Caucasian	3:52	Abdominal aortic aneurysm
AMD 3	M	72	Caucasian	4:57	Esophagus cancer
AMD 3	F	73	Caucasian	5:17	Congestive heart failure
AMD 3	M	75	Caucasian	3:21	Chronic obstructive pulmonary disease
AMD 3	F	76	Caucasian	6:27	Sepsis
AMD 3	F	78	Caucasian	3:48	Acute cardiac crisis
AMD 3	M	82	Caucasian	5:18	Coronary artery disease
AMD 3*	F	90	Caucasian	3:02	Gastrointestinal bleed
AMD 3	M	94	Caucasian	4:30	Acute cardiac crisis
AMD 4	F	76	Caucasian	2:52	Chronic obstructive pulmonary disease
AMD 4	M	80	Caucasian	2:24	Pneumonia
AMD 4	M	81	Caucasian	4:09	Metastatic cancer
AMD 4	F	85	Caucasian	3:00	Renal failure
AMD 4	M	88	Caucasian	4:52	Lung cancer
AMD 4	F	93	Caucasian	3:15	Coronary artery disease
AMD 4	F	94	Caucasian	3:05	Chronic obstructive pulmonary disease

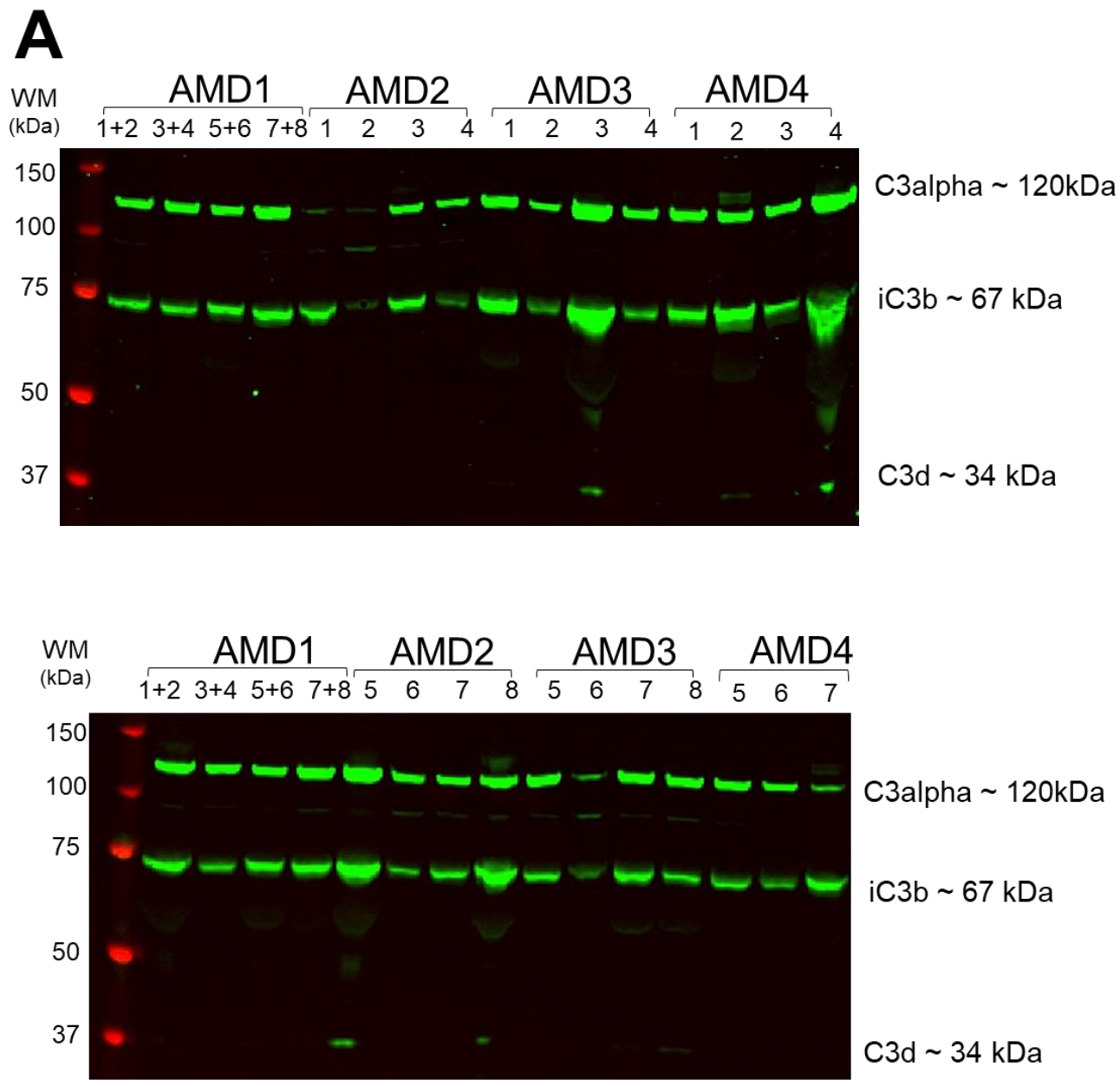
*The donor had prior intravitreal anti-VEGF treatments.

Supplementary Table S4. Incidence of mRNA detection by anatomical locations in the macular neural retina and RPE/choroid.

	Alternative pathway																															
	Terminal pathway																										Classical					
	C3				***CFB***				***CFD***				***CFH***				***CFI***				***CFP***				***C5***				***C9***				***C1QA***			
AMD Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
GCL	3/3	1/1	2/2	4/4	2/3	1/1	2/2	3/4	0/3	0/1	0/2	0/4	0/3	0/1	0/2	3/4	3/3	1/1	2/2	4/4	3/3	1/1	2/2	3/4	2/3	1/1	1/2	0/4	0/3	0/1	0/2	0/4	1/2	1/1	2/2	3/4
INL	2/3	1/1	1/2	4/4	2/3	1/1	2/2	3/4	0/3	0/1	0/2	0/4	0/3	0/1	0/2	0/4	3/3	1/1	2/2	4/4	3/3	1/1	2/2	3/4	2/3	1/1	2/2	3/4	0/3	0/1	0/2	0/4	1/2	1/1	2/2	3/4
ONL	1/3	0/1	1/2	4/4	0/3	0/1	0/2	2/4	0/3	0/1	0/2	0/4	0/3	0/1	0/2	0/4	0/3	0/1	1/2	4/4	3/3	0/1	1/2	0/4	2/3	1/1	1/2	0/4	0/3	0/1	0/2	0/4	0/2	0/1	1/2	2/4
RPE	0/3	0/1	2/2	4/4	0/3	0/1	1/2	2/4	0/3	0/1	0/2	0/4	2/3	1/1	2/2	4/4	3/3	1/1	2/2	3/4	0/3	0/1	0/2	0/4	1/3	0/1	0/2	0/4	0/3	0/1	0/2	0/4	2/2	0/1	1/1	0/4
CC	3/3	1/1	2/2	4/4	0/3	0/1	1/2	3/4	0/3	0/1	1/2	2/4	3/3	1/1	2/2	4/4	3/3	1/1	2/2	3/4	2/3	0/1	0/2	0/4	0/3	0/1	0/2	0/4	0/3	0/1	0/2	0/4	2/2	1/1	1/1	4/4

First number in each cell is the number of eyes detected with any convincing positive signal for the indicated complement gene in each AMD grade. The second number is the total number of donor eyes examined per AMD grade. Incidence of mRNA detection is not a quantitative measurement. Description of retinal regions: GCL, ganglion cell layer (from nerve fiber layer to inner plexiform layer); INL, inner nuclear layer (from inner nuclear layer to outer plexiform layer); PR, photoreceptor layer (outer nuclear layer and inner and outer segments); RPE, retinal pigmented epithelium (visualized in RPE and pigmented cells in the region of the RPE layer); CC, choroidal capillaries, or choroid.

Supplementary Figure S1



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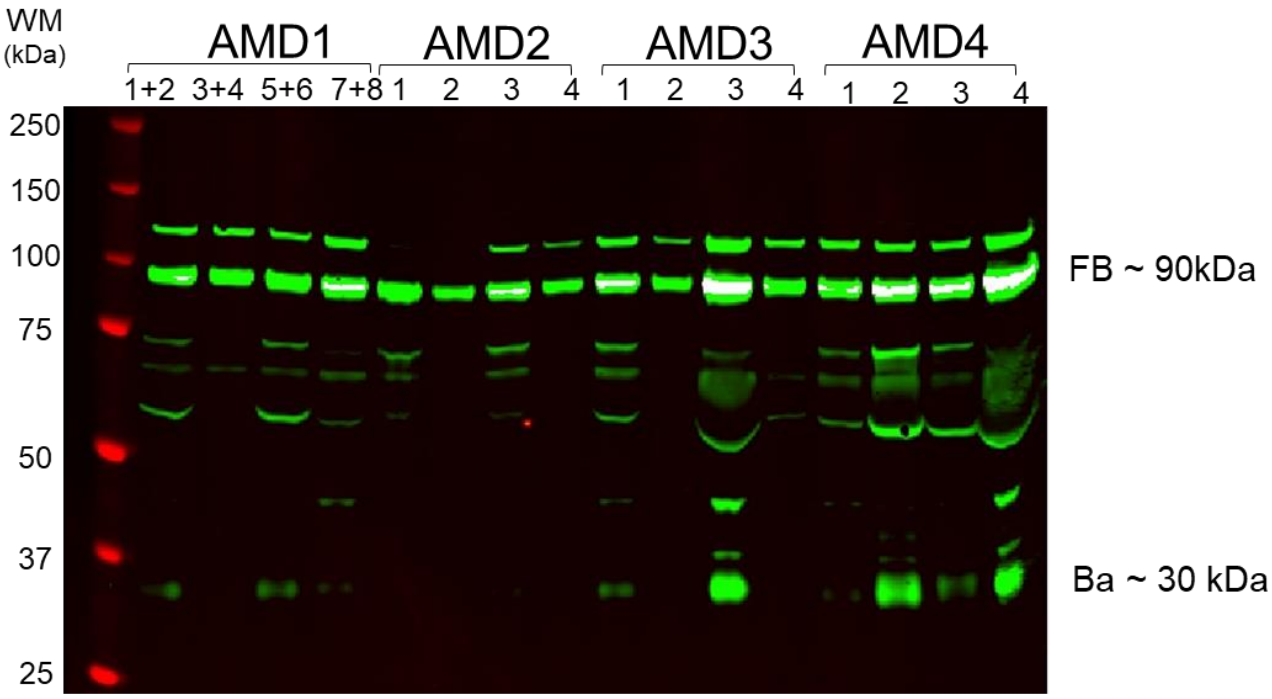
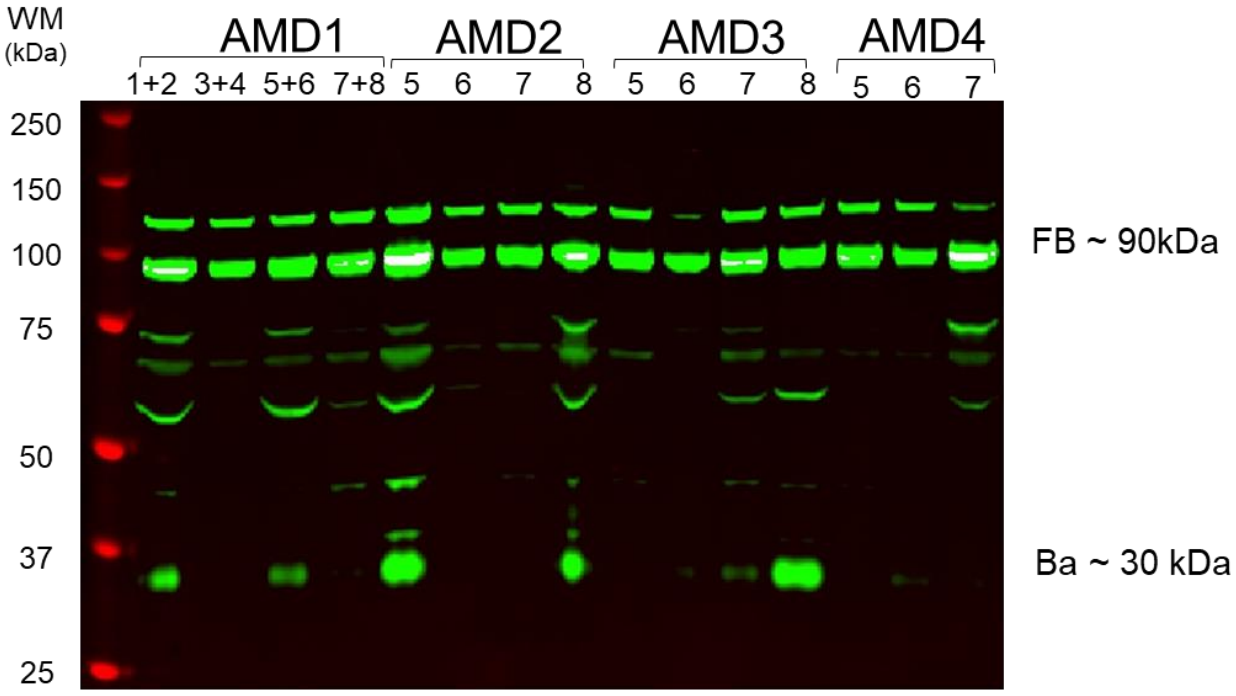
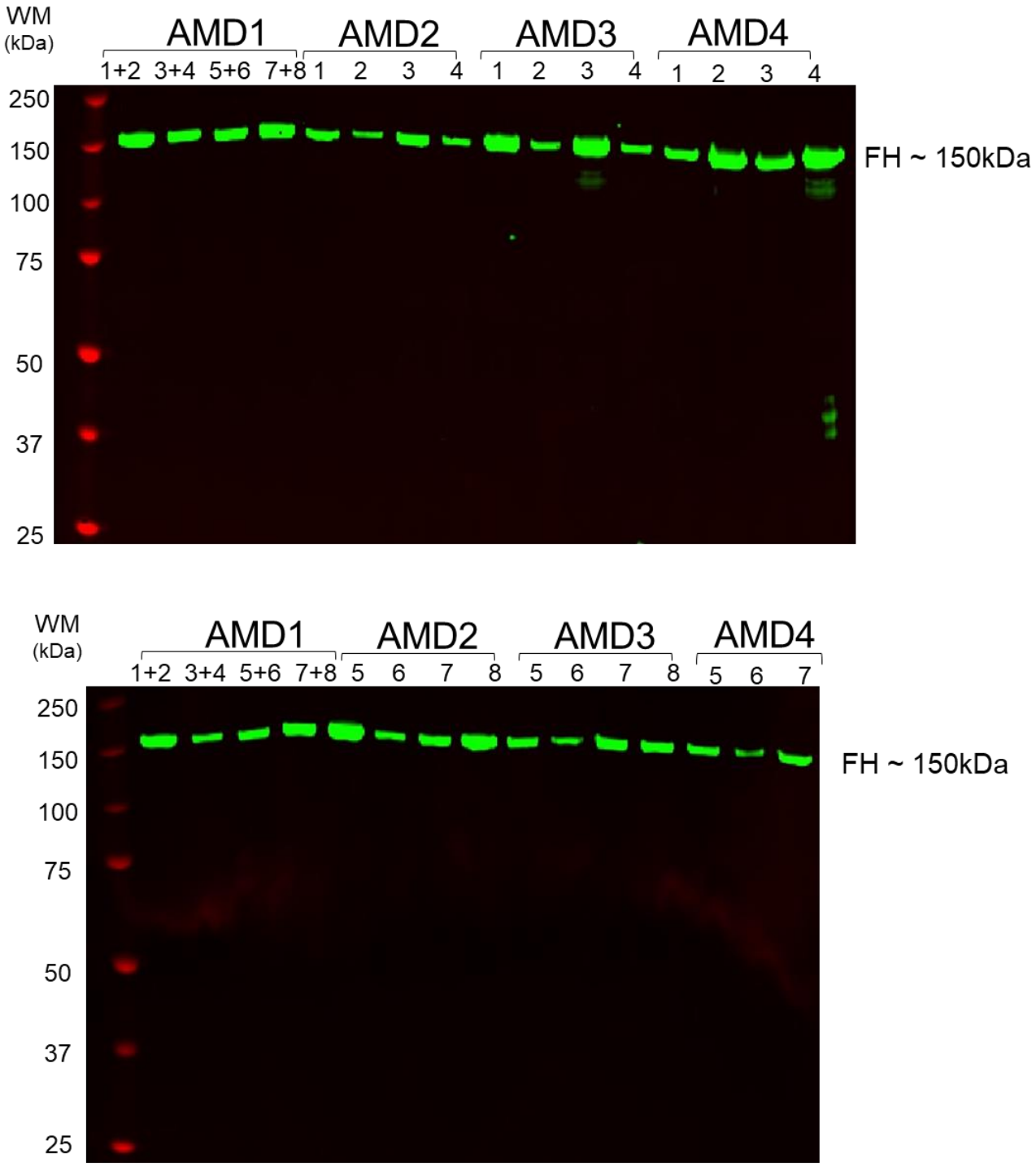
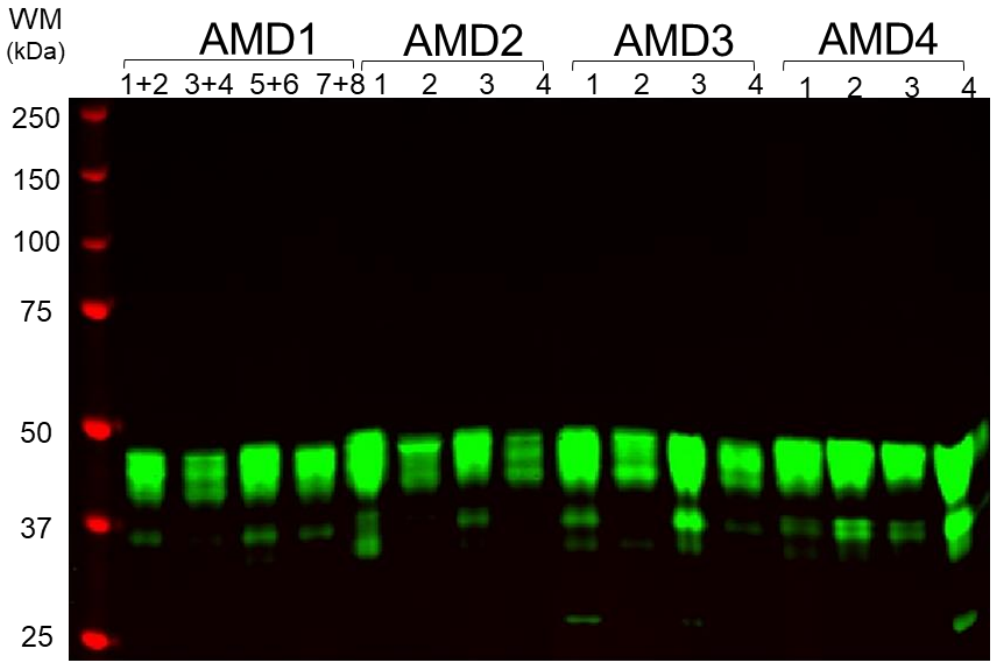

WM
(kDa)
AMD1
AMD2
AMD3
AMD4
1+2 3+4 5+6 7+8 1 2 3 4 1 2 3 4 1 2 3 4
250
150
100
75
50
37
25
FB ~ 90kDa
Ba ~ 30 kDa


WM
(kDa)
AMD1
AMD2
AMD3
AMD4
1+2 3+4 5+6 7+8 5 6 7 8 5 6 7 8 5 6 7
250
150
100
75
50
37
25
FB ~ 90kDa
Ba ~ 30 kDa

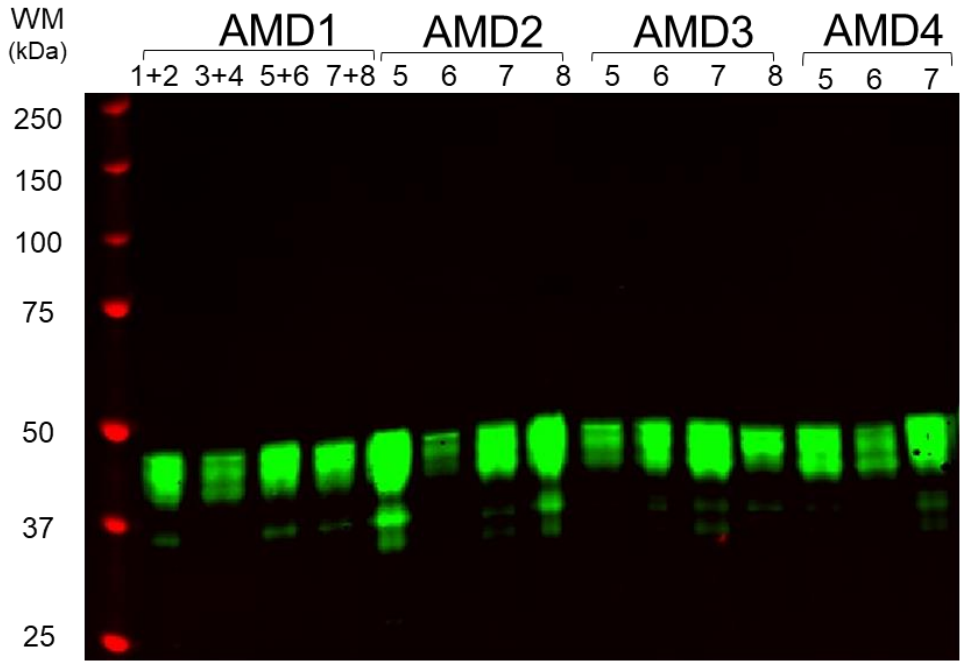
C


WM
(kDa)
AMD1
AMD2
AMD3
AMD4
1+2 3+4 5+6 7+8 1 2 3 4 1 2 3 4 1 2 3 4
250
150
100
75
50
37
25
FH ~ 150kDa
WM
(kDa)
AMD1
AMD2
AMD3
AMD4
1+2 3+4 5+6 7+8 5 6 7 8 5 6 7 8 5 6 7
250
150
100
75
50
37
25
FH ~ 150kDa

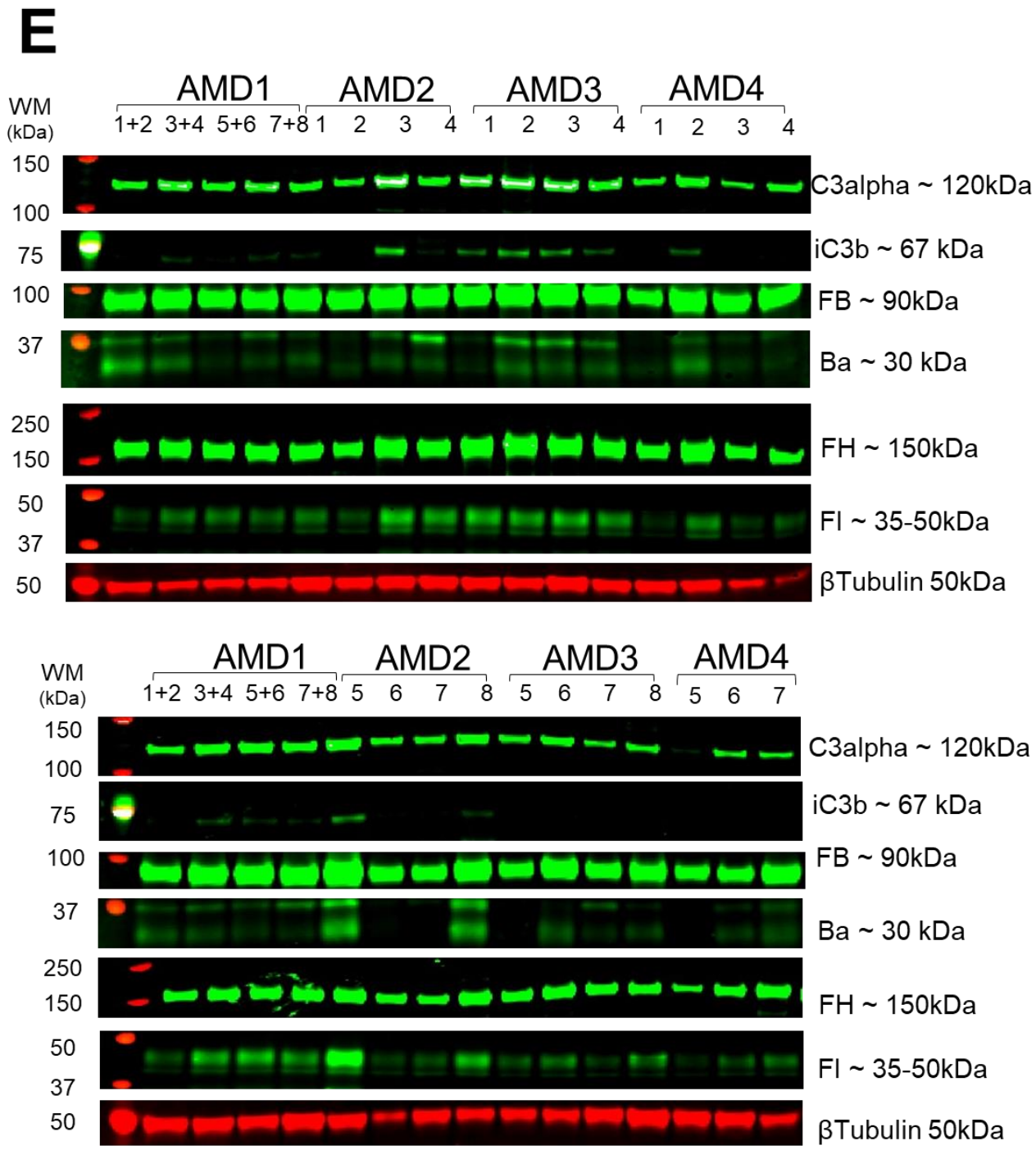
D



FI ~ 35-50kDa



FI ~ 35-50kDa


E
AMD1
AMD2
AMD3
AMD4
WM (kDa)
1+2 3+4 5+6 7+8 1 2 3 4 1 2 3 4 1 2 3 4
150
100
75
100
37
250
150
50
37
50
C3alpha ~ 120kDa
iC3b ~ 67 kDa
FB ~ 90kDa
Ba ~ 30 kDa
FH ~ 150kDa
FI ~ 35-50kDa
βTubulin 50kDa
AMD1
AMD2
AMD3
AMD4
WM (kDa)
1+2 3+4 5+6 7+8 5 6 7 8 5 6 7 8 5 6 7
150
100
75
100
37
250
150
50
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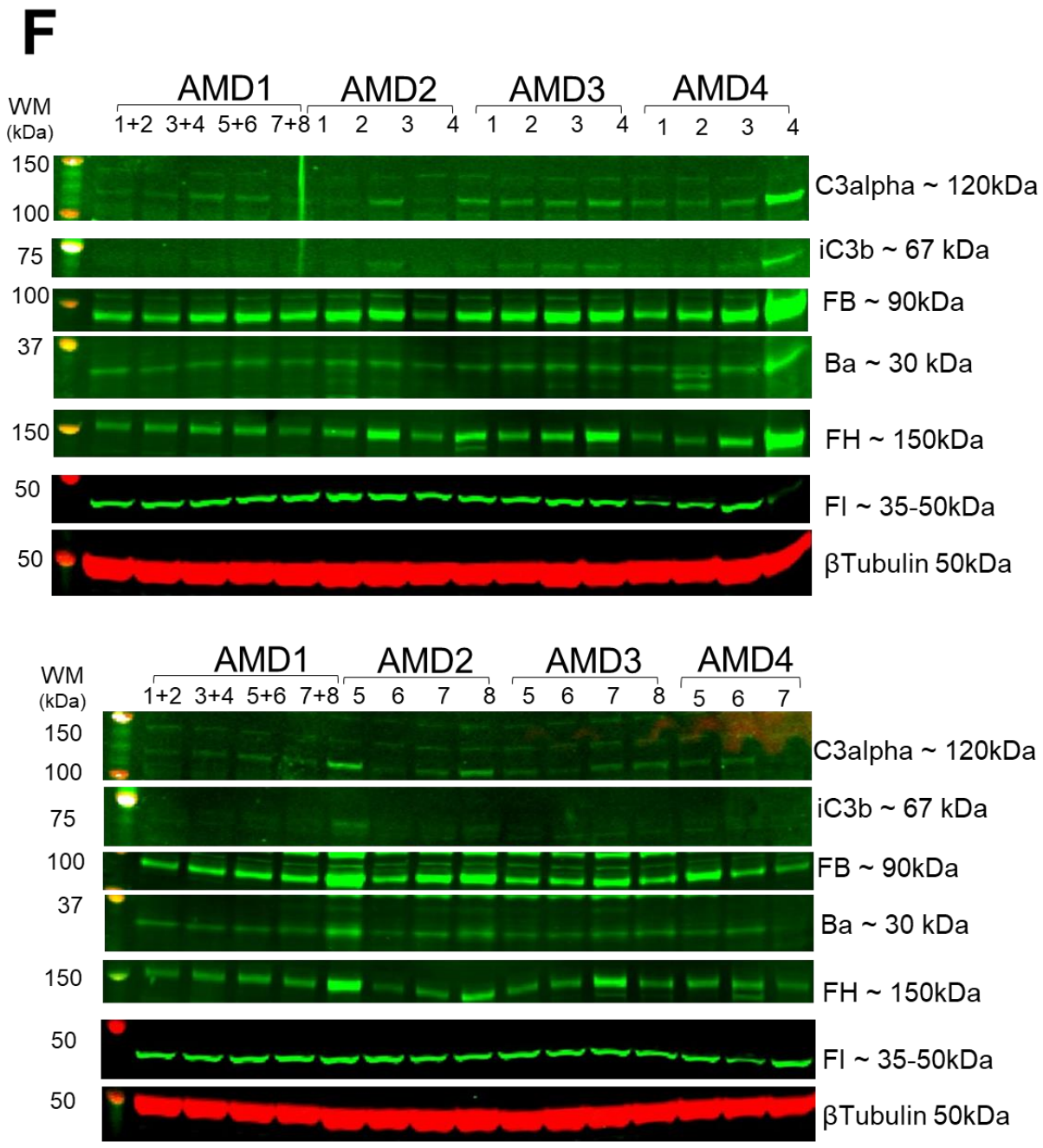
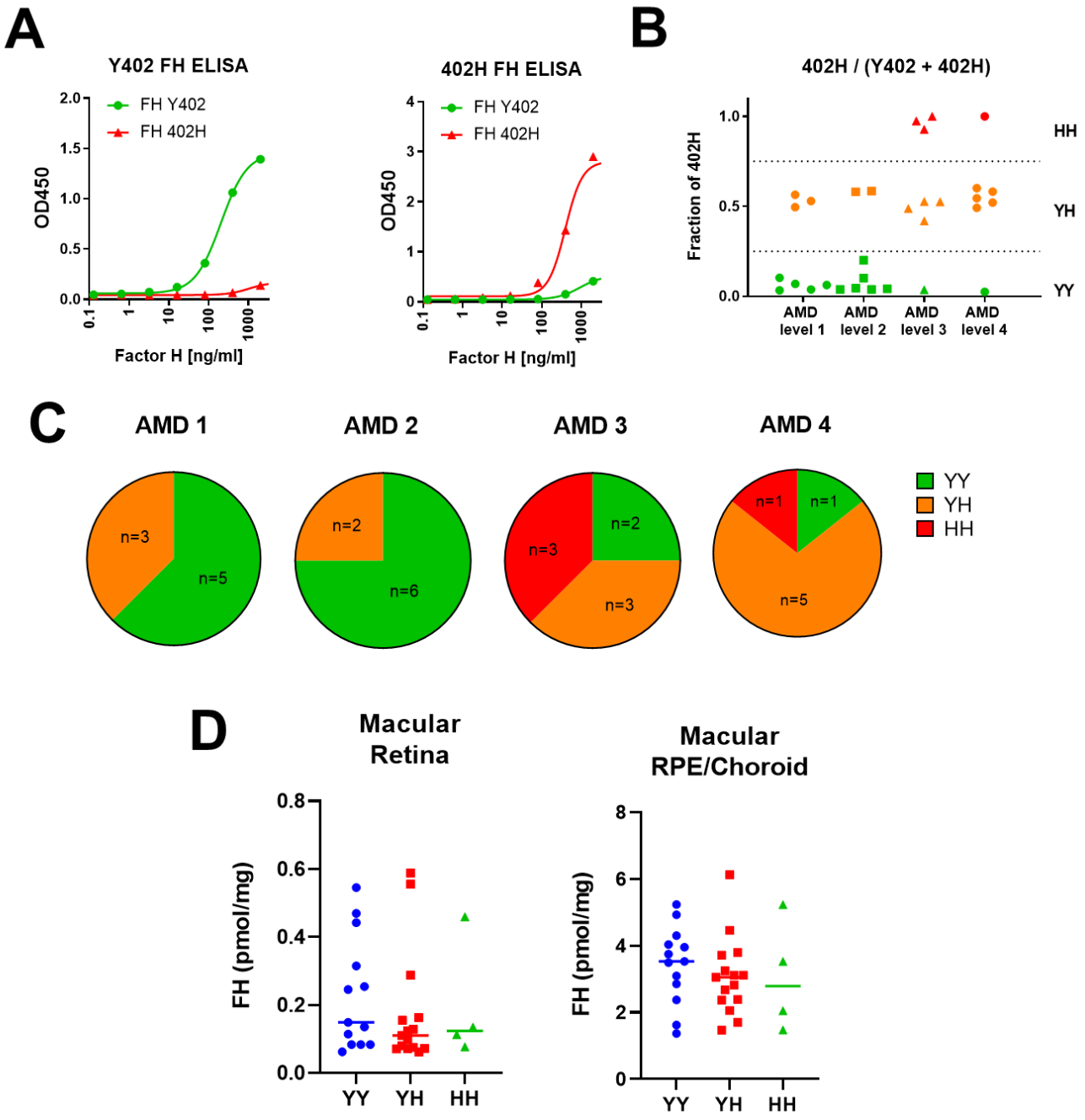
FB ~ 90kDa
Ba ~ 30 kDa
FH ~ 150kDa
FI ~ 35-50kDa
βTubulin 50kDa



Supplementary Figure S1 Western blots of complement C3, FB, FH, and FI in vitreous humor, macular retina, and macular RPE/choroid from donor eyes with all 4 AMD grades. For each complement protein analysis on 16-well gels plus one well of protein molecular weight standard, equal amounts of two AMD 1 samples were pooled and loaded on two gels. The first four AMD2, 3, and 4 samples were run on the first gel and the rest was run

on the second gel. All western blot values were normalized by the average AMD1 value from the same gel. Vitreous humor samples were diluted directly in 4x gel loading buffer, loaded 10 µL (7.5 µL vitreous) per well, and probed with antibodies against C3 (**A**), FB (**B**), FH (**C**), and FI (**D**). Expected protein sizes were labeled on the right. Macular retina (**E**) and macular RPE/choroid (**F**) tissue lysates (50 µg/per well) were probed for anti-C3, FB, FH, and FI antibodies and adjusted for loading control anti-beta tubulin.

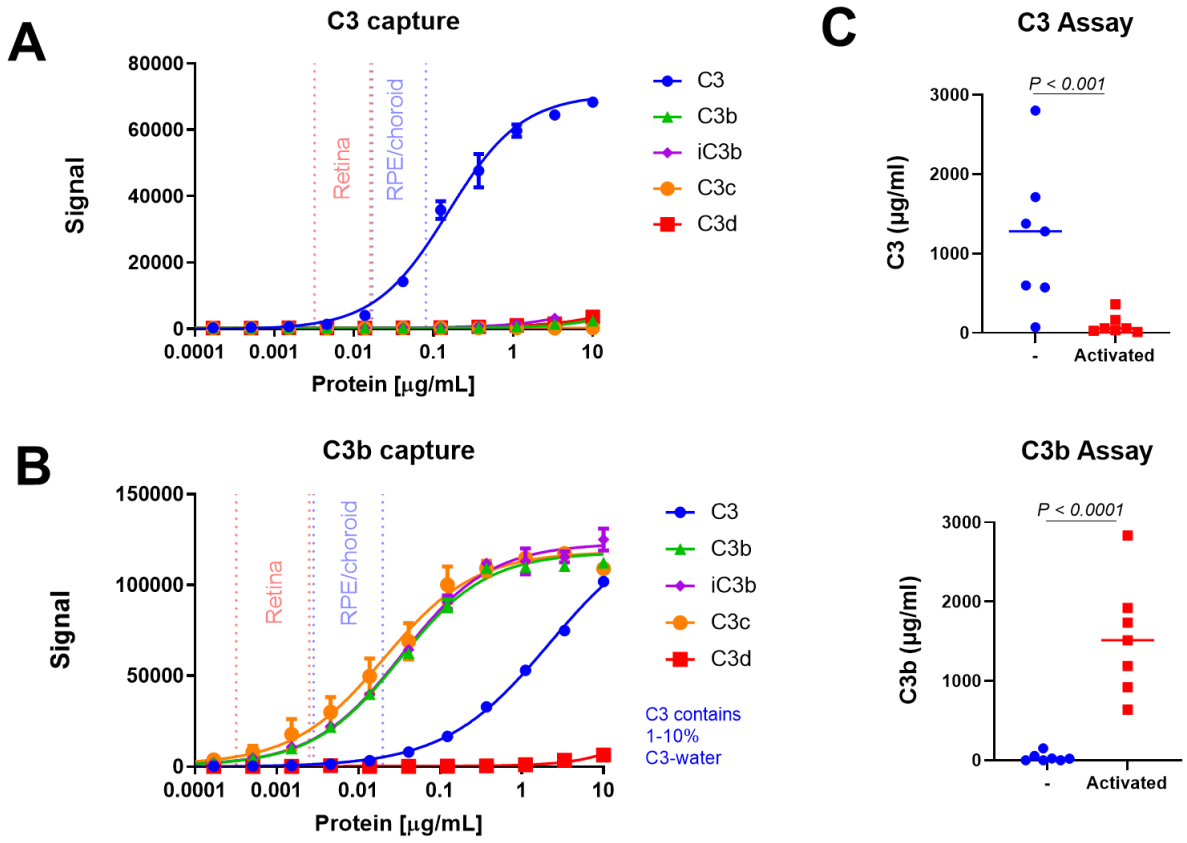
Supplementary Figure S2



Supplementary Figure S2 CFH Y402H genotype is associated with AMD severity but not with ocular FH protein levels. Protein lysates from peripheral RPE/choroid were genotyped for

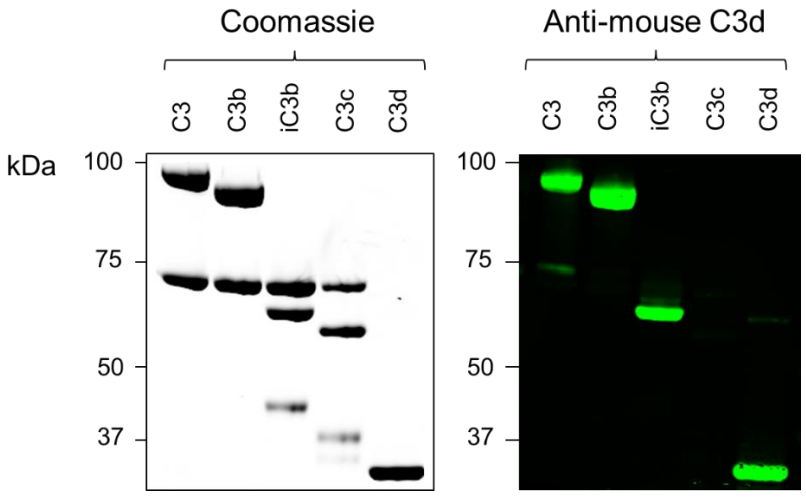
the AMD-risk common Y402H variant in CFH gene using selective ELISAs (SKU: HK353, Hycult Biotech, Wayne, PA, USA) per manufacturer's instructions. (**A**) Titration curves of Y402 and 402H FH ELISAs using Y402 and 402H FH protein standards. CFH genotype was determined by the extrapolated levels of 402H relative to total FH levels (Y402 + 402H) in each sample, grouped by each AMD grade (**B**), and also graphed in pie charts (**C**). Donors in AMD3 and AMD4 grades had higher incidence of 402H variants than donors in AMD 1 and AMD 2 grades. Levels of FH (**D**) and other complement proteins (not shown) were compared between Y402H genotypes in either macular neural retina or macular RPE/choroid. There was no statistically significant difference in levels which may be due to low sample size.

Supplementary Figure S3



Supplementary Figure S3 Selectivities of C3 and C3b MDS assays Selectivity of C3 (**A**) and C3b (**B**) MSD assays were determined, with extrapolated ranges for unknown tissue samples highlighted. C3 MSD assay captures only C3 full-length not C3b, iC3b, C3c, or C3d. C3b MSD assay captures C3b, iC3b, or C3c, but not C3d. Purified C3 is prone to spontaneous conversion to C3-water, which alters the C3 conformation to a "C3b-like" conformation, so the C3b assay will pick up these trace contaminants (1-10%). (**C**) Confirmation of C3 and C3b selectivities by measuring C3 and C3b levels in complement-activated normal human serum (NHS). Cobra Venom Factor (CVF, Qiudel, SanDiego, Califonia) is the complement-activating protein from cobra venom. C3 levels were reduced to less than 1% in CVF-activated serum (10ug/ml, 5 mM Mg^{2+}/Ca^{2+}, 2 hours) compared to non-activated serum (10 mM EDTA) while C3b levels were increased by over 40-fold, confirming the conversion of C3 to C3b breakdown products.

Supplementary Figure S4



Supplementary Figure S4 Cross-reactivity of an anti-mouse C3d to human C3d containing C3 proteins Purified human proteins were purchased from Complement Technology Inc. (Texas, USA): C3 (A113c), C3b (A114), iC3b (A115), C3c (A116) and C3d (A117). C3 proteins were loaded into two 4-12% Bis-Tris gels, 5 µg of each protein was loaded on the gel for imaging with a Coomassie stain, and 1µg was used on the other gel for a western blot with the anti-mouse C3d antibody (AF2655, R&D). As shown in supplemental Figure S4, the anti-mouse C3d antibody cross-reacts with C3d epitopes within the alpha chains of human C3, C3b, iC3b, as well as C3d protein. It does not bind to human C3beta and C3c proteins.