Mouse genetics and proteomic analyses demonstrate a critical role for complement in a model of DHRD/ML, an inherited macular degeneration

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Macular degenerations, inherited and age related, are important causes of vision loss. Human genetic studies have suggested perturbation of the complement system is important in the pathogenesis of age-related macular degeneration. The mechanisms underlying the involvement of the complement system are not understood, although complement and inflammation have been implicated in drusen formation. Drusen are an early clinical hallmark of inherited and age-related forms of macular degeneration. We studied one of the earliest stages of macular degeneration which precedes and leads to the formation of drusen, i.e. the formation of basal deposits. The studies were done using a mouse model of the inherited macular dystrophy Doyne Honeycomb Retinal Dystrophy/Malattia Leventinese (DHRD/ML) which is caused by a p.Arg345Trp mutation in EFEMP1. The hallmark of DHRD/ML is the formation of drusen at an early age, and gene targeted Efemp1R345W/R345W mice develop extensive basal deposits. Proteomic analyses of Bruch’s membrane/choroid and Bruch’s membrane in the Efemp1R345W/R345W mice indicate that the basal deposits comprise normal extracellular matrix (ECM) components present in abnormal amounts. The proteomic analyses also identified significant changes in proteins with immune-related function, including complement components, in the diseased tissue samples. Genetic ablation of the complement response via generation of Efemp1R345W/R345W:C3−/− double-mutant mice inhibited the formation of basal deposits. The results demonstrate a critical role for the complement system in basal deposit formation, and suggest that complement-mediated recognition of abnormal ECM may participate in basal deposit formation in DHRD/ML and perhaps other macular degenerations.

INTRODUCTION

Macular degenerations are important causes of vision loss. Inherited macular degenerations often affect individuals in the first two decades of life (1). Age-related macular degeneration (AMD) is the leading cause of vision loss in individuals over 65 years of age in the Western world and is increasing in prevalence in other parts of the world (2–5). AMD is a complex disorder with age as the major risk factor (6,7). Genetics and other factors such as smoking, hypertension, obesity and diet also contribute to the risk of the disease (6,8–10). Genetic association studies have identified 19 loci that are associated with the risk of developing AMD (11). These loci include genes involved with lipid metabolism, angiogenesis, extracellular matrix (ECM)
remodeling and the complement system. Variants of complement genes and the ARMS2/HTRA1 locus on chromosome 10q26, account for the majority of the genetic risk for AMD (7,11).

Human genetic studies identified variants in genes for complement factor H (CFH), complement C3 (C3), complement C2 (C2), complement factor B (CFB), complement factor D (CFD) and complement factor I (CFI) that alter the risk of AMD or the progression to advanced AMD (10,12–23). The complement system can be activated through three major pathways: classical, lectin and alternative pathways (24,25). Complement C3 is central to all three pathways. C2 is a component of the classical and lectin pathways and CFB, CFI and CFD are components of the alternative pathway. It is not known which complement pathway initiates the involvement of complement in AMD or whether more than one pathway is involved. The link between AMD and variants of CFH, a regulator of the alternative pathway, seems to be stronger than that of the other complement proteins, suggesting that the alternative pathway may be the major pathway involved (12–15). The specific mechanisms by which complement contributes to the pathogenesis of AMD are not known. It is, also, not known if complement has a major role in the pathogenesis of inherited macular degenerations. An inhibitor of the activation of complement C3 leads to a regression of drusen in nonhuman primates (26). Drugs have been designed and some are in trials that target additional complement components as a way of limiting inflammation which is thought to be a major factor in drusen formation and AMD pathogenesis (27–30).

The presence of drusen is one of the first clinical indicators of a risk for developing macular degeneration and a characteristic clinical finding in the maculae of patients with both age-related and inherited macular degenerations (31,32). Drusen are extracellular accumulations of proteins and lipid between the retinal pigment epithelia (RPE) and Bruch’s membrane (BrM) (33–35). Many components of drusen have been identified, but the sources of these components and the mechanisms of drusen formation are not fully understood (27,36–46). The presence of complement proteins in drusen led to the original hypothesis that the immune system and inflammation were involved (27,28). The location of drusen between the RPE and BrM suggests that the components may be primarily derived from the RPE, but there is evidence that choroid and possibly the retina may also contribute to drusen formation (45,47).

Basal deposits, microscopic accumulations of material under the RPE, have been implicated as precursors to drusen and as indicators of risk for developing AMD (33,35,48–50). The mechanisms involved in the formation of basal deposits are, however, not understood. Basal laminar deposits were reported to contain long-spaced collagen, carbohydrate structures, laminin, fibronectin, collagen type IV and vesicles (51,52). Studies of AMD patients and studies on transgenic mice suggest a role of lipid metabolism and high-fat diets in basal deposit formation (40,43,53). It is known that several genetically engineered mouse strains used to study AMD, each with a different targeted gene, develop basal deposits (54–59). Thus, there may be common mechanisms involved in deposit formation.

We investigated the formation of basal deposits using an animal model of the inherited macular dystrophy, Doyne Honeycomb Retinal Dystrophy/Malattia Leventinese (DHRD/ML) (60). DHRD/ML is the inherited macular dystrophy that is phenotypically most similar to the more common AMD (57,61,62). A hallmark of DHRD/ML is extensive drusen which can be present as early as the second decade of life (60). DHRD/ML is caused by a p.Arg345Trp mutation in epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1) (56,63).

EFEMP1, also known as fibulin 3, is a member of the fibulin family of seven extracellular matrix proteins (64–67). Variants in FBLN5 (fibulin 5), but not in EFEMP1, have been associated with the risk of AMD (61). EFEMP1 is ubiquitously expressed in epithelial and endothelial cells and is a component of BrM (64,66–68). EFEMP1 has been shown to have a role in elastic fiber integrity, in signal transduction by binding and activating EGF receptors and in altering tumor growth by modulating the extracellular environment (67,69–72). In addition, EFEMP1 is a binding partner of TIMP3 which, when mutated, causes the inherited Sorsby’s fundus dystrophy (73,74).

Gene targeted mice (Efemp1<sup>R345W/R345W</sup>) develop features of macular degeneration including the formation of extensive basal deposits with age and RPE abnormalities including vacuoles and loss of basolateral infoldings (56,58). These are the result of the p.Arg345Trp mutation in Efemp1 as Efemp1 knock-out mice do not have a retinal phenotype (69). Increased levels of complement C3 between the RPE and BrM were present in the mutant mice implicating the complement system in the pathology of the mutation in Efemp1 (56). Thus, the Efemp1<sup>R345W/R345W</sup> knock-in mice provide a useful model for studying the formation of basal deposits as potential precursors to drusen formation and the potential involvement of the complement system.

In this study, we used two approaches, proteomics and mouse genetics, to investigate basal deposit formation and the role that the complement system may have in this process in the Efemp1<sup>R345W/R345W</sup> mice. The proteomics analyses of BrM and the Bruch’s membrane/choroid (BrCh) of mutant and control mice further implicate complement and immune processes in the pathogenesis of the p.Arg345Trp mutation in Efemp1. The genetic studies functionally validated a critical role for complement in the formation of basal deposits in the Efemp1<sup>R345W/R345W</sup> mice.

RESULTS

Proteomic analyses

The mechanisms of basal deposit formation in the Efemp1<sup>R345W/R345W</sup> mice were investigated by analyzing the proteins in BrCh and BrM using mass spectrometry. BrCh from 8, 14 and 24 month old mice was analyzed in order to follow protein changes during the time that the basal deposits have formed (8 months) until the time they are extensive and can be a few microns thick (24 months) (56,58). BrM samples (24 months) were analyzed to more specifically investigate the composition of the basal deposits, as the deposits remained with BrM after the removal of the RPE cells (Fig. 1). The numbers of proteins identified for each of the eight samples studied are listed in Table 1. A total of 1062 proteins were identified among the eight samples. Fifty percent of these proteins were present in at least seven of the eight samples. Complete lists of the proteins identified and estimates of their abundances based on normalized spectral counts for each protein are
Elastin becomes cross-linked but even in the absence of cross-linking there are very few tryptic peptides from elastin that are present at lower abundance. Elastin was not detected in BrM.

Figure 1. Electron micrograph of basal deposits associated with BrM after the removal of RPE cells as described in Materials and Methods. Wide-spaced collagen is indicated by the white arrow.

Table 1. Number of proteins identified in each sample

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Sample</th>
<th>Mice</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>BrCh</td>
<td>Efemp1-R345W</td>
<td>844</td>
</tr>
<tr>
<td>8</td>
<td>BrCh</td>
<td>wt</td>
<td>824</td>
</tr>
<tr>
<td>14</td>
<td>BrCh</td>
<td>Efemp1-R345W</td>
<td>889</td>
</tr>
<tr>
<td>24</td>
<td>BrCh</td>
<td>Efemp1-R345W</td>
<td>795</td>
</tr>
<tr>
<td>24</td>
<td>BrCh</td>
<td>wt</td>
<td>654</td>
</tr>
<tr>
<td>24</td>
<td>BrM</td>
<td>Efemp1-R345W</td>
<td>727</td>
</tr>
<tr>
<td>24</td>
<td>BrM</td>
<td>wt</td>
<td>606</td>
</tr>
</tbody>
</table>

Total number of proteins identified in all samples 1062
Number of proteins identified in seven of eight samples 530

Identification of proteins associated with the mutant phenotype

Principal component analysis

Principal component analysis (PCA) was used to analyze the normalized spectral counts for the eight sets of data listed in Supplementary Material, Table S1 (8, 14, 24 months for BrCh and 24 months for BrM) to determine the relationships among these sets of data. PCA is an exploratory method that makes no a priori decisions about the relationships among the data (80). The correlated multivariate data are reduced to uncorrelated variables, called the principal components (PCs), based on the variance of the data. The general trends in a data set can usually be seen in plots of the first two or three PCs. The contributions of each protein to the PCs are obtained from their respective PCA loadings (81). PCA is now frequently used to analyze proteomic data (82,83).

A PCA scores plot for the eight sets of data is shown in Figure 2A. PC1 (x-axis) accounts for 31% of the variance and is roughly correlated with the age of the mice. An additional 21% of the variance is accounted for by PC2 (y-axis) and is correlated with the mouse genetics (wt versus Efemp1R345W/R345W mice) for the 24 months mice. Simplistically, the distance between data points provides a relative measure of similarity among the data sets, with closer data points being more similar. The plot clearly shows that at 8 months there is little effect of the mutation in Efemp1 on the proteins in BCh samples. The effect of the mutation begins to be apparent by 14 months as the distance between the data points is slightly greater. At 24 months, the difference between the samples from mutant and wt mice is pronounced. There is a wide separation between the data points for the mutant and the wt mice for both the BCh samples and the BrM samples. PC2 is highly correlated with the difference between the wt and mutant mice at 24 months and, thus, is most associated with the basal deposit phenotype.

Prior to age (PC1) and genetics (PC2) based on the loadings are listed in Tables 2 and 3. Each list in Tables 2 and 3 contains the top 60 proteins that contribute the most and account for about half of the variance in PC1 and in PC2 (see Materials and Methods and Supplementary Material, Fig. S1).

Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) using a standard clustering algorithm (K nearest neighbor) was performed as an additional approach to verify the relationships among the sets of data (Fig. 2B) (84). The observed pattern for HCA agrees with that found using PCA. The 8 months samples (mutant and wt) and the 14 months samples (mutant and wt) are linked first to each other indicating a high degree of similarity. At 24 months, the mutant BCh and BrM samples were linked, indicating that they are more similar to each other than to their respective wt samples, confirming that the basal deposits altered protein composition most at 24 months of age.

Matched pair analysis

Matched pair analysis was also used to identify biologically significant protein changes associated with the development of basal deposits in the Efemp1R345W/R345W mice (85). This
BrM data. (wt is wild type, ki is knock-in) from each other, with the ki BrCh data being most similar to the 24-month-old ki samples. That is, the 24-month-old ki and wt samples are most different. These analyses showed the same relationships between samples as that observed.

K-nearest-neighbor method with 1 PC and normalized mean-centered data. This was based on the following observations. The PCA score plot (Fig.2A) on the fold changes. Those protein changes that were >3 SD from the mean of the differences were considered significant.

The SD calculated for the 8 months data was used to define the random differences of those proteins unaffected by the genetic changes. These random differences include natural biological, sample preparation and instrumental factors. This was based on the following observations. The PCA score plot (Fig. 2A) showed strong similarity between the data for the wt and mutant samples at 8 months. The Pearson correlation coefficient between proteins from wt and mutant mice was 0.984 indicating a strong correlation. The slope for the plot of the normalized spectral counts for the proteins in wt versus the mutant mice was 0.975 ± 0.026 (data not shown). These three results suggest that the levels of the majority of the proteins were highly correlated and were unaffected at 8 months by the p.Arg345Trp mutation in Efemp1. In fact, only 21 of the 832 proteins analyzed in the 8 months data set were outside 3 SD.

Based on the SD of protein variation established using the 8 months data set (SD = 0.298), the proteins exceeding the 3 SD limit for the other data sets were identified and their deviation was assumed to arise from the mutation. The numbers of proteins >3 SD at 14 and 24 months were 66 and 88, respectively, for BrCh and 65 for 24 months BrM. The proteins for 24 months, BrCh and BrM, are listed in Tables 4 and 5, respectively.

The matched pair difference approach is biased toward high-abundance proteins. In contrast, the use of ratios or fold changes is biased toward low abundance proteins. Thus, high-abundance proteins with large changes in their normalized spectral counts may be considered significant even though the fold change is not large. Likewise, changes in low abundance proteins may not be considered significant when they may, in fact, be biologically significant. More emphasis was placed on the ratios of normalized spectral counts between the wt and mutant mice for the low abundance proteins. Examples for low abundance proteins with significantly altered ratios in the mutant mice include collagen IV, alpha 1 which was decreased 10-fold and tenascin C and complement C4 which were increased 4.9- and 4.3-fold, respectively.

**Biological processes of significantly altered proteins in mutant mice**

Each protein in Tables 2–5 was annotated according to its associated biological processes using the Panther Classification system (86) and Mouse Genome Informatics (87). Immune system processes, cell–cell and cell–matrix adhesion, signal transduction and intracellular protein transport contributed the most to the differences observed in the mutant mice. Other biological processes such as proteolysis, apoptosis, lipid-related processes, cellular morphogenesis and angiogenesis were represented but to a much lesser extent. The number of proteins that contributed to each of these four major biological processes and the gene symbol for all of the proteins included for each biological process are summarized in Figure 3. The majority of proteins identified by the matched pair analysis overlap with those identified as major contributors in the PCA. As examples of proteins in these four categories, the following changes were observed.

**Immune system processes**

Immune system processes include complement activation, macrophage activation, B-cell-mediated immunity and antigen presentation and processing. Over 150 proteins with roles in these processes were identified in the eight data sets. Of these, 43 proteins were significantly altered in 24 months mutant mice or contributed significantly to PC1 and PC2 (Fig. 3). A few of these proteins changed progressively with age from 8 to 24 months. Thrombospondin 1 increased 13-fold in the mutant mice. Leukotriene A4 hydrolase was 8-fold higher in the mutant mice as a result of a comparable decrease with age in the wt mice. Macrophage migration inhibitory factor also decreased nearly 10-fold in the wt mice from 8 to 24 months.
The locations of complements C3 and C4, CFH, MBL and thrombospondin 1 in BrM were validated using immunofluorescence (Fig. 5). In addition, the relative levels of staining were consistent with the results obtained by mass spectrometry (Table 6). Staining for C3 was more intense in the mutant mice than in wt mice as previously reported (56), staining was more intense for C4 in mutant mice, and staining was comparable for CFH in both wt and mutant mice. MBL was weakly detectable in both the wt and mutant mice but there were a few regions of intense staining in the mutant mice.

### Table 2. Proteins that contribute most significantly to PC1

<table>
<thead>
<tr>
<th>Loadings</th>
<th>Proteins 1–30</th>
<th>Gene</th>
<th>Loadings</th>
<th>Proteins 31–60</th>
<th>Gene</th>
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<tr>
<td>0.169</td>
<td>Milk fat globule-EGF factor 8 protein</td>
<td>Mfge8</td>
<td>0.075</td>
<td>Macrophage migration inhibitory factor</td>
<td>Mif</td>
</tr>
<tr>
<td>0.144</td>
<td>Trifunctional protein, alpha subunit</td>
<td>Hauda</td>
<td>0.073</td>
<td>Perlecian (heparan sulfate proteoglycan 2)</td>
<td>Hspg2</td>
</tr>
<tr>
<td>0.138</td>
<td>Trypsin 10</td>
<td>Try10</td>
<td>0.073</td>
<td>Synaptophysin-like protein</td>
<td>Syp1</td>
</tr>
<tr>
<td>0.138</td>
<td>Thrombospondin 1</td>
<td>Thbs1</td>
<td>0.072</td>
<td>Cell adhesion molecule 1/nectin-like 2</td>
<td>Cadm1</td>
</tr>
<tr>
<td>0.129</td>
<td>Galectin-3</td>
<td>Lgal3</td>
<td>0.069</td>
<td>Immunoglobulin heavy chain complex</td>
<td>Ig8</td>
</tr>
<tr>
<td>0.128</td>
<td>Cathepsin B</td>
<td>Ctsb</td>
<td>0.068</td>
<td>Fibrinogen alpha chain</td>
<td>Fga</td>
</tr>
<tr>
<td>0.120</td>
<td>Collagen, type VI, alpha 3</td>
<td>Col6a3</td>
<td>0.068</td>
<td>Methyltransferase like 7A1</td>
<td>Mettl7a1</td>
</tr>
<tr>
<td>0.118</td>
<td>Cytochrome c oxidase subunit I</td>
<td>Mito-co1</td>
<td>0.067</td>
<td>Decorin</td>
<td>Dcn</td>
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<tr>
<td>0.115</td>
<td>Efemp1</td>
<td>Efemp1</td>
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<td>EH-domain containing 3</td>
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<td>Bgn</td>
<td>0.067</td>
<td>Ras homolog gene family, member C</td>
<td>Rhec</td>
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<td>Actin, beta-like 2</td>
<td>Actb2</td>
<td>0.067</td>
<td>RAB5A, member RAS oncogene family</td>
<td>Rab5a</td>
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<td>0.106</td>
<td>Tubulin beta-2A chain</td>
<td>Tubb2a</td>
<td>0.066</td>
<td>Fibrinogen gamma chain</td>
<td>Fgg</td>
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<td>0.103</td>
<td>Lysozyme 2/lysozyme C-2 precursor</td>
<td>Lys2</td>
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<td>Glycoprotein (transmembrane) mb</td>
<td>Gpmb</td>
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<td>0.101</td>
<td>Protease, serine, 1 (trypsin 1)</td>
<td>Prss1</td>
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<td>Ap1</td>
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<td>0.099</td>
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<td>0.066</td>
<td>PRA1 family protein 2</td>
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<td>0.065</td>
<td>Actin, beta</td>
<td>Actb</td>
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<td>0.096</td>
<td>Sec61 alpha 1 subunit (S. cerevisiae)</td>
<td>Sec61a1</td>
<td>0.065</td>
<td>Translocase, outer mitochondrial membrane 40</td>
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<td>0.096</td>
<td>ATPase, H+ transporting, lysosomal V0 subunit C</td>
<td>Ap6v0c</td>
<td>0.065</td>
<td>Myosin, light chain 12B, regulatory</td>
<td>Myl12b</td>
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<tr>
<td>0.088</td>
<td>Histidine triad nucleotide-binding protein 1</td>
<td>Hnt1</td>
<td>0.064</td>
<td>ATPase, H+ transporting, lysosomal V0 subunit D2</td>
<td>Ap6v0d2</td>
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<td>0.085</td>
<td>Syndecan-binding protein</td>
<td>Sdcbp</td>
<td>0.063</td>
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<tr>
<td>0.083</td>
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<td>Ig mu chain C region secreted form</td>
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<tr>
<td>0.082</td>
<td>Heat-shock protein 2</td>
<td>Hspa2</td>
<td>0.063</td>
<td>ADAMTS-like protein 5 isoform 1 precursor</td>
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<tr>
<td>0.081</td>
<td>6-Phosphogluconolactonase</td>
<td>Pgls</td>
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<td>0.078</td>
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<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5</td>
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<td>0.061</td>
<td>RAB32, member RAS oncogene family</td>
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<tr>
<td>0.075</td>
<td>Actin, alpha, cardiac muscle 1</td>
<td>Actc1</td>
<td>0.061</td>
<td>Offactory receptor 810</td>
<td>Olf810</td>
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</tbody>
</table>

Eleven of the major components of the three complement pathways were identified in the eight data sets although, as expected, the abundance was low (Table 6). C3 was increased (1.8-fold) in the Efemp1R345W/R345W mice in 24 months BrM and was increased at younger ages in BrCh in the Efemp1R345W/R345W mice. CFH was present at comparable levels in the 24 months mice, wt and mutant. Complement C1q (C1q) and mannose-binding lectin (MBL) are recognition molecules for the classical and lectin pathways, respectively, were both identified and were present at low but comparable levels.

Of particular interest was the contribution of C4 to immune processes and signal transduction for PC2 (Fig. 3B). C4, a relatively low abundance protein, was increased >4-fold in the BrCH of mutant mice at 24 months and was present at the same increased level in BrM in the mutant mice. An increase in C4 in the mutant relative to the wt mice was apparent by 14 months in BrCh.

The peptides identified in complements C3 and C4 in 24 months BrM are in Supplementary Material, Table S3 and their locations in the proteins are illustrated in Figure 4. In C3, the majority of the peptides (11 of 15) detected in the wt and mutant mice were from the fragment C3dg. The C4 peptides detected were only present in the mutant mice and were located within the fragment C4d.

### Adhesion and signal transduction

The numbers of proteins involved in cell–cell and cell–matrix adhesion and in signal transduction that contributed significantly to the basal deposit phenotype and the gene symbols for these proteins are shown in Figure 3. ECM and ECM-associated proteins comprise the majority of the proteins that are associated with adhesion and signaling among those proteins that contribute most significantly to PC1 and PC2 and for those proteins that undergo the largest changes in BrCh and BrM as a result of the mutation in Efemp1. Many of the same proteins contribute to both adhesion and signaling processes. EFEMP1, collagens type VI, alpha 3 and alpha 1, thrombospondin 1 and milk fat globule-EGF factor 8 are among the proteins in each category.
Intracellular protein transport

Proteins annotated as having roles in intracellular protein transport include proteins known to be associated with lysosomes, with endocytic and exocytic processes, and in vesicle-mediated transport. The numbers of proteins involved in intracellular protein transport contributed almost equally to PC1 and PC2 and to the 24 months BrCh and BrM samples. However, only 7 of the proteins are the same for both PC1 and PC2, but 12 proteins are the same for BrM and BrCh (Fig. 3). Lamins (alpha 5 and beta 2), associated with endocytic processes, were two of only five ECM proteins associated with intracellular protein transport and both were decreased at 24 months. EH-domain containing protein 4, involved with regulation of early endosomal transport, and clathrin, which functions in receptor-mediated transport, contributed to PC1, BrM and BrCh but not PC2. Both were increased (88,89).

Role of complement in basal deposit formation

We chose to test the functional significance of our data analyses by focusing on the relevance of the complement system to a major histopathologic feature of the R345W/R345W mice, i.e. the age-dependent formation of basal laminar deposits. This choice was based on the following: (1) the increased levels of C3 and C4 in BrM in the R345W/R345W mice, which contributed most significantly to adhesion and signaling processes. Each of these was increased. The relative increase of thrombospondin 1 in mutant mice is evident in Figure 5. The matricellular proteins, decorin and biglycan, which have roles in both adhesion and signaling were increased. The lamins were decreased. Perlecans which contributed most significantly to adhesion and signaling only in PC1 (age) was notable in that it decreased progressively with age in the wt mice and decreased further in the mutant mice to 20% of the level at 8 months (Supplementary Material, Table S1).

For both adhesion and signaling processes. Each of these was increased. The relative increase of thrombospondin 1 in mutant mice is evident in Figure 5. The matricellular proteins, decorin and biglycan, which have roles in both adhesion and signaling were increased. The lamins were decreased. Perlecans which contributed most significantly to adhesion and signaling only in PC1 (age) was notable in that it decreased progressively with age in the wt mice and decreased further in the mutant mice to 20% of the level at 8 months (Supplementary Material, Table S1).
at the point of complement C3. Thus, in the absence of an active complement C3, the complement system could not be activated. The mice were assessed at 14 months for basal laminar deposits using transmission electron microscopy. In the Efemp1<sub>R345W/R345W</sub> mice (Fig. 6A, panels A and B), contiguous basal laminar deposits were present. In addition, vacuoles are noted in the RPE and basal infoldings were not present. In contrast, basal deposits were absent in the homozygous Efemp1<sub>R345W/R345W</sub> double-mutant mice (Fig. 6, panel C). Some vacuoles could be seen in the double-mutant mice but basal infoldings were preserved and the RPE cells had a more normal appearance.

A quantitative comparison of the mean areas of the deposits for the different mouse strains was performed as described in Materials and Methods. The total area of basal deposits in each image was determined for each mouse (average of 65 images per mouse) for each strain (Fig. 6). The means of the cumulative image was determined for each mouse (average of 65 images per mouse) for each strain (Fig. 6). The means for the different mouse strains were determined and are compared in Figure 6E. There was a significant decrease in the area of basal deposits in the Efemp1<sub>R345W/R345W</sub> double-mutant mice. The differences between the means for the Efemp1<sub>R345W/R345W</sub> and the Efemp1<sub>R345W/R345W</sub> mice were significant to >95% confidence interval (CI) and the differences between Efemp1<sub>R345W/R345W</sub> mice and control mice were significant at 94% CI. The difference between the means of the areas for the double-mutant mice and control mice (wt and C3<sup>−/−</sup>) was not statistically significant. The area of deposits in one of the double-mutant mice was determined statistically to be an outlier but the data were still included in this analysis.

**DISCUSSION**

The results presented in this study provide insight into the role of complement in the early stages of macular degenerations and define the pathology in the BrM caused by the p.Arg345Trp mutation in Efemp1. A critical role for an active complement system in the formation of basal deposits was clearly demonstrated. Genetic disruption of complement C3 inhibited the formation of basal deposits, preserved the basal infoldings and reduced the RPE pathology in the Efemp1<sub>R345W/R345W</sub> mice. As basal deposits are considered precursors to drusen the results of this study provide evidence of how complement may contribute to drusen formation. The mutation in EFEMP1 causes the presence of extensive drusen in the inherited disorder, DHRD/ML, thus, a role for the complement system is implicated in the pathogenesis of DHRD/ML (60). Drusen are also a characteristic feature in

### Table 4. BrCh proteins: standard deviations (absolute) for differences between wt and Efemp1<sub>R345W/R345W</sub> mice

<table>
<thead>
<tr>
<th>Proteins 1–35</th>
<th>Gene</th>
<th>SD</th>
<th>Proteins 36–69</th>
<th>Gene</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin, beta 6 class V</td>
<td>Tubb6</td>
<td>2.43</td>
<td>Eukaryotic translation elongation factor 1 alpha 1</td>
<td>Eef1a1</td>
<td>4.8</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>2.30</td>
<td>Efemp1</td>
<td>Efemp1</td>
<td>4.8</td>
</tr>
<tr>
<td>Actin, alpha cardiac muscle 1</td>
<td>Actc1</td>
<td>1.68</td>
<td>Glutathione S-transferase, pi 1</td>
<td>Gsta1</td>
<td>4.6</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Bgn</td>
<td>1.41</td>
<td>Protease, serine, 1 (trypsin 1)</td>
<td>Prss1</td>
<td>4.6</td>
</tr>
<tr>
<td>Tubulin, alpha 1A</td>
<td>Tuba1a</td>
<td>1.17</td>
<td>Aldo-keto reductase family 1, member A1</td>
<td>Akr1a4</td>
<td>4.5</td>
</tr>
<tr>
<td>Collagen, type VI, alpha 1</td>
<td>Col6a1</td>
<td>1.10</td>
<td>Heat-shock protein 90 alpha (cytosolic)</td>
<td>Hsp90ab1</td>
<td>4.3</td>
</tr>
<tr>
<td>Milk fat globule-EGF factor 8 protein</td>
<td>Mfge8</td>
<td>0.98</td>
<td>Receptor accessory protein 5</td>
<td>Reep5</td>
<td>4.3</td>
</tr>
<tr>
<td>scel alpha 1 subunit (S. cerevisiae)</td>
<td>Sec61a1</td>
<td>0.92</td>
<td>Cathepsin L</td>
<td>Ctsl</td>
<td>4.2</td>
</tr>
<tr>
<td>Monoglycereide lipase</td>
<td>Mgl</td>
<td>0.89</td>
<td>Myosin, light chain 12B, regulatory</td>
<td>Myl12b</td>
<td>4.2</td>
</tr>
<tr>
<td>CD63 antigen</td>
<td>Cd63</td>
<td>0.84</td>
<td>Myosin, light polypeptide 9, regulatory</td>
<td>Myl9</td>
<td>4.2</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>Lgals3</td>
<td>0.81</td>
<td>EH-domain containing 4</td>
<td>Ehd4</td>
<td>4.0</td>
</tr>
<tr>
<td>Collagen, type VI, alpha 3</td>
<td>Col6a3</td>
<td>0.80</td>
<td>Signal sequence receptor, delta</td>
<td>Ssrd</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycoprotein (transmembrane) mnb</td>
<td>Gpnmnb</td>
<td>0.79</td>
<td>Surfeit gene 4</td>
<td>Syngr1</td>
<td>4.0</td>
</tr>
<tr>
<td>Surfeit gene 4</td>
<td>Surf4</td>
<td>0.77</td>
<td>Caenorhabditis elegans</td>
<td>Caenorhabditis elegans</td>
<td>3.9</td>
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<tr>
<td>Tubulin beta-2A chain</td>
<td>Tubb2a</td>
<td>0.76</td>
<td>Vesicle amine transport1 homolog (T. californica)</td>
<td>Vat1</td>
<td>3.6</td>
</tr>
<tr>
<td>Cathepsin b</td>
<td>Ctsb</td>
<td>0.74</td>
<td>Guanine nucleotide-binding protein (G protein), b1</td>
<td>Gna1b</td>
<td>3.9</td>
</tr>
<tr>
<td>Olfactory receptor 485</td>
<td>Olfr485</td>
<td>0.74</td>
<td>Perlecan (heparan sulfate proteoglycan 2)</td>
<td>Eng</td>
<td>3.9</td>
</tr>
<tr>
<td>Actin, cytoplasmic 1</td>
<td>Actb</td>
<td>0.71</td>
<td>Fibrillin 1</td>
<td>Fbn1</td>
<td>3.5</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>Tbps1</td>
<td>0.70</td>
<td>Guanine nucleotide-binding protein (G protein), b2</td>
<td>Gna2b1</td>
<td>3.5</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Vim</td>
<td>0.70</td>
<td>Leukotriene A4 hydrolase</td>
<td>Lta4h</td>
<td>3.5</td>
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<tr>
<td>Clathrin, heavy polypeptide (Hc)</td>
<td>Cltc</td>
<td>0.63</td>
<td>ADP-riboisolation factor-like protein 8B</td>
<td>Arf8b</td>
<td>3.4</td>
</tr>
<tr>
<td>Actin-related protein 2/3 complex, subunit 5-like</td>
<td>Arpc5l</td>
<td>0.59</td>
<td>Cytochrome b-5</td>
<td>Cyb5a</td>
<td>3.4</td>
</tr>
<tr>
<td>Collagen, type VI, alpha 2</td>
<td>Col6a2</td>
<td>0.59</td>
<td>PREDICTED high-mobility group protein B1-like</td>
<td>Hmgbl</td>
<td>3.4</td>
</tr>
<tr>
<td>Laminin, gamma 1</td>
<td>Lams</td>
<td>0.59</td>
<td>Integrin beta 4</td>
<td>Itgb4</td>
<td>3.4</td>
</tr>
<tr>
<td>Pyridoxal (pyridoxine, vitamin B6) kinase</td>
<td>Pdxk</td>
<td>0.59</td>
<td>Lumican</td>
<td>Lum</td>
<td>3.4</td>
</tr>
<tr>
<td>Myosin regulatory light chain 2-A (R. norvegicus)</td>
<td>Myl12a</td>
<td>0.59</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>Ppia</td>
<td>3.4</td>
</tr>
<tr>
<td>Syndecan-binding protein</td>
<td>Sdcbp</td>
<td>0.58</td>
<td>Novel protein similar splicing factor, arg/ser-rich 3</td>
<td>Srsf3</td>
<td>3.4</td>
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<tr>
<td>Histocompatibility 2, D region locus 1</td>
<td>H2-D1</td>
<td>0.57</td>
<td>Transketolase</td>
<td>Tkt</td>
<td>3.4</td>
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<tr>
<td>6-Phosphogluconolactonase</td>
<td>Pgl</td>
<td>0.56</td>
<td>Tryptase beta 2</td>
<td>Tpsb2</td>
<td>3.4</td>
</tr>
<tr>
<td>Ubiquitin C</td>
<td>Ub</td>
<td>0.54</td>
<td>Vesicle-associated membrane protein 2</td>
<td>Vamp2</td>
<td>3.4</td>
</tr>
<tr>
<td>Decorin</td>
<td>Den</td>
<td>0.52</td>
<td>Heat-shock protein 90, beta (G3pdh), member 1</td>
<td>Hsp90ab</td>
<td>3.3</td>
</tr>
<tr>
<td>Tubulointerstitial nephritis antigen-like 1</td>
<td>Tnagl1</td>
<td>0.52</td>
<td>Laminin, alpha 5</td>
<td>Lama5</td>
<td>3.3</td>
</tr>
<tr>
<td>Moesin</td>
<td>Msna</td>
<td>0.49</td>
<td>RAB1B, member RAS oncogene family</td>
<td>Rab1b</td>
<td>3.1</td>
</tr>
<tr>
<td>ADP-ribosylation factor 3</td>
<td>Arf3</td>
<td>0.48</td>
<td>Clusterin</td>
<td>Clu</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The 19 mitochondria proteins with SD >3 were not included in this list. They did not contribute to the four biological processes.
involved in basal deposit formation by identifying those proteins.

A major goal of this study was to determine the mechanisms

Proteomics as well.

AMD, suggesting that the results are relevant to that disorder

as well.

Proteomics

A major goal of this study was to determine the mechanisms

involved in basal deposit formation by identifying those proteins

that were altered in response to the mutation in \textit{Efemp1}. The

proteomic experiments were optimized to solubilize and identify

as many of the proteins as possible in the BrCh and BrM samples

including the ECM proteins. The ECM and ECM-associated pro-

teins comprised

including the ECM proteins. The ECM and ECM-associated pro-

teins. While the majority of the proteins detected in the BrM

samples are thought to be from BrM itself, some proteins
detected may be from the choriocapillaris which forms one

layer of the BrM or from membrane fragments and cell debris

trapped in basal deposits within a compromised BrM.

Biologically significant changes

Defining the biologically significant changes that were the result

of the mutation in \textit{Efemp1} presented a challenge. Since one focus

of the study was on BrM, an ECM, labeling techniques were not

considered feasible. Spectral counting is a successful method for

quantifying proteins and normalized spectral counts have been

successfully used to quantify cilia proteins and were used in this

study (75,76,90). Replicates were not done at any one age;

instead, data were collected at three ages and a comparison

between BrCh and BrM was done at one age as this approach

provided further confidence in the validity of the approaches used.

We chose to use PCA to identify trends and those proteins that

contribute to those trends (80). In addition, we chose to use the

matched pair difference approach and the SD of differences to

further analyze and understand the biological significance in

the data (85). When the matched pair difference approach was

applied to wt aging data, i.e. differences between 8 and 24

months, those proteins outside 3 SD were many of the same pro-

teins that contributed to PC1 (age) (data not shown). This pro-

vided further confidence in the validity of the approaches used.

Some proteins were only identified in either wt or mutant

samples at a given age and could reflect a technical issue

rather than a biological difference between the mutant and

wt samples. The matched pair difference approach provided a

means of identifying whether any of those differences were

significant. As indicated in the results, the matched pair differ-

ence approach is biased toward low abundance proteins.

ence approach is biased toward high-abundance proteins and the

use of ratios is biased toward low abundance proteins. More emphasis

was placed on the ratios of normalized spectral

consideration.
counts between the wt and mutant for the low abundance proteins. Two such significant examples for low abundance proteins with increased ratios in the mutant mice were complement C4 and C3. The significance of the changes in C3 and C4 based in this case on ratios was evident from the demonstration that complement has a necessary role in basal deposit formation.

Complement

While it was clearly shown in this study that active complement C3 is necessary for basal deposit formation in the Efemp1R345W/R345W mice, the underlying mechanisms by which the complement system contributes to basal deposit formation remain to be identified. The complement pathway that initiates the involvement of the complement system in basal deposit formation in the Efemp1R345W/R345W mice is not known. There are three pathways by which the complement system is typically activated, the classical, lectin and alternative pathways (24,25,9,12). The classical pathway is typically activated by immune complexes, the lectin pathway is activated by surface patterns and the alternative pathway is constitutively active at a low level. All three pathways can be activated by altered patterns caused by foreign proteins or damaged self (93).

Genetic studies which showed that variants of complement factors H and B correlated with a risk of developing AMD focused attention on the importance of the alternative pathway (12–17,19,21). The alternative pathway has also been
considered key in other human diseases (24,25,94). We found no direct evidence to suggest the alternative pathway was or was not involved in basal deposit formation in the \( \text{Efemp1}^{R345W/R345W} \) mice. Complement factor B was not detected and the levels of complement factor H were comparable in both the wt and mutant mice (Table 5 and Fig. 4). A role for either the classical or the lectin pathway in initiating the involvement of the complement system was suggested by two observations: (1) low levels of the recognition molecules for both the classical and lectin pathways, C1q and MBL, respectively, were present, and (2)

![Figure 4](https://example.com/image4)

**Figure 4.** Depiction of activation fragments of C4 and C3 with the location of peptides identified by mass spectrometry indicated. Black lines: C3 peptides identified in wt mice. Red lines: C3 and C4 peptides identified in \( \text{Efemp1}^{R345W/R345W} \) mutant mice. Asterisk indicates the location of the reactive thiol ester. The fragmentation pattern for C4 is modeled after human C4B.

![Figure 5](https://example.com/image5)

**Figure 5.** Confocal fluorescent images of cryosections stained with antibodies to the indicated proteins on the left of the figure. Sections were from 19-month-old mice. (wt is wild type, ki is \( \text{Efemp1}^{R345W/R345W} \) knock-in mice.) Scale bar is 10 \( \mu \text{m} \).

![Figure 6](https://example.com/image6)

**Figure 6.** Basal laminar deposit formation is inhibited in \( \text{Efemp1}^{R345W/R345W} \) mutant mice that are null for complement C3. Transmission electron micrographs are shown for 14-month-old mice. (A and B) Basal laminar deposits are present in \( \text{Efemp1}^{R345W/R345W} \)-mutant mice between the RPE and BrM. The deposits are outlined in (B). (C and D) \( \text{Efemp1}^{R345W/R345W, C3 }^{−/−} \) double mutant mouse (C) and wt mice of the same age (D) do not have basal deposits. (E) The mean area of basal deposits is plotted for each mouse strain: control mice (wt and C3 null), small hatch bars; \( \text{Efemp1}^{R345W/R345W} \) mutant mice (ki), black bar; and \( \text{Efemp1}^{R345W/R345W, C3 }^{−/−} \) double mutant mice (ki:C3 null), striped bar. The error bars are standard error of the mean. Double asterisks indicate the difference between the \( \text{Efemp1}^{R345W/R345W} \)-mutant mice and the \( \text{Efemp1}^{R345W/R345W, C3 }^{−/−} \) double mutant mice was significant (>95% CI). The difference between the \( \text{Efemp1}^{R345W/R345W} \) and control mice was significant to 94% CI. The number of mice is indicated above the error bars. The mean area of deposits for the double mutant mice is markedly reduced relative to that for the \( \text{Efemp1}^{R345W/R345W} \) mutant mice and is comparable to that for the wt mice.
levels of C4 were increased in BrM in the mutant mice. C4 participates in the activation of both the classical and lectin pathways. Evidence obtained using a cell culture model of human RPE cells that mimics the formation of drusen, suggested activation in that system was initiated by the classical pathway through binding of C1q to lipid rich protein deposits (95).

We propose that the altered components of BrM in the mutant mice led to the involvement of immune processes and the activation of the complement system by exposing new surfaces or neo-epitopes that then interact with the recognition molecules of the appropriate complement pathway. Increased levels of C3 and C4 peptides were found in BrCh by 14 months, and were derived from the activation fragments of C3 and C4, C3dg and C4d, respectively, in 24 months BrM. C3dg and C4d contain the reactive thiol ester that can covalently attach C3 and C4 to target sites (96–99). This implies that BrM components in the Efemp1R345W/R345W mice are recognized by the complement system, and may indicate that this recognition plays a role in deposit formation by activating the complement system.

It is known that alterations in the integrity of ECM and variants in components of ECM contribute to the pathogenesis of AMD (49,100,101). Further, mutations in EFEMP1, TIMP3 and CTRP5, all of which encode ECM proteins, lead to inherited macular degenerations (56,63,74,102). Models of the inherited macular degenerations caused by mutations in each of these genes develop basal deposits, suggesting that the underlying mechanisms may be similar and may involve complement (54–56,59,103).

Altered biological processes

The assignment of biological processes to those proteins that undergo large changes as a result of the mutation provides some assessment of what functions may be impacted by the p.Arg345Trp mutation in Efemp1. However, each protein may be assigned to more than one biological process and the major biological role of that protein may not be discernible from the assignments. Regardless, it provides direction and the basis of future investigations into how the Efemp1R345W/R345W mutation affects the function of the RPE/BrM/Choroid complex and leads to basal deposit formation. The largest impact appeared to be on immune system processes, cell–cell and cell–matrix adhesion, signal transduction and intracellular protein transport (Fig. 3). The role of the immune system in drusen formation was implicated previously (27). The results of this study support and extend the role of the immune system, in particular, the complement system in drusen formation.

Changes in the level of proteins with roles in intracellular protein transport are evidence that exo- and endocytic processes, vesicle transport and possibly lysosomal function are impacted by the mutation in Efemp1. This is consistent with the presence of vacuoles in the RPE as early as 2 months in the mutant mice and their enlargement with increasing age (56).

ECM and ECM-associated proteins are prominently represented in each of these biological processes and are prominent among the proteins with the major changes induced by the mutation in Efemp1. As would be expected for an ECM, the altered levels of ECM proteins lead to disruption of cell–cell and cell–matrix adhesion and the inherent signaling processes that rely on the structural integrity of the ECM (77). How these changes specifically lead to basal deposit formation is not clear. Four ECM and ECM-associated proteins stand out as being particularly significant in the mutant based on their large increase in the BrM of mutant mice. These are EFEMP1, thrombospondin 1, milk fat globule-EGF factor 8 and collagen VI. As described briefly below, each is involved in multiple cellular processes and/or the structure and function of the ECM.

**EFEMP1**

The phenotypic effects of the Arg345Trp mutation in EFEMP1 can be seen by 2 months when vacuoles are present in the RPE and wide-spaced collagen is present in BrM. Basal deposits become visible shortly thereafter. Yet the number of proteins increased or decreased in mutant BrCh is small even at 8 months. The major protein changes are not seen in the mutant mice until after 14 months with large changes observed in BrM proteins at 24 months. EFEMP1 accumulates in BrM of mutant mice. The accumulation in BrM could be due to increased expression and secretion or decreased turnover. Decreased secretion of the protein from cells in culture was reported but EFEMP1 appears to be efficiently secreted in the mutant mice (57,104,105). The Arg345Trp mutation in EFEMP1, which leads to increased levels of EFEMP1, may also lead to an altered conformation of the protein or altered interactions with components of BrM, any of which may cause the disrupted structure of BrM.

EFEMP1 is a binding partner to TIMP3 which is an inhibitor of matrix metallo-proteinases, but it is not known whether the Arg345Trp mutation influences the interaction with TIMP3 and the turnover of matrix components including EFEMP1 (73). EFEMP1 has function in the integrity of elastic fiber integrity in some tissues, and is also important in tumor growth where it activates signaling pathways through an interaction with EGF receptors and may alter cell motility (67,69–72). The specific mechanisms by which the Arg345Trp mutation in EFEMP1 leads to the pathology are under investigation.

**Thrombospondin 1**

The increase in thrombospondin 1 is the largest observed for any protein in the Efemp1R345W/R345W mice. Thrombospondin 1, a multi-domain protein, activates TGFbeta, initiates signal transduction, functions in the immune system and is involved in inflammation and wound healing (106–108). It is secreted by the RPE, is a component of BrM, choriocapillaris and larger vessels, and is important for retinal and choroidal vascular homeostasis (109–112). Thrombospondin 1 modulates angiogenesis (106–108). Mimetics of the type 1 repeats and the procollagen domains are anti-angiogenic and are being used to treat neovascular disease (113). A role of thrombospondin 1 in AMD is suggested by its presence in drusen (114). What function the increase in the Efemp1R345W/R345W mice may have and what stimulates its increase are not known but these questions warrant further study.

**Milk fat globule-EGF factor 8 protein**

Milk fat globule-EGF factor 8 protein (MFGE8), also known as lactadherin, is a pleiotropic, secreted glycoprotein that is a
component of BrM. The contribution of MFGE8 to PC1 and PC2 was highly significant and the protein was one of the most increased in the mutant mice. Its role in BrM in the mutant mice is totally unknown but a few of its reported functions may be significant in the mutant mice. MFGE8 is a ligand for alphavbeta5 integrin and is involved in the phagocytosis of photoreceptor outer segment discs by the RPE (115). MFGE8 limits inflammation and promotes tissue homeostasis through its role in clearing apoptotic bodies by bridging apoptotic bodies and phagocytic cells. MFGE8 is also thought to negatively regulate fibrosis by facilitating the removal of accumulated collagen, a function which may be relevant to BrM (116).

Collagen VI
Collagen VI comprises three chains, alpha 1, alpha 2 and alpha 3, and ultimately forms tetramers. This protein is an important component of extracellular matrices and is found in most interstitial tissues (117). Biglycan and decorin, both of which were increased, bind collagen VI and are involved in fibril formation (118-120). Collagen VI is an abundant component of the BrM proteome as reported here and was observed in BrM by immunostaining (121). In the Efemp1R345W/R345W mice, collagen VI was the only collagen that was not decreased and was slightly increased. It will be important to our understanding of basal deposit formation to determine if collagen VI is a major component of deposits and, if it is, what leads to its altered structure and increased presence.

Basal deposits
New proteins in the BrM of Efemp1R345W/R345W mice that could explain the composition of the basal deposits were not identified in the proteomic analyses reported here. This led us to propose that the basal deposits comprise proteins normally present in BrM but with altered levels and/or structures. For example, as early as 2 months, wide-spaced collagen was observed in BrM but with altered levels and/or structures. For example, as early as 2 months, wide-spaced collagen was observed in BrM by immunostaining (121). In the Efemp1R345W/R345W mice, collagen VI was only collagen that was not decreased and was slightly increased. It will be important to our understanding of basal deposit formation to determine if collagen VI is a major component of deposits and, if it is, what leads to its altered structure and increased presence.

Wide-spaced collagen has been observed in other ocular tissues such as epiretinal membranes and tissue samples from patients with AMD and Sorsby’s fundus dystrophy (52,122).

Several structural studies on the wide-spaced collagen in these tissues led to the conclusion that collagen VI is at least one component of these structures (52,122-124). Collagen type IV, laminin, and fibronectin have been described as components of basal laminar deposits (51). In the present study, collagen type IV and laminins were less abundant in the mutant mice than in wt mice, whereas collagen VI, an abundant protein in the BrM proteome, was increased in the mutant mice. While it is tempting to speculate that an altered structure of collagen VI may be a major component of basal laminar deposits and the wide-spaced collagen, additional evidence for this is needed.

The proteomic analyses identified structural proteins that are present in deposits such as collagens; however, we also think that a subset of proteins in the deposits may have a causative role in deposit formation. The detection of multiple complement system components in the deposits led us to the hypothesis that complement activation could be a causal factor in deposit formation. This hypothesis turned out to be true, and the results suggest an early role for perturbations to the complement system in deposit formation and macular degeneration pathogenesis. Studies of additional deposit components will be needed to identify other causative factors in deposit formation. It is also possible that factors which are not deposit components could play a role in initiating deposit formation, such as alterations in RPE function.

Comparison with human study
A proteomic study of human BrCh identified 901 proteins that were presence with varied frequency in 24 patients with AMD (125). A similar number of proteins (1062) were identified in this study in three ages of BrCh and 24 months BrM from wt and Efemp1R345W/R345W mice. About 50% of the proteins identified in the human BrCh were also identified in the mouse samples, which is excellent concordance for proteomics experiments using somewhat different methods (125). Most importantly, similar trends in the data between the two studies were observed. Seven of the 14 proteins that were less abundant in AMD were also decreased in the 24-month-old mutant mice. Nineteen of the 45 proteins that were more abundant in AMD were identified and 25% of those were increased in the mutant mice. Five proteins that were less abundant in early-to-mid AMD were also decreased in mutant mice. Four proteins that were increased in more advanced AMD, dry and neovascular, were likewise increased in the mutant mice. These were complement C3, vitronectin, galectin 3 and Ig mu chain C region, all of which have roles in the immune system. Complements C3 and C4 were elevated to a greater extent in the mutant mice than in the AMD samples. CFH was not increased in the mutant mice but CFH was slightly increased in the AMD samples. While there were quantitative differences in the results between these two studies, there was a remarkable concurrence of results between a mouse model of an inherited macular dystrophy and human BrCh in AMD. This concurrence provides validation for the use of the Efemp1R345W/R345W mouse model to study the pathogenesis of basal deposit formation in early macular degenerations.

MATERIALS AND METHODS
Mice
The use of the mice in this study was approved by the Massachusetts Eye and Ear Infirmary and Harvard Medical School Animal Care and Use Committees. The point mutation knock-in mice (Efemp1R345W/R345W mice) made in this laboratory were previously described (56). C3 null mice (C3−/−) had 105 bp of exon 1 and 2.3 kb of the 5′ flanking region disrupted with a neo-cassette transcriptionally opposite the C3 gene (126). Messenger RNA and protein were not expressed in the liver, there was no circulating C3, and the mice were functionally null for C3. A nonfunctional C3 protein expressed in other tissues is missing the leader sequence preventing native processing and secretion of the protein (126). C3 was not properly processed in our C3 null mice; the 10K fragment was not generated as seen in C3+/+ mice (Supplementary Material, Fig. S2).
Homozygous Efemp1<sup>R345W/R345W</sup>·C<sup>2−/−</sup> double-mutant mice were generated by crosses of the two strains. The double-mutant mice were viable, grossly normal, and had a normal lifespan. Control C3 null mice were obtained during the generation of the double-mutant mice and were subsequently bred. Littermate wt mice were used when possible but this was not always possible considering some of the mice were aged 24 months. In those cases, the wt mice were from the same colony but different breeders.

Genotyping
The primers used to genotype the mice are shown in Supplementary Material, Table S4. Genomic DNA was prepared from tail snips or ear clips using the DNaseqykit following the manufacturer’s instructions (Qiagen, Valencia, CA, USA). TaqMan polymerase, dNTP mix and reaction buffer were from Roche Applied Science (Indianapolis, IN, USA) and primers were from Integrated DNA Technologies (Coralville, IA, USA). PCR reactions for Efemp1 ki and wt and for C3 null were performed using 50 ng genomic DNA in 25 μl containing 25 nmol primers. PCR for C3 wt was done using 25 nmol primers in Green PCR Master Mix following the manufacturer’s protocol (Fermentas Life Sciences, Lafayette, CO, USA). PCR conditions for Efemp1 ki and wt were as follows: 94°C for 3 min and 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min followed by 94°C for 5 min. PCR conditions for C3 null and wt were as follows: 94°C for 3 min and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by 72°C for 2 min. PCR products were electrophoresed in a 1.5% agarose gel and visualized by SYBR Safe DNA gel stain (Invitrogen, Life Technologies, Grand Island, NY, USA).

Electron microscopy
Fourteen-month-old mice were euthanized with CO2 gas and perfused transcardially with 10 ml fresh 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The eyes were removed and placed in the same fixative for 3 h on ice. Eye cups were prepared by cutting just below the ora serrata and removing the anterior segment. After washing twice for 5 min in 0.1 M phosphate buffer (pH 7.4), the retina was cut into vertical oriented sections that extended from the optic nerve to the retinal periphery. These were left in phosphate buffer at 4°C overnight in a humid chamber. Cuts were made to flatten the eye cup. The eye cup was vortexed briefly in PBS. Remaining RPE cells were removed, washed with PBS and cleaned of tissue. The whole eyes were placed in OCT (Electron Microscopy Sciences, Hatfield, PA, USA) and quick frozen. Twelve-micron thick cryosections were cut, dried onto Colorfrost Plus slides and stored at −80°C until use.

Antibody reactivity
Anti-C3 (Ab11862), anti-C4 (Ab11863), anti-CFH (Ab53800), anti-MBL (Ab106046) and anti-thrombospondin (A6.1) were from Abcam Inc. (Cambridge, MA, USA). Cryosections were dried at room temperature for 1 h. The tissue samples were fixed with cold acetone for 10 min and were rehydrated with PBS for 10 min. Samples were blocked with 1% BSA in PBS for 1 h at room temperature. Primary and secondary antibodies were diluted in the sample buffer. Incubation with the primary antibody was performed at 4°C overnight in a humid chamber. Samples were washed three times for 10 min each with PBS. Incubation with secondary antibodies was done for 1 h at room temperature. Samples were again washed three times for 10 min each with PBS. Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was added and a coverslip was placed onto the slide.

Confocal microscopy
Confocal microscopy was performed using a Leica SP5 microscope and LASAF software (Leica Microsystems Inc., Buffalo Grove, IL, USA). All sections were analyzed at 7% laser power except when using antibodies to CFH when the laser power was 40%. The objective used was ×63 oil.

Sample preparation
For consistent sample preparation, all samples were dissected and prepared by the same person. BrCh, BrM and basal deposits (BrM with deposits) were prepared as follows. Eye cups were made and the neural retina was peeled out. The eye cups were filled with PBS (without Mg<sup>2+</sup> and Ca<sup>2+</sup>) and frozen at −80°C until used. Room temperature PBS was added to thaw the sample. Cuts were made to flatten the eye cup. The eye cup was vortexed briefly in PBS. Remaining RPE cells were removed by washing the BrM using a stream of PBS. The BrCh was manually peeled from the sclera. Basal deposits remained associated with the BrM (Fig. 1). BrM samples were prepared by dissecting out the BrM and manually removing the remaining choroid from the BrM. An electron micrograph of isolated BrM is in Supplementary Material, Figure S3. The proteins in the BrCh and BrM preparations were solubilized in 1% SDS/8 M urea/50 mM DTT/25 mM EDTA by short bursts of sonication at 4°C. Insoluble material was removed by centrifugation twice for 40 min at 14 000 g.
The solubilized proteins obtained in the cleared supernatants from the mutant and control tissue samples were electrophoresed 2 cm into 1 mm SDS–PAGE gels, 4–12% acrylamide. The amounts of protein loaded onto the gels for each sample were comparable as determined by scanning the lanes of colloidal Coomassie stained gels (Invitrogen, Carlsbad, CA, USA) (128). Each gel lane was cut into 1 mm slices and the proteins in each slice were digested in-gel with trypsin. Each slice was treated as an individual sample, destained, dried in a Speedvac, reduced using 100 μl of 40 mM iodoacetamide in 25 mM ammonium bicarbonate (pH 8.0) and alkylated using 100 μl of 40 mM iodoacetamide in 25 mM ammonium bicarbonate (pH 8.0). After thorough washing, gel pieces were dried, rehydrated with 20 μl of 0.02 mg/ml modified trypsin (Promega, Madison, WI, USA) (pH 8.0) and incubated using 100 μl of Tris (2-carboxyethyl) phosphine hydrochloride in 25 mM ammonium bicarbonate (pH 8.0) and alkylated using 100 μl of 40 mM iodoacetamide in 25 mM ammonium bicarbonate (pH 8.0). After thorough washing, gel pieces were dried, rehydrated with 20 μl of 0.02 mg/ml modified trypsin (Promega, Madison, WI, USA) in 40 mM ammonium bicarbonate, and incubated overnight with shaking at 37°C. The gel slices were extracted with 20 μl of 40 mM ammonium bicarbonate, supernatants were combined and digestion was stopped by adding 4 μl concentrated acetic acid.

**Mass spectrometry**

Tryptic digests were analyzed by injecting 8 μl onto a 15-cm nanocapillary reverse-phase column (New Objective PicoFrit 75 μm column) terminating in a nanospray 15 μm tip self-packed with Microm Magic C18 AQ 200A, 5 μm resin, which was directly coupled to a ThermoElectron Orbitrap XL or ThermoElectron FTICR mass spectrometer. Peptides were eluted at a flow rate of 300 nl/min using 0.1% formic acid in MilliQ water as solvent A and 0.1% formic acid in acetonitrile as solvent B and a gradient consisting of: 3–50% B over 65.5 min, 50–80% B over 5 min, and a 5 min hold at 80% B. Mass spectral (MS) and MS/MS data were acquired in data-dependent mode with full MS scans from m/z = 300 to 2000 at 60 000 resolution using a top six method with a minimum MS signal threshold of 1000.

A customized database was created using the UniRef mouse database (downloaded multiple times until December 2011) with common contaminants such as trypsin, keratins and other environmental proteins added. A reverse database was produced by inverting each protein sequence and these sequences were appended in front of the forward sequences. The database was indexed using partial trypsin specificity, variable methionine sulfoxide oxidation and static cysteine modification as carboxyamidomethyl cysteine. MS/MS data were searched against these databases using the SEQUEST search engine within ThermoFisher Bioworks (Version 3.3.1) with a precursor mass tolerance of 100 ppm and fragment ion tolerance of 1 amu. Results were further processed using DtaSelect (versions 1.3 and 2.0). With DtaSelect, data were filtered using 10 ppm, full tryptic specificity, ΔCn ≥ 0.05, and grouped peptides into consensus proteins and compared spectral counts between sample and control.

The output from Sequest was further analyzed using Scaffold software (Proteome Software, Inc., Portland, OR, USA). Proteins were identified by two or more peptides. Probabilities of peptide and protein identifications were >95 and 99%, respectively. Estimates of protein levels (abundance) were made from unweighted spectral counts and normalized using the number of theoretical tryptic peptides between m/z range of 750 and 3000 (76). The nuclear proteins were not included in the PCA.

**Data analysis**

PCA was applied to normalized spectral counts using Solo (Eigenvector Research Inc., Wenatchee, WA, USA) (80). The analysis yielded both the score plot and the loadings. Loadings are the measure of the contribution of each variable (each protein) to each PC (81). The percent variance in the loadings was calculated by squaring the loadings, summing the results for the desired number of proteins (variables) and multiplying by 100. HCA was performed using Solo (Eigenvector Research Inc.). The analysis was done on normalized spectral counts using a standard clustering algorithm (K nearest neighbor) (84).

Standard deviation of differences for proteomic data was done as follows. The mean and SD were determined using the 8 months data as control data for the other data sets. The mean of the differences for each protein was determined for these data and the SD of the distance from the mean for each protein was determined. Those proteins with differences that were >3 SD from the mean were considered to be proteins that were affected by the mutation or were outliers. For 8 month mice only 28 proteins were outside 3 SD from the mean (>99% CI). These proteins were removed and the calculations were repeated. The mean was 0.068. The SD of 0.298 was used to determine those proteins in the 14- and 24-month data sets that were >3 SD.

ECM and ECM-associated proteins were identified from the matrisome database (http://web.mit.edu/hyneslab/matrisome/). The biological processes associated with proteins were identified using the Panther database (www.pantherdb.org) and the Mouse Genome Informatics (MGI) Web, The Jackson Laboratory, Bar Harbor, ME, USA, World Wide Web (www.informatics.jax.org).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** J.D.L. is the inventor of patents and/or patent applications that describe the use of complement inhibitors for therapeutic purposes and is the founder of Amyndas Pharmaceuticals which is developing complement inhibitors for clinical applications.

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