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Bestrophinopathy: An RPE-Photoreceptor Interface Disease

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Bestrophinopathy: An RPE-Photoreceptor Interface Disease

Abstract

Bestrophinopathies, one of the most common forms of inherited macular degenerations, are caused by mutations in the BEST1 gene expressed in the retinal pigment epithelium (RPE). Both human and canine BEST1-linked maculopathies are characterized by abnormal accumulation of autofluorescent material within RPE cells and bilateral macular or multifocal lesions; however, the specific mechanism leading to the formation of these lesions remains unclear. We now provide an overview of the current state of knowledge on the molecular pathology of bestrophinopathies, and explore factors promoting formation of RPE-neuroretinal separations, using the first spontaneous animal model of BEST1-associated retinopathies, canine Best (cBest). Here, we characterize the nature of the autofluorescent RPE cell inclusions and report matching spectral signatures of RPE-associated fluorophores between human and canine retinæ, indicating an analogous composition of endogenous RPE deposits in Best Vitelliform Macular Dystrophy (BVMD) patients and its canine disease model. This study also exposes a range of biochemical and structural abnormalities at the RPE-photoreceptor interface related to the impaired cone-associated microvillar ensheathment and compromised insoluble interphotoreceptor matrix (IPM), the major pathological culprits responsible for weakening of the RPE-neuroretina interactions, and consequently, formation of vitelliform lesions. These salient alterations detected at the RPE apical domain in cBest as well as in BVMD- and ARB-hiPSC-RPE model systems provide novel insights into the pathological mechanism of BEST1-linked disorders that will allow for development of critical outcome measures guiding therapeutic strategies for bestrophinopathies. © 2017 Elsevier Ltd

Keywords

Bestrophinopathies; Canine model; hiPSC-RPE; Lipofuscin; Macula; RPE apical microvilli; RPE-Photoreceptor interface

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ABSTRACT

Bestrophinopathies, one of the most common forms of inherited macular degenerations, are caused by mutations in the *BEST1* gene expressed in the retinal pigment epithelium (RPE). Both human and canine *BEST1*-linked maculopathies are characterized by abnormal accumulation of autofluorescent material within RPE cells and bilateral macular or multifocal lesions; however, the specific mechanism leading to the formation of these lesions remains unclear. We now provide an overview of the current state of knowledge on the molecular pathology of bestrophinopathies, and explore factors promoting formation of RPE-neuroretinal separations, using the first spontaneous animal model of *BEST1*-associated retinopathies, canine Best (cBest). Here, we characterize the nature of the autofluorescent RPE cell inclusions and report matching spectral signatures of RPE-associated fluorophores between human and canine retinae, indicating an analogous composition of endogenous RPE deposits in Best Vitelliform Macular Dystrophy (BVMD) patients and its canine disease model. This study also exposes a range of biochemical and structural abnormalities at the RPE-photoreceptor interface related to the impaired cone-associated microvillar ensheathment and compromised insoluble interphotoreceptor matrix (IPM), the major pathological culprits responsible for weakening of the RPE-neuroretina interactions, and consequently, formation of vitelliform lesions. These salient alterations detected at the RPE apical domain in cBest as well as in BVMD- and ARB-hiPSC-RPE model systems provide novel insights into the pathological mechanism of *BEST1*-linked disorders that will allow for development of critical outcome measures guiding therapeutic strategies for bestrophinopathies.

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1. Introduction

The retinal pigment epithelium (RPE) is an organized monolayer of highly specialized cells responsible for a sustained interaction with photoreceptors, a mission pivotal for normal visual function and retinal preservation (Hageman and Johnson, 1991; Bok, 1993; Strauss, 2005). These diverse functions are orchestrated by a broad array of molecules designed to access specific cascades of intra- and intercellular interactions in the retina (reviewed in: Steinberg, 1985; Strauss, 2005; Kevany and Palczewski, 2010; Bonilha, 2014). One of such RPE-specific molecules is BESTROPHIN 1 (OMIM #607854), a transmembrane channel protein encoded by *BEST1* gene and associated with the RPE basolateral membrane (Marmorstein et al., 2000; Sun et al., 2002; Gomez et al., 2013). Although BESTROPHIN 1 has been extensively studied and described as a multifunctional protein implicated in mediating anion transport, regulation of calcium signaling and cell volume (Rosenthal et al., 2005; Hartzell et al., 2008; Strauss et al., 2014; Kane Dickson et al., 2014; Yang et al., 2014; Milenkovic et al., 2015), its multifaceted nature and complex interactions with photoreceptors in health and disease still remain elusive.

Mutations in *BEST1* have been causally associated with several clinically heterogeneous retinal disorders, collectively termed bestrophinopathies (Petrukhin et al., 1998; Seddon et al., 2001; Yardley et al., 2004; Schatz et al., 2006; Burgess et al., 2008; Davidson et al., 2009; Boon et al., 2009a). The *BEST1* mutational spectrum underlying these retinopathies varies greatly, and involves over 200 distinct mutations (Boon et al., 2009a; Pasquay et al., 2015; Yang et al., 2015). Additionally, a considerable variability in age at onset, rate of disease progression and phenotypic expression has been reported, not only between unrelated patients harboring the same mutation, but also among affected individuals within a single family (Kramer et al., 2000; Kinnick et al., 2011; Bitner et al., 2012; MacDonald et al., 2012; Boon et al., 2013). This phenomenon of phenotypic and allelic heterogeneity highlights a significant phenotypic overlap among *BEST1*-linked disorders, posing a challenge in both determining the diagnostic specificity as well as predicting the outcomes of visual impairment. In recent years, the complexity of genotype-phenotype correlation in bestrophinopathies has proven difficult to explain with traditional models of disease pathogenesis (Allikmets et al., 1999; Boon et al., 2007; Yu et al., 2007; Querques et al., 2009; Booij et al., 2010; Liu et al., 2016), indicating a presence of potential genetic modifiers or still undetermined Best1 protein interactors, involvement of environmental components or a combination of both. This likely would point to a complex interplay of genetic susceptibility factors and modifiable environmental stimuli utilizing novel signaling pathways in the retina.

The broad spectrum of clinical presentations in bestrophinopathies ranges from the widespread symptoms affecting peripheral

retina and vitreous in a rare condition of vitreoretinchoroidopathy (ADVIRC; OMIM#193220) to the well-defined clinical abnormalities restricted to the macula and central retina in Best Vitelliform Macular Dystrophy (BVMD) and autosomal recessive bestrophinopathy (ARB). BVMD (a.k.a. VMD2, OMIM#153700), inherited as an autosomal dominant trait with incomplete penetrance, and the recessive form (ARB; OMIM#611809) are the most common and best explored juvenile macular dystrophies among bestrophinopathies, characterized by a markedly abnormal electrooculogram (EOG) accompanied by an excessive accumulation of lipofuscin material within RPE cells, formation of focal and multifocal subretinal lesions, and consequently, loss of central vision (Pianta et al., 2003; Boon et al., 2009b; Pasquay et al., 2015; Fung et al., 2015).

An abnormal accumulation of lipofuscin is a major risk factor implicated in different forms of macular degeneration (Delori et al., 1995a; Marmorstein et al., 2002; Gerth et al., 2007; Biarnes et al., 2015), and also the most notable and consistent pathological finding in *BEST1*-linked maculopathies, serving as an indirect biomarker of metabolic activity between the photoreceptor outer segment (POS) turnover and RPE phagocytosis (Bakall et al., 2007; Piñeiro-Gallego et al., 2011; Lei et al., 2013; Singh et al., 2013a). Recent advances with noninvasive retinal imaging modalities have enabled detailed mapping and quantification of fundus autofluorescence (FAF) *in vivo*, and its correlation with increased levels of lipofuscin components in the aged and diseased retinae (Delori et al., 1995a, 1995b; Brunk and Terman, 2002; Boon et al., 2008; Duncker et al., 2014); however, the polymorphous nature of lipofuscin material and consequences of its buildup in the retina are still controversial.

To begin to address these questions, we used the spontaneous canine *BEST1* disease model (cBest a.k.a. canine multifocal retinopathy, *cmr*) (Guziewicz et al., 2007, 2011; Zangerl et al., 2010; Beltran et al., 2014; Singh et al., 2015) to characterize lipofuscin fluorophores in the cBest1 mutant RPE, and explore factors leading to the formation of subretinal lesions in *BEST1*-associated maculopathies. This study highlights matching spectral profiles of the native lipofuscin autofluorescence between human and dog bestrophinopathies, as well as robust biochemical and structural alterations at the RPE-photoreceptor interface that trigger formation of vitelliform lesions.

2. Canine models of human *BEST1*-related dystrophies

Over the recent decades, hundreds of spontaneous genetic conditions have been described in dogs, and most of them with clinically and genetically close counterparts to the human disorders (OMIA: <http://omia.angis.org.au>). The naturally occurring canine models of inherited retinal disorders in man have proved crucial in the investigation of disease mechanisms and development of new therapeutic strategies (Acland et al., 2001; Komáromy et al., 2010;

Beltran et al., 2012; Downs et al., 2016).

We have recently characterized a naturally occurring autosomal recessive disorder in dogs, canine Best (cBest a.k.a. canine multifocal retinopathy, *cmr*), which is caused by the same genetic defects as human bestrophinopathies, and captures the full range of clinical manifestations observed in patients (Guzewicz et al., 2007, 2011; Zangerl et al., 2010; Beltran et al., 2014; Singh et al., 2015). To date, cBest retinopathy affects thirteen dog breeds worldwide and results from any of the three distinct mutations identified in the canine *BEST1* (*cBEST1*) gene. The premature stop mutation located in the first coding exon of *cBEST1* (c.73C > T/p.Arg25* *alias* R25X) was recognized among Mastiff-related breeds (Guzewicz et al., 2007, 2011); a missense change (c.482G > A/p.Gly161Asp *alias* G161D) affecting cBest1 protein folding and trafficking was discovered in a rare breed of Coton de Tulear (Guzewicz et al., 2007, 2012); whereas the frameshift mutation (c.1388delC/p.Pro463fs *alias* P463fs), truncating BESTROPHIN 1 C-terminus, was shown to segregate in Lapponian Herders (Zangerl et al., 2010). All three mutations lead to the consistent clinical phenotype in homozygous affected dogs, and model all major aspects of the disease-associated mutations as well as their molecular consequences described in man (Guzewicz et al., 2007, 2011, 2012; Beltran et al., 2014; Singh et al., 2015).

The spectrum of clinical and molecular features recapitulated, including the salient predilection of lesions to the canine macular region, makes cBest an extremely attractive model system not only for addressing principles behind the molecular pathology of bestrophinopathies, but also for validating new therapeutic strategies (Guzewicz et al., 2013; Beltran et al., 2014; Singh et al., 2015). Currently, the loss of function model (cBest1-R25X), the C-terminal deletion (cBest1-P463fs), and the compound heterozygous model (cBest1-R25X/P463fs) harboring both mutations in a heterozygous state, are available for research study. We used these translational models as a platform for in-depth investigation of the pathogenic sequence in *BEST1*-related disorders, and addressed the central question in the disease pathogenesis, the intrinsic susceptibility of the macula to its primary detachment in bestrophinopathies.

3. Lipofuscin as a pathological agent in *BEST1*-associated maculopathies

3.1. Excessive buildup of lipofuscin within RPE: a hallmark of canine and human bestrophinopathies

Retinal pigment epithelium has been recognized as a primary target site for many retinal degenerative disorders, including bestrophinopathies. Although the disease-causative gene product, BESTROPHIN 1, has been associated with basolateral membrane of RPE cells (Marmorstein et al., 2000; Strauss et al., 2014), the predominant clinical manifestations in BVMD and ARB patients pertain to the abnormal apposition of RPE apical surface to the neurosensory retina, delineated by intense FAF signals *in vivo* and aggregation of autofluorescent material when examined *ex vivo* (O'Gorman et al., 1988; Spaide et al., 2006; Bakall et al., 2007; Sparrow et al., 2016). As the canine Best's disease model mirrors both the altered topographic cSLO-FAF distribution as well as the end-stage pathological findings described in patients (Guzewicz et al., 2007; Beltran et al., 2014; Singh et al., 2015), this model was used to examine the spectral nature of the RPE-associated autofluorescent constituents using Hyperspectral Autofluorescence Imaging (HAI) technology (Gao et al., 2012; Smith et al., 2014).

Baseline evaluation of the wild-type canine retinae (age range: 10 mos-6 yrs) demonstrated a gradual increase in native autofluorescence within RPE cells (Fig. 1A). This slow yet steady augmentation of granular yellow to brown-tan deposits reflects the

natural aging process of the canine retina and, consistent with the studies in human, increases over the dog's lifespan in a linear manner (Fig. 1A). Furthermore, in agreement with clinical assessments in both BVMD and ARB patients (Boon et al., 2013; Sparrow et al., 2016), the cBest model exhibits an abnormal distribution of FAF intensities associated with focal and multifocal retinal detachments observed by *in vivo* imaging (Fig. 1B,C). Canine retinae harboring a nonsense mutation (c.73C > T/p.R25X) in *cBEST1* gene do not express BESTROPHIN 1 protein, and show both, increased FAF signals associated with areas of separations of the neural retina from the underlying RPE, and an excessive accumulation of lipofuscin-like material within RPE when compared to the age-matched controls (Fig. 1B–E). Representative H&E and DIC images of cBest1-R25X-affected and control retinae are shown in Fig. 1D,E. Canine cBest1-R25X/P463fs compound heterozygous and cBest1-P463fs homozygous models (Zangerl et al., 2010; Beltran et al., 2014) display similar pathological characteristics.

To investigate the nature of these RPE inclusions and address the question of whether the buildup of the lipofuscin-like deposits promotes development of focal to multifocal retinal detachments, cBest samples with early pre-vitelliform and vitelliform lesions from supero-temporal quadrant were selected for HAI and IHC analyses (*for details, see Supplementary Materials and Methods*). The HAI-determined spectral signatures derived from canine Best1-R25X mutant samples were then compared to the HAI emission profiles of human BVMD (hBest1-T6R and -Y227N) RPE (Mullins et al., 2005, 2007), as well as to the healthy controls from both species (Fig. 2). Comparative HAI analysis of endogenous RPE fluorophores confirmed the excessive accumulation of autofluorescent material in *BEST1* mutant as a common feature of both human and canine bestrophinopathies (Fig. 2A–E), and further revealed not only matching spectral profiles between Best1-affected and control samples of dog (Fig. 2B,D) and human (Fig. 2E), but also analogous emission profiles between human and canine bestrophinopathies (Fig. 2F,G). Overall, the endogenous RPE deposits emitted intense fluorescent signals across the recorded spectrum, with emission intensity, I_{em} , significantly higher in cBest1-R25X *versus* age-matched wild-type (WT) control ($I_{em} \geq 3$ fold; $***p < 0.000002$) across the interval as depicted in a box plot (Fig. 2C). The spectral signature was highly comparable among all samples examined, with a major emission peak at 536–569 nm consistent with lipofuscin (Ben Ami et al., 2016), followed by a shoulder peak at 650 nm (Fig. 2B–G).

Because I_{em} amplitude was considerably higher in samples from older human donor eyes *versus* young canine retinae, to directly compare the individual HAI emission data points across all wavelength intervals tested, irrespective of the emission intensity, we used a linear regression to identify a monotonous transformation to boost the amplitude of the baseline I_{em} signal without affecting the overall shape of the profile (*for details, see Supplementary Materials and Methods*). As indicated by the initial HAI analysis, the emission peak profiles of the cBest1-R25X mutant and canine WT-adjusted were nearly identical, and fell within the SEM range of each other over most wavelength intervals (Fig. 2D). An analogous linear regression equation was identified for cBest1-R25X mutant (cBest1-R25X adjusted) for its direct comparison to the HAI emission pattern derived from BVMD (hBest1-T6R and -Y227N averaged), revealing a highly overlapping emission spectral profile of the RPE-associated lipofuscin constituents between human and canine *BEST1*-maculopathies (Fig. 2G).

In conclusion, consistent with the findings in a healthy human retina (Kennedy et al., 1995; Delori et al., 2001), we now show that lipofuscin granules accumulate as a function of age in a normal canine RPE. Interestingly, no apparent disease-specific HAI emission peaks in either canine or human Best1-mutant RPE were

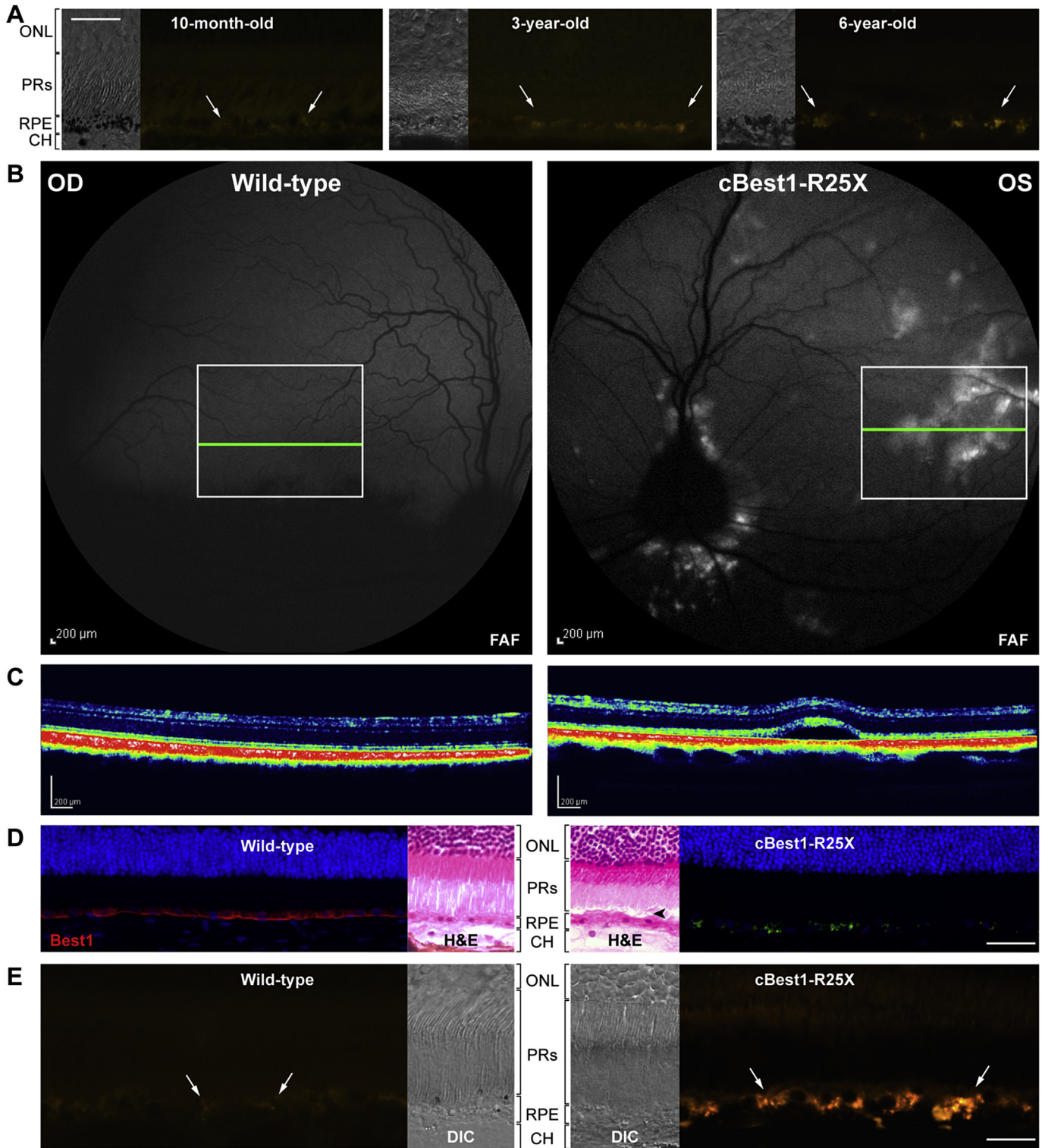


Fig. 1. Abnormal buildup of autofluorescent material within RPE cells is a hallmark of canine bestrophinopathies. (A) Accumulation of autofluorescent debris (arrows) is an age-dependent process in normal canine RPE cells. (B) Confocal scanning laser ophthalmoscopy fundus autofluorescence (cSLO FAF) images of a 20-month-old affected (OS) and an age-matched wild-type control eye (OD). Note the increased fundus autofluorescence signals scattered throughout the supero-temporal quadrant and adjacent to the optic disc in the mutant retina. Boxed areas identify regions analyzed *ex vivo* by Hyperspectral Autofluorescence Imaging (HAI) and immunohistochemistry (IHC). Note the missing autofluorescence over the central lesion (OS). (C) Corresponding SD-OCT images taken at the position of the green line in the above images (B) of the temporal area. A characteristic bullous separation of the neuroretina from the RPE is demonstrated in the mutant retina; the structurally intact age-matched wild-type control retina is shown for comparison. (D) Immunostaining against Best1 protein (red) in cryosections from canine wild-type and cBest1-R25X-affected retinas. Note the presence of native autofluorescent granules (green) and absence of Best1 labeling within affected RPE cells, which appear hypertrophic on the corresponding H&E image; there is a discreet separation of the outer segments from the apical RPE in the affected retina (arrowhead). Nuclei were stained with DAPI. (E) Confocal microscopy images of a 20-month-old mutant and an age-matched wild-type retinas showing an excessive accumulation of autofluorescent deposits (arrows) within the affected RPE. ONL: outer nuclear layer; PRs: photoreceptors; RPE: retinal pigment epithelium; CH: choroid; Scale bars: 10 μ m applies to all photomicrographs in (A) and (E); 40 μ m applies to all (D) panels.

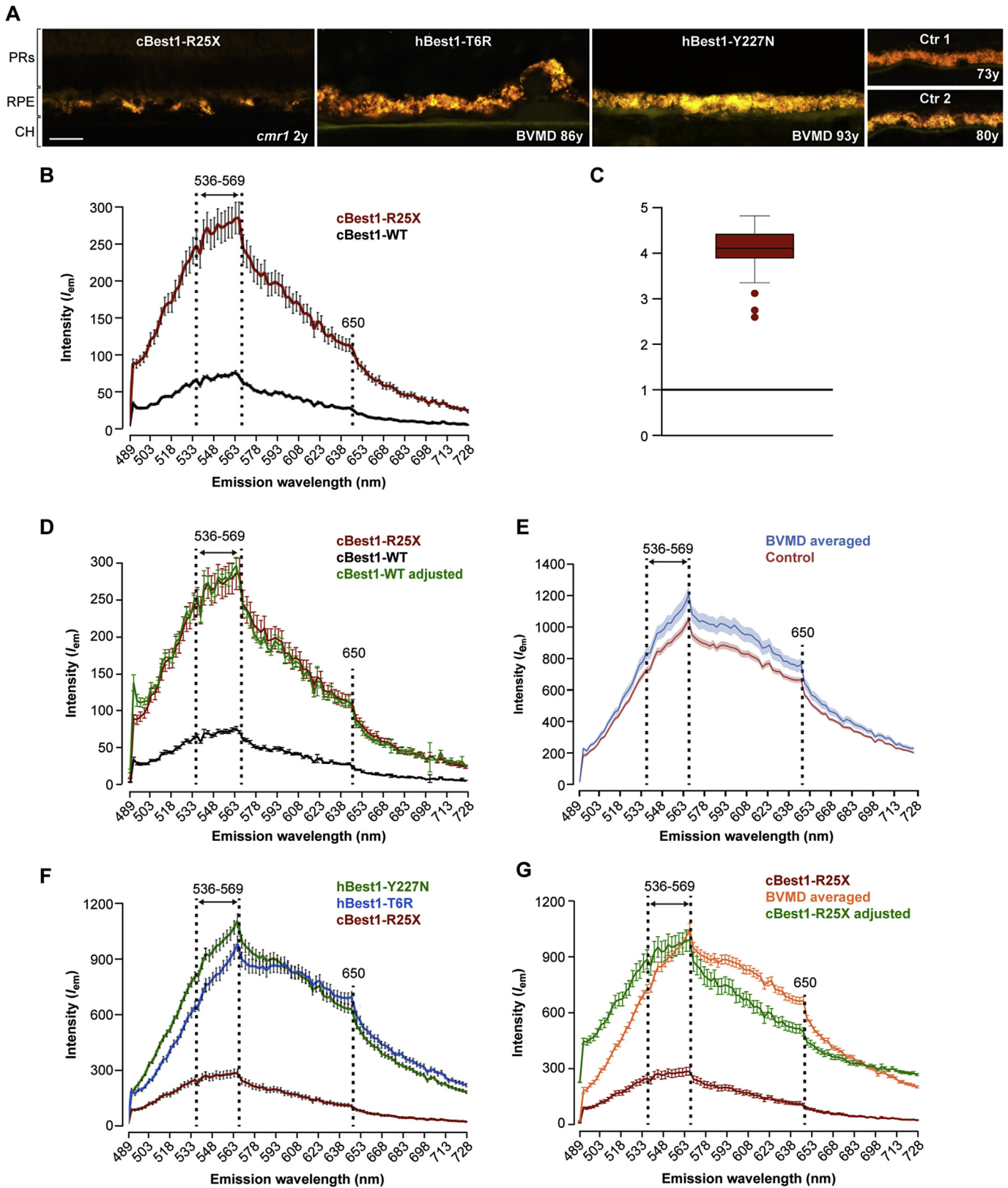


Fig. 2. Hyperspectral autofluorescence imaging (HAI) reveals analogous emission profile between human and canine bestrophinopathies. (A) Representative confocal microscopy images of the native RPE autofluorescence in cryosections from BVMD patients (hBest1-T6R and -Y227N), control subjects (Ctr) of comparable age, and canine bestrophinopathy model (cBest1-R25X) by HAI analysis. Abundant accumulation of autofluorescent material within RPE cells is a common feature in both human and canine bestrophinopathies. PRs: photoreceptors; RPE: retinal pigment epithelium; CH: choroid. (B) HAI analysis of endogenous RPE fluorophores in canine cBest1-R25X-affected and age-matched WT RPE revealed a major emission peak between 536 and 569 nm consistent with lipofuscin signals, followed by a shoulder peak at 650 nm. (C) Statistical analysis confirmed accelerated accumulation of lipofuscin granules in the affected tissue with emission intensity (I_{em}) 3-fold higher in the mutant vs age-matched control ($***p < 0.000002$). (D) Direct comparison of the individual HAI emission data points from cBest1-R25X and cBest1-WT RPE using linear regression across all wavelength intervals. After enhancing the amplitude of the baseline

detected when compared to age- and retinal location-matched normal controls (Fig. 2). Although this disease aspect should be investigated further using other advanced RPE diagnostic tools, our HAI data strongly suggest that no intrinsic disease-specific RPE fluorophores underlie pathophysiology of bestrophinopathies, and the anomalous increase of lipofuscin bisretinoids observed in *BEST1*-retinopathies reflects rather an inability of RPE cells to properly metabolize POS (Singh et al., 2013a), and, in essence, manifests as a premature senescence of the affected RPE cell monolayer.

Furthermore, HAI and linear regression analyses exposed matching spectral signatures between cBest1-R25X and hBest1-T6R and -Y227N affected RPE, suggesting a similar composition of endogenous RPE-associated fluorophores between humans and dogs. These data support the recent *ex vivo* findings from a set of HAI-profiled healthy human eyes, assigning the primary spectral emission peak to the abundant lipofuscin-melanolipofuscin signal, and suggesting a presence of a third candidate family of RPE-fluorophores that requires further biochemical characterization (Ben Ami et al., 2016). Considering the corresponding hyperspectral characteristics, the dog should serve as an excellent model system assisting in further studies on the biochemical composition of the undefined families of RPE fluorophores.

3.2. Bisretinoids-mediated cholesterol accumulation in BVMD and cBest model

Studies in polarized ARPE-19 cells demonstrated that A2E, the most prominent lipofuscin fluorophore, contributes to the accumulation of free and esterified cholesterol, perturbing its metabolism (Lakkaraju et al., 2007). The mechanism of this bisretinoid-induced cholesterol accumulation within RPE cells was further explored, signifying the role of trapped cholesterol macromolecules in the disruption of RPE autophagy (Toops et al., 2015; Tan et al., 2016). It is unclear however, whether the A2E-mediated cholesterol accumulation reported *in vitro* contributes to the pathogenesis and progression of other RPE-linked disorders such as BVMD. To that end, we examined if the chronic accumulation of lipofuscin bisretinoids in the *Best1*-mutant RPE is indeed associated with increased levels of cholesterol and/or abnormal cholesterol trafficking in human and canine bestrophinopathies (Fig. 3). We found that lipofuscin granules and free cholesterol (filipin-positive puncta) were strongly positively associated in the RPE cells of both human BVMD (Pearson's correlation coefficient $r = 0.85 \pm 0.03$) and canine *Best1*-mutant RPE ($r = 0.77 \pm 0.02$) (Fig. 3A,B). This positive correlation was in agreement with the emission profile analyses where the somewhat less intense blue fluorescent signals of filipin-positive aggregates were co-distributed with the bright green lipofuscin granules (Fig. 3B).

This strong positive correlation between lipofuscin-derived autofluorescence and cholesterol levels detected *ex vivo* in canine and human bestrophinopathies supports the original *in vitro* observations by Lakkaraju et al. (2007), and strongly suggests that the perturbed cholesterol metabolism and its extensive downstream consequences (Toops et al., 2015; Tan et al., 2016) contribute to the homeostatic imbalance in cBest- and BVMD-RPE. It has been shown

that the tight control of intracellular cholesterol levels is essential for proper autophagosome-lysosome interactions, and that excess of cholesterol within RPE can interfere with intracellular trafficking, inhibiting lysosomal degradation and consequently, the autophagic clearance (Lakkaraju et al., 2007; Toops et al., 2015). Furthermore, as demonstrated in the recent study by Tan et al. (2016), overload of bisretinoids/cholesterol compromises the protective responses against complement-mediated damage, leading to mitochondrial fragmentation and oxidative stress in the RPE cells (Tan et al., 2016).

Detailed experiments focused on the gene expression controlling cholesterol metabolism and dysregulation of lipid homeostasis in *Best1*-mutant RPE are beyond the scope of this review. Such analyses, however, are in progress and constitute the main objective of the next phase of research. As there is no doubt that the overload of both bisretinoids and cholesterol leads to the severe perturbations of RPE cell homeostasis, a chronic accumulation of lipofuscin has been implicated in the pathology of numerous inherited retinal disorders, including AMD and Stargardt disease (Spaide, 2003; Cideciyan et al., 2004; Lakkaraju et al., 2007; Petrukhin, 2007; Tan et al., 2016), and as such constitutes a non-specific disease biomarker that is unlikely to trigger *per se* the retinal separation in bestrophinopathies.

4. Dysregulation of lipid metabolism and its impact on subretinal microenvironment in cBest

Besides the increase in unesterified cholesterol clearly associated with an excess of bisretinoid compounds within *Best1*-mutant RPE, comparable distribution and intensity of filipin staining was observed across the rest of the tissue in the wild-type and cBest retinae (Fig. 4A-C). Consistent with the studies on filipin staining patterns in human retina, free cholesterol, by far the most predominant form of sterols in the vertebrate retina (Fliesler and Bretilton, 2010), was broadly distributed throughout all layers of the canine retina with no difference detected in the cBest1-R25X-affected vs age-matched WT controls (Fig. 4A,B). However, we did observe a noticeable difference in the esterified cholesterol (EC) levels between cBest model and WT controls after subjecting the samples to ethanol-based extraction followed by cholesterol esterase pretreatment (for details, see Supplementary Materials and Methods) to visualize the EC content (Fig. 4D,E). While in a healthy human retina EC is mainly associated with Bruch's membrane (Rudolf and Curcio, 2009; Zheng et al., 2012), in a normal canine retina this form of sterol was faintly detected within choroid (Fig. 4D), whereas in cBest retina a substantial increase of filipin-stained EC fluorescence was associated with photoreceptor outer segment (Fig. 4E, inset). Because filipin-sterol fluorescence undergoes rapid photobleaching, it is not quantifiable; therefore, the EC findings were verified by alternative and more stable neutral lipid stains, a fluorescent probe BODIPY 493/503 and the classic lipid-soluble dye Oil Red O (ORO), both known to stain EC (Rudolf and Curcio, 2009).

A series of experiments with BODIPY 493/503 as well as ORO confirmed the EC staining pattern in the *Best1*-mutant retina (Fig. 4G-L). The bright green fluorescent signals emitted by BODIPY 493/503 were present across all layers in both the normal control

I_{em} signal (cBest1-WT adjusted), the overlapping emission peak profiles of cBest1 mutant and cBest1-WT fell within the SEM range of each other over most wavelength intervals. (E) HAI of the native RPE autofluorescence from BVMD patients (hBest1-T6R and -Y227N averaged) and control donor eyes demonstrating parallel RPE emission spectra profiles. Data analysis was performed on hyperspectral datasets derived from a mean of four BVMD-affected or control retinae with three cryosections per eye and six ROIs per section ($n_{BVMD} = 72$ and $n_{Ctrl} = 72$); the color-corresponding shaded areas represent mean \pm SEM across the wavelength intervals. (F) HAI-profiled intrinsic RPE fluorescence from patients harboring either hBest1-Y227N or -T6R mutation (plotted individually) and cBest1-R25X model illustrating matching spectral profiles among the disease samples from both species, varying only in the emission intensity, consistently higher in samples from elderly BVMD donors than that of young cBest retinae. (G) HAI emission peak signatures of BVMD (hBest1-T6R and -Y227N averaged) and cBest1-R25X (cBest1-R25X adjusted) indicating similar profiles of RPE-associated autofluorescence constituents in human and canine bestrophinopathies. Scale bar: 10 μ m applies to all (A) panels.

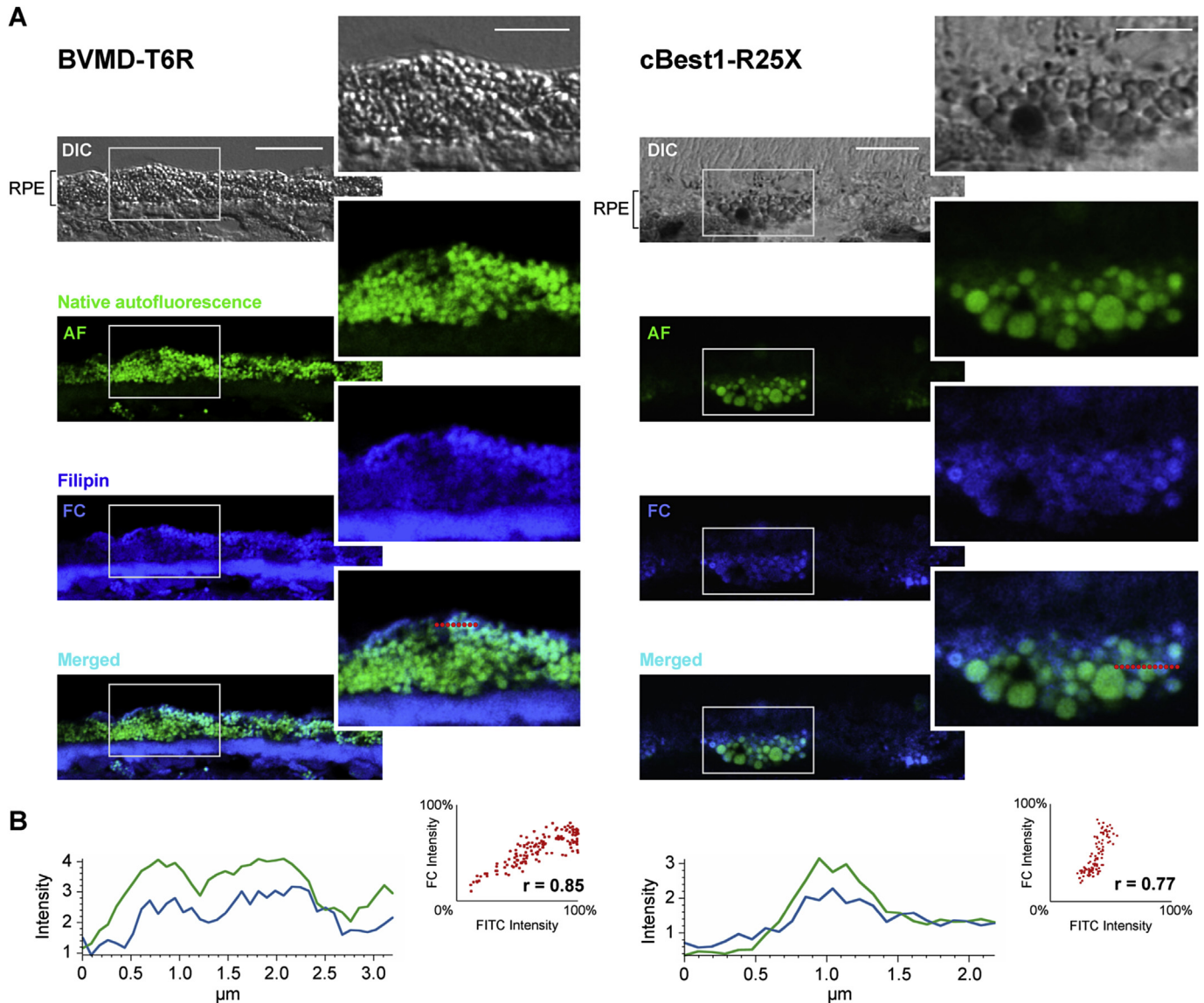


Fig. 3. Bisretinoids-mediated intracellular cholesterol accumulation in BVMD- and cBest-affected RPE. (A) Representative DIC, native autofluorescence (AF), filipin-stained free cholesterol (FC), and AF/FC-merged confocal photomicrographs of RPE cells from human BVMD (hBest1-T6R) and canine model (cBest1-R25X). Lipofuscin granules (green) and free cholesterol (filipin-positive puncta, cobalt blue) were highly associated in BVMD and cBest RPE cells. (B) Both the analysis of AF and FC emission profiles as well as Pearson correlation coefficient 'r' values indicated a strong positive correlation between endogenous lipofuscin fluorophores (FITC) and filipin-stained-FC in BVMD-T6R and canine cBest1-R25X RPE ($r = 0.85 \pm 0.03$ and $r = 0.77 \pm 0.02$, respectively). Scale bars: 10 μm ; magnified images: 5 μm .

and cBest retinae (Fig. 4G,H). In the cBest retina, however, a significant increase ($*p < 0.05$) of EC-BODIPY 493/503 was detected in the photoreceptor outer segment layer with the highest staining intensity in the region bordering the inner segment (IS) and at the OS tips (Fig. 4H,I). Similar EC distribution profile in the mutant retina was observed with ORO assay where, in addition, numerous EC-rich inclusions were found within cBest1-R25X mutant RPE and in the subretinal space as ORO-positive intracellular and extracellular deposits (Fig. 4K arrows, Fig. 4L, higher magnification). Note the subtle RPE detachment from the retinal surface in cBest retina (Fig. 4K,L, arrowheads).

To determine whether the observed dysregulation of cholesterol homeostasis in the mutant retina is associated with enhanced lipid peroxidation, we examined the levels of 4-hydroxy-2-nonenal (HNE), one of the most abundant by-products of lipid peroxidation (Esterbauer et al., 1985). As shown in Fig. 4, increased levels of HNE-adducts were present in cBest vs age-matched control retinae

(Fig. 4M–O). While in the WT canine retina HNE was primarily associated with RPE and IS/OS border, in the mutant retina, the staining pattern of HNE showed in addition a patchy distribution across OS layer that was characteristic for the retinal regions located within close proximity to the areas of established subretinal lesions, and associated with hypertrophic RPE cells featuring their abnormal scalloped apical membrane (Fig. 4O, arrows).

Numerous studies have documented the essentiality of cholesterol and its tight homeostatic regulation in the maintenance of outer segment structure and function (Fliesler and Bretillon, 2010; Albert et al., 2016). Metabolic defect in any aspect of this process can lead to abnormal accumulation of cholesterol and its toxic derivatives resulting in retinal degeneration impacting both the structural integrity and function of POS (Fliesler and Bretillon, 2010). Such alterations are likely due to a combination of factors, including the requirement for cholesterol in rhodopsin activation, formation of cholesterol-rich signaling microdomains as well as the

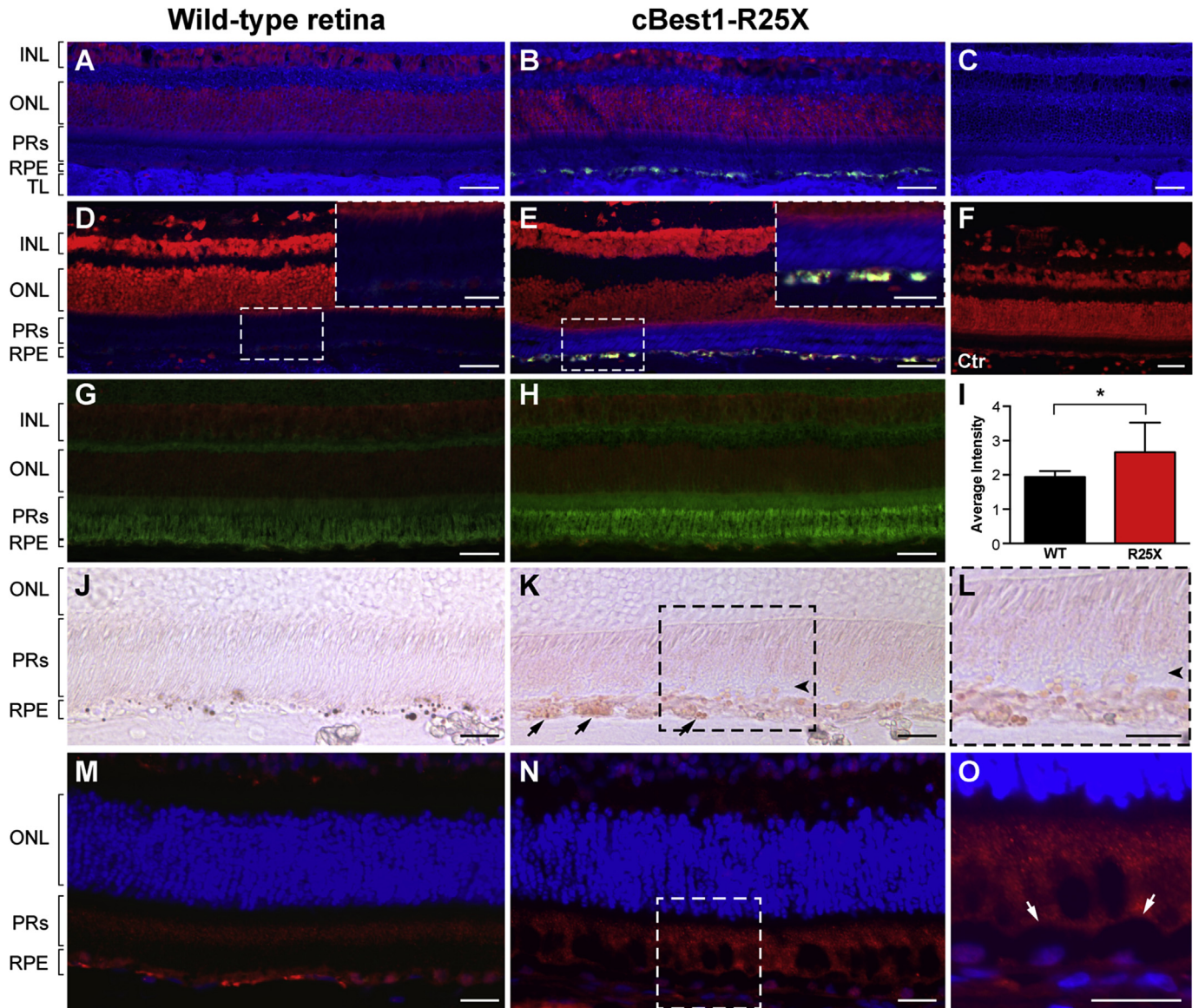


Fig. 4. Dysregulation of lipid metabolism defines canine bestrophinopathy as an RPE-photoreceptor interface disease. Spatial distribution of unesterified (free) cholesterol visualized by sterol-binding probe filipin (cobalt blue) in a normal (A) and cBest1-R25X-affected (B) retinas. Note the excess of autofluorescent RPE deposits in the diseased tissue (B). Filipin-bound free cholesterol was found to be widely distributed across all layers of the canine retina, and appeared to be primarily associated with plasma membrane in ONL and INL, in contrast to the remaining retinal layers where it was found in both cell membranes and somata (C); *tapetum lucidum* (TL), a choroidal modification characteristic to carnivores, showed the most intense staining (A–C). (D, E) Histochemical detection of esterified cholesterol (EC, cobalt blue) in a 12-month-old cBest1-affected vs age-matched control retina. Note the abnormal buildup of autofluorescent material (yellow-green) within RPE and an increase of EC-filipin associated with photoreceptor layer in the mutant (E, close-up). (F) Non-enzyme control: unesterified cholesterol was extracted with ethanol and sections stained with filipin, but omitting the cholesterol esterase treatment (no filipin labeling). (G, H) Representative retinal cryosections from cBest (H) and age-matched control (G) stained with a fluorescent neutral lipids tracer dye BODIPY 493/503 (green). (I) Quantification of EC-BODIPY 493/503 signals in photoreceptor outer segment layer between wild-type and cBest1-R25X-affected retinas. The observed difference was assessed as statistically significant using unpaired *t*-test (**p* < 0.05). (J–L) EC distribution profile in canine wild-type and cBest1-affected retinas assayed with a lysochrome Oil Red O (ORO, rose). ORO-positive inclusions within the affected RPE (arrows) and in the subretinal space are shown in (K) and (L, close-up); arrowheads point to the discreet separation of the outer segments from the RPE apical surface in cBest (K, L). (M–O) Anti-4-HNE labeling (red) in the mutant and control retinas. A scattered distribution of HNE-adducts within outer segments was observed in the affected retina (N and O, close-up) outlining the apical contour of the hypertrophic RPE cells (O, arrows). Nuclei were counterstained with propidium iodide (A, B, D–H) or DAPI (M–O). INL: inner nuclear layer; ONL: outer nuclear layer; PRs: photoreceptors; RPE: retinal pigment epithelium; TL: *tapetum lucidum*; Scale bars: 40 μ m (A–H), and 20 μ m (D–E close-ups, and J–O).

role of cholesterol in regulating pools of phosphoinositides (Albert et al., 2016). One such cholesterol-derived compound, 7-ketocholesterol (7KCh), is an oxidized form of cholesterol produced by non-enzymatic oxidation at C7 (Moreira et al., 2009; Rodriguez and Fliesler, 2009). This oxysterol is considered a potent pro-inflammatory agent playing a role in activation of NLRP3 inflammasome and associated with choroidal neovascularization, formation of AMD drusen and aging (Rodriguez

and Fliesler, 2009; Moreira et al., 2009; Rodriguez et al., 2014). At the molecular level, excess 7KCh not only alters the biophysical properties of plasma membranes (Bach et al., 2008; Ruggiero et al., 2012), but also constitutes a ready substrate for esterification, producing a highly unstable esterified 7-keto molecule that re-releases 7KCh. The higher the degree of fatty acid unsaturation, the more unstable the esterified 7-KCh molecule is (Bretillon et al., 2008; Lee et al., 2015). Thus, the highly unsaturated fatty acids of

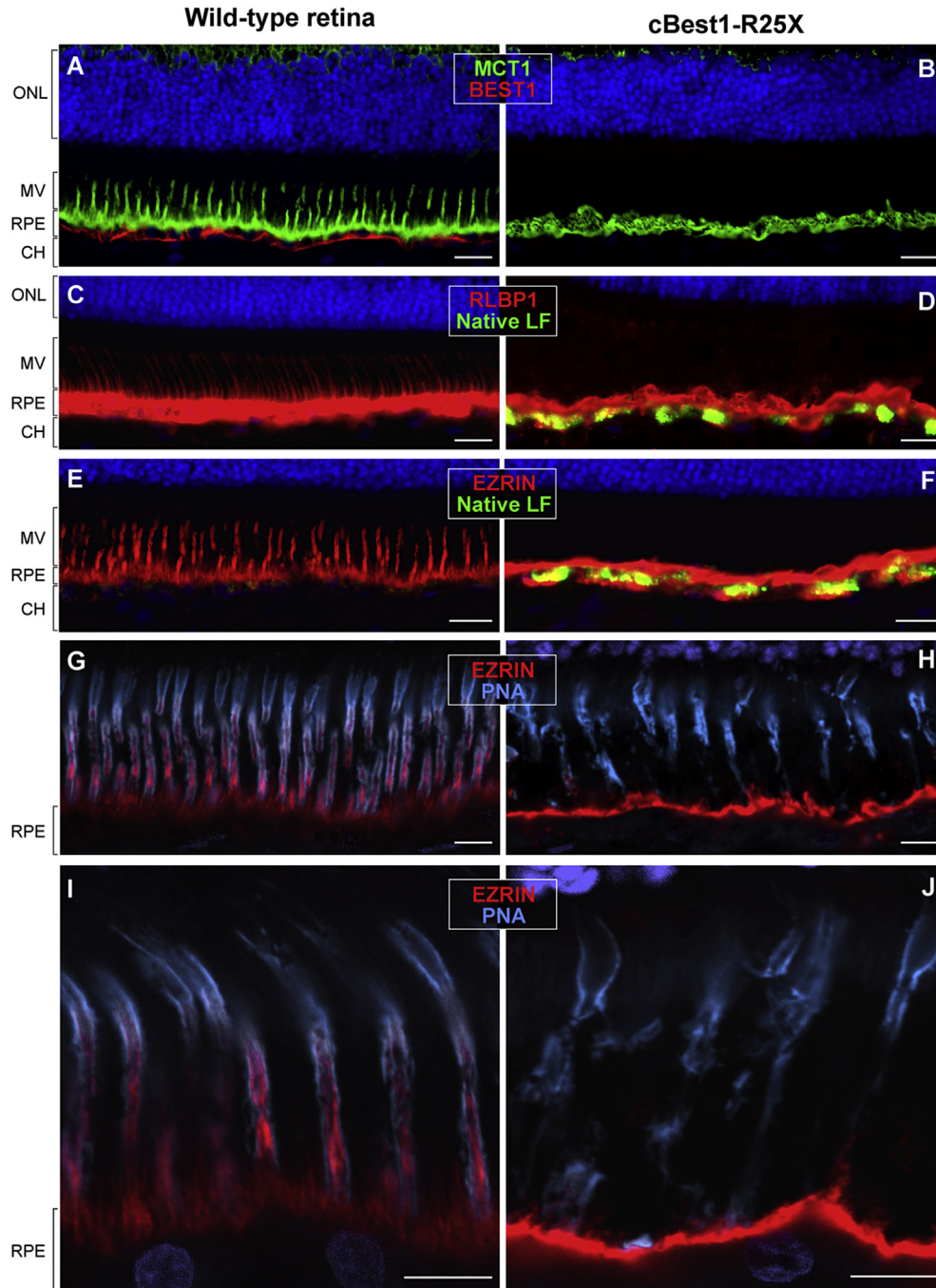


Fig. 5. Compromised cone-associated RPE apical domain and insoluble interphotoreceptor matrix (IPM) in canine bestrophinopathy. (A–J) A striking absence of cone-associated RPE apical microvilli was observed across cBest1-R25X-affected retinæ as confirmed by immunolabeling against various RPE-specific markers. (A, B) Anti-MCT1 (green) and anti-BEST1 (red) double IHC labeling; Note the absence of BESTROPHIN 1 basolateral labeling in the mutant retina, accompanied by a dramatic retraction of RPE apical projections (B). (C, D) Representative images of RLBP1 (a.k.a. CRALBP) immunolabeling (red); native lipofuscin fluorescence is visualized as yellow-green RPE inclusions in the mutant RPE (D). (E, F) Direct comparison of anti-EZRIN immunostaining (red) between canine wild-type and cBest1-R25X-affected retinæ. The massive inclusions (yellow-green) within the mutant RPE represent native lipofuscin (F). (G–J) Confocal photomicrographs of anti-EZRIN (red) and peanut agglutinin lectin (PNA, cyan) double-labeling in mutant (H and J) and age-matched controls (G and I). Note that in the WT retinæ PNA-stained insoluble extracellular matrix closely invest cone-associated RPE apical microvilli (G–I); in the cBest mutant, however, the structure of both the intrinsic RPE-associated- and the external IPM-derived cone-specific domain is largely compromised (H, J), affecting the adhesive forces of RPE-photoreceptor complex. ONL: outer nuclear layer; MV: microvilli; RPE: retinal pigment epithelium; CH: choroid. Scale bars: 20 μ m (A–F) and 10 μ m (G–J).

the photoreceptor OS would provide a highly unstable variant of this compound, compromising the subretinal microenvironment, and consequently, the interplay of RPE-photoreceptor complex. Such changes in concentration of metabolites surrounding POS may also disturb the hydration, bonding properties and viscosity of the interphotoreceptor matrix (IPM), and misregulate the volume of

subretinal space affecting retinal adhesion (Marmor, 1994; Pianta et al., 2003; Milenkovic et al., 2015).

It is possible, therefore, that the altered distribution and increased levels of cholesterol esters and HNE-adducts within photoreceptor layer of the cBest1-affected retinæ may indeed indicate a compromised IPM domain, exposing thereby a new role

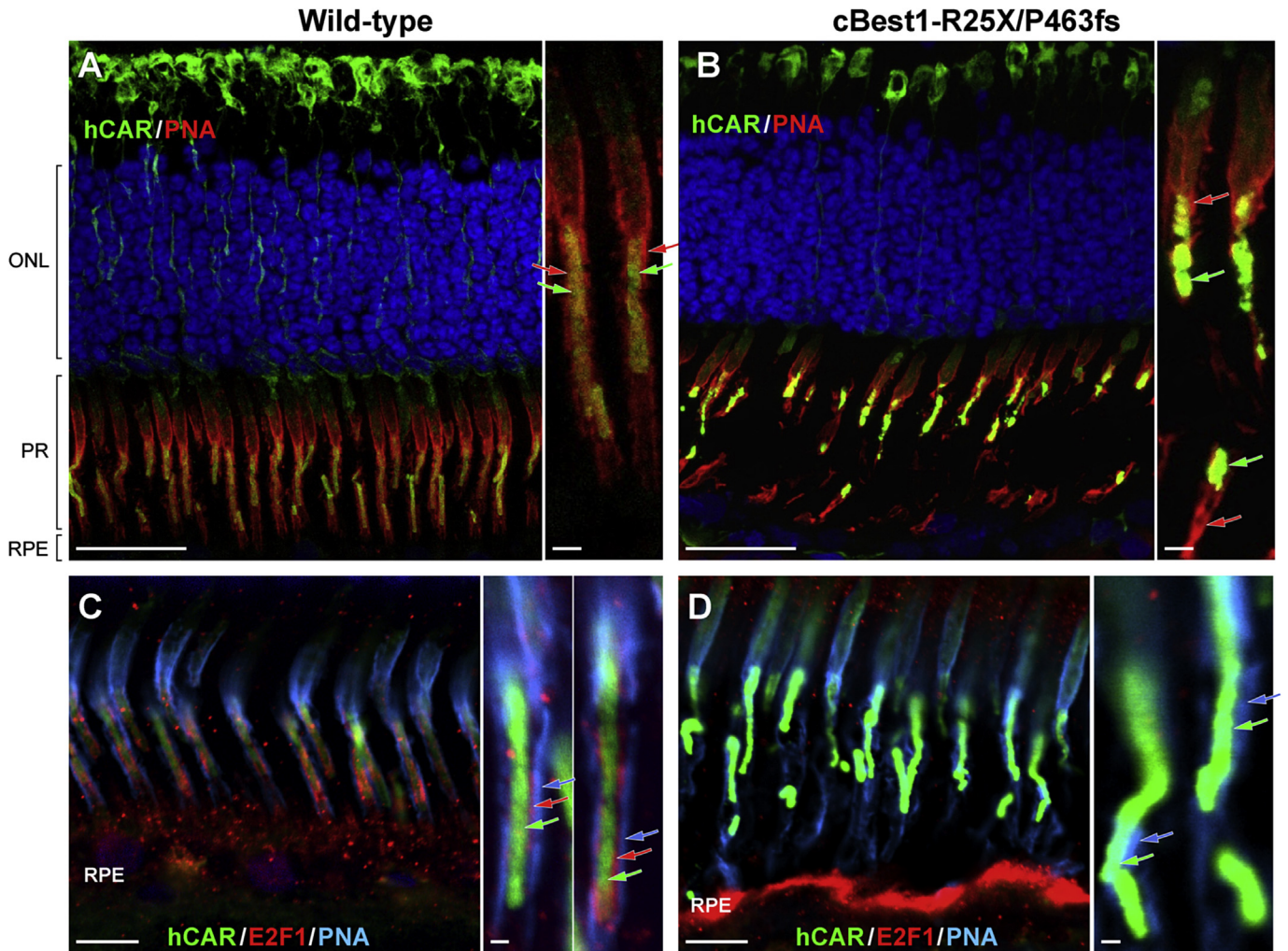


Fig. 6. Impaired RPE-cone interaction and photoreceptor alignment in canine bestrophinopathy. (A, B) Confocal photomicrographs of canine wild-type (A) and cBest1-R25X/P463fs-affected (B) retinæ immunolabeled for human cone arrestin (hCAR, green) and PNA lectin (red). PNA-positive cone extracellular matrix sheaths tightly embrace the E2F1-labeled cone-specific RPE projections, extending along the outer- and toward the inner segment of cone photoreceptor cells in the control retina (A, close-up); in the cBest mutant, this IPM domain appears ruptured, exposing OS to the extracellular space (B, close-up). Together with the impairment in structural integrity of cone-specific IPM escheatment, cone photoreceptor OS lose their proper orientation (B), necessary for the normal interactions with RPE cells. (C, D) Multi-label IHC images of canine wild-type (C) and cBest mutant (D) retina illustrating bilayered extracellular compartmentalization of the cone-specific escheatment responsible for a normal apposition of RPE-cone OS (C, inset). This well-defined extracellular structure was lost in the affected retinæ (D), revealing a dearth of the intrinsic RPE-associated apical microvillus layer, accompanied by rather fragmented external insoluble cone-specific matrix domain (D, close-up). Cone OS stripped of their protective sheaths appeared undermined with no direct contact with the underlying RPE (B, D). hCAR: human cone arrestin (shown in green A–D, and delineated by green arrows on the magnified images); PNA: peanut agglutinin lectin (shown in red A–B, and defined by red arrows on the corresponding close-ups; PNA in C–D is represented in cyan, and by cyan-colored arrows on the magnified photomicrographs); E2F1: E2F transcription factor 1 (shown in red C–D, and indicated by red arrows in C inset). ONL: outer nuclear layer; PRs: photoreceptors; RPE: retinal pigment epithelium. Scale bars: 20 μm (A, B), 10 μm (C, D), and 1 μm (applies to all magnified images). Photomicrographs C & D: modified from Guziewicz et al. *Adv. Exp. Med. Biol.* (in press).

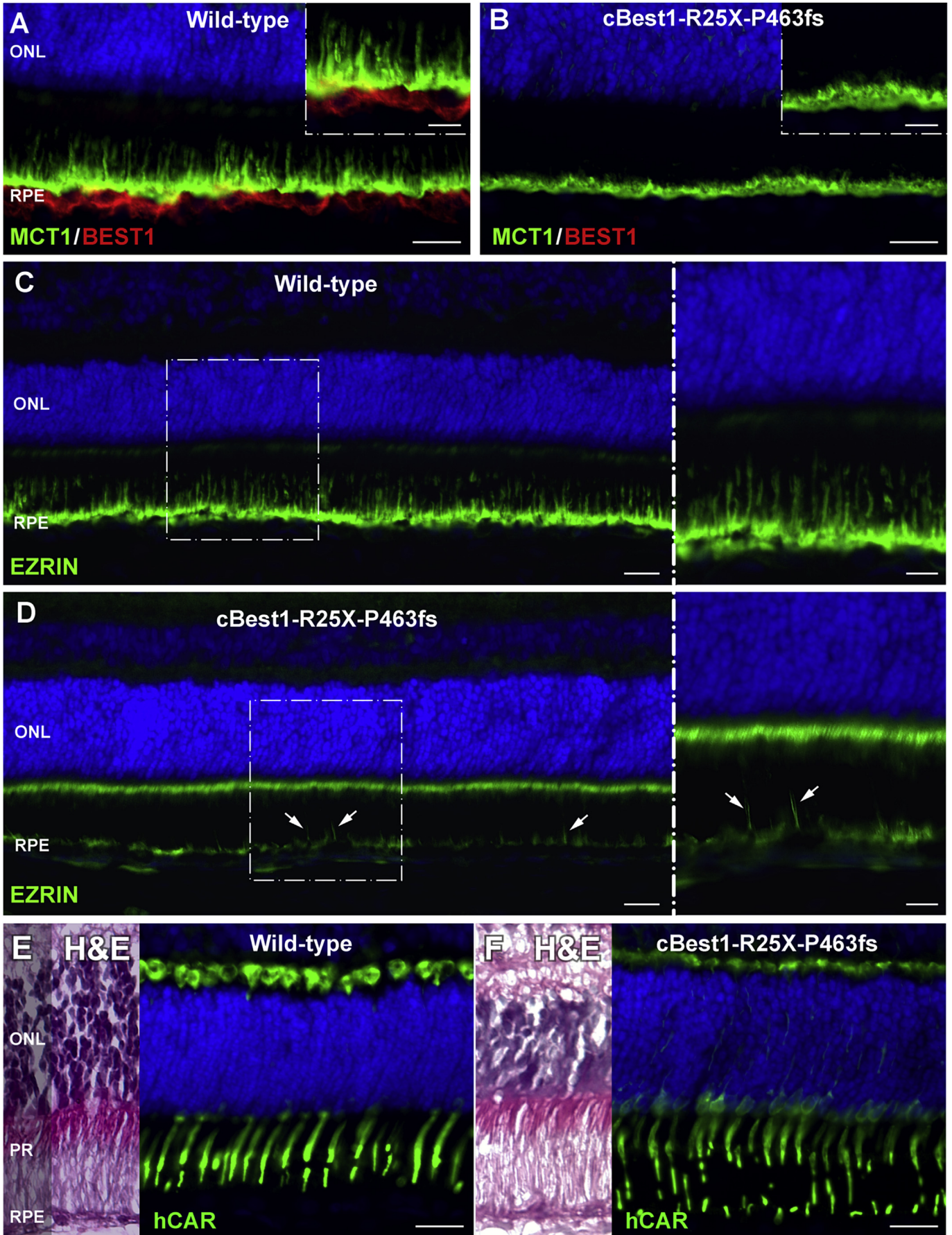
of Best1 in modulating the homeostatic milieu of subretinal space. While the excess of EC could function as an atypical chronic inflammatory stimulus interrelated to the impaired calcium signaling and fluid flow, such as 7-KCh (Lakkaraju et al., 2007; Moreira et al., 2009; Wang et al., 2010; Singh et al., 2013a; Strauss et al., 2014), the lipoperoxidation product, HNE, may act as a stress signaling molecule (Shen et al., 2007; Perluigi et al., 2012), both predisposing retina to the loss of adhesive forces in response to these pathological stimuli.

5. Bestrophinopathy: an RPE-photoreceptor interface disease

5.1. Compromised RPE-photoreceptor interaction in cBest

The RPE-photoreceptor interface is an area of crucial importance to proper retinal function (Hageman and Johnson, 1991).

Dysregulation of lipid metabolism documented in cBest1-mutant retina pointed to the presence of biochemical perturbations in the subretinal space reflecting homeostatic imbalance in the delicate IPM microenvironment. To gain additional insight into the spectrum of abnormalities at the RPE-photoreceptor interface in cBest, and identify further potential factors that may play a role in lesion formation in bestrophinopathies, a set of RPE-, photoreceptor- and IPM-specific markers were used for IHC evaluation of cBest vs control retinæ (Fig. 5). A striking difference between the two was the retraction of RPE apical projections (a.k.a. microvilli, MV) in all cBest1-R25X-affected retinæ as evidenced by the absence of MV labeling using a set of markers known to be expressed at the RPE apical membrane and along its extensions (Fig. 5A–F). Specific immunostaining against MCT1 (Monocarboxylate Transporter, Member 1), RLBP1 (Retinaldehyde Binding Protein 1), and EZRIN was present in both the structurally normal



and mutant retinae, but in the latter the immunolabeling was limited to the apical surface of RPE monolayer, which lacked the MV extensions that interdigitated with POS (Fig. 5B, D, and F). This finding was age- and sex-independent and observed in all canine *BEST1* mutant eyes examined after disease onset, in both the central retina as well as in the periphery. In contrast, the immunolabeling pattern of EZRIN, RLBP1 and MCT1 in cBest carrier retinae was comparable to that of the WT (*data not shown*). Confocal imaging of the mutant retinae immunolabeled by anti-EZRIN in combination with PNA lectin, known for its selective binding to the cone insoluble extracellular matrix microdomains (Johnson and Hageman, 1991; Mieziowska et al., 1993), confirmed MV retraction and revealed disordered and considerably disrupted cone-associated IPM ensheathment (Figs. 5G–J, and 6A–D). While the retraction of the longer RPE processes embracing cones was evident throughout the cBest1 mutant retinae, in both regions with bullous detachments as well as in non-detached areas, the much shorter residual MV associated with the longer rod outer segments were occasionally seen. However, the RPE apical extensions associated with rods are much shorter and finer than the cone-ensheathing processes, hence somewhat more difficult to visualize and track; yet at higher magnification, these small structures of rod-associated RPE extensions appeared disordered and interlaced (Fig. 5J). An expanded immunohistochemical study defined a bilayered extracellular compartmentalization of the cone specific escheatment responsible for a normal apposition of RPE-cone OS (Fig. 6C–D). This well-defined extracellular structure was lost in the cBest1-affected retinae, revealing a dearth of the intrinsic RPE-associated apical microvillus layer accompanied by a fragmented external cone-specific matrix domain (Fig. 6C–D, insets). As a result, cone OS stripped of their protective sheaths appeared undermined with no direct contact with the underlying RPE (Fig. 6).

The structural abnormalities related to the impaired microvillar ensheathment of photoreceptor outer segment tips and compromised insoluble IPM constitute two major pathological culprits responsible for weakening of the RPE-neuroretina physical and electrostatic interactions (Mieziowska, 1996; Bonilha et al., 2006). Despite the fact that IPM occupies a strategic location in the retina, for many years it was considered as an amorphous substance of unknown function and significance. We now know that this intricate yet highly organized structure with interconnected domains designed to meet the specific needs of both rods and cones, is critical for maintaining the homeostatic milieu of subretinal space and formation of an adhesive bond between RPE and photoreceptors (Johnson and Hageman, 1991; Mieziowska et al., 1993; Ishikawa et al., 2015). The key location between RPE apical surface and photoreceptors allows IPM to fulfill a range of biochemical and physical tasks fundamental for normal retinal function, among which the most critical ones like regulation of oxygen, nutrients and retinoid transport or preservation of cytoskeleton and retinal adhesion by providing electrostatic support for photoreceptors (Johnson and Hageman, 1991; Mieziowska et al., 1993; Hauck et al., 2005; Ishikawa et al., 2015). It is therefore not unexpected that any genetic or metabolic defect found in the soluble and insoluble components of IPM play a role in the etiology of retinal degenerative disorders, such as IMPG1- and IMPG2-associated vitelliform

macular dystrophy (Manes et al., 2013; Meunier et al., 2014; Ishikawa et al., 2015).

The dramatic retraction of the intrinsic RPE apical microvilli that underlies the loss of bilayered extracellular compartmentalization is of particular importance to the molecular pathology of bestrophinopathies. As highlighted earlier, the RPE performs a series of highly specialized tasks essential for homeostasis and structural integrity of the neural retina, including bidirectional transepithelial transport, supplying nutrients into and eliminating waste products from the photoreceptor layer, daily POS phagocytosis, light absorption, transport and regeneration of visual pigment; and all of these functions involve RPE microvilli that normally extend from its apical surface interdigitating with and tightly investing the POS (Bok, 1993; Bonilha et al., 2004, 2006). It is also important to note that these RPE projections greatly increase the apical surface area and, consequently, the number of transport and signaling molecules it contains, thereby enhancing the epithelial functional capacity (Bonilha et al., 2004; Miura et al., 2015). Thus, it is very likely that most of the major hallmarks of bestrophinopathies, e.g. disrupted ion and fluid transport (Sun et al., 2002; Milenkovic et al., 2015), impaired phagocytosis (Singh et al., 2013a), abnormal photoreceptor equivalent thickness (PET) documented in BVMD patients (Kay et al., 2012), an excessive build-up of autofluorescent debris in the subretinal space, and finally, the RPE-neuroretinal detachment and formation of serous lesions (Boon et al., 2009a, 2009b), arise directly from the dearth of properly formed microvilli, and impaired interaction/adhesiveness with OS. We posit that these salient interface defects may also account for the abnormal EOG and Arden ratio present clinically in bestrophinopathy patients (Pianta et al., 2003; Boon et al., 2013).

5.2. Underdeveloped RPE apical domain and lesion formation in bestrophinopathies

Based on these fresh insights from the spontaneous canine model of *BEST1*-maculopathies, it is reasonable to hypothesize that the cone-associated microvilli as well as the insoluble cone matrix sheaths are more sensitive to the biochemical changes in the delicate subretinal microenvironment and retract first, which would explain the predilection of the highly metabolically active cone-packed macular region to its primary detachment in human and canine bestrophinopathies. Alternatively, it is possible that the *BEST1* mutations impair the ability of RPE apical cone sheaths to form along with the shorter rod-associated MV processes. To test the premise, comparable IHC studies were performed in young (6-week-old) cBest retinae before disease onset, yet at the time point when the canine retina completes its development (Acland and Aguirre, 1987).

As shown in Fig. 7, both the immunolabeling against MCT1 and EZRIN revealed an underdeveloped RPE apical domain in the young cBest retinae, with only a few longer RPE microvilli and a highly disorganized distribution of EZRIN (Fig. 7A–D). In comparison to the age-matched controls, the 6-week-old cBest retina showed greatly diminished expression of EZRIN at the RPE apical surface, accompanied by an anomalous increase of EZRIN-positive signals associated with microvilli of Müller cells (Fig. 7D, close-up). While

Fig. 7. Underdeveloped RPE apical domain underlies formation of subretinal lesions in canine bestrophinopathies. (A–D) IHC evaluation of 6-week-old cBest1-R25X/P463fs-affected and age-matched wild-type control retinae showed underdevelopment of RPE apical domain in cBest mutant, as demonstrated by comparative labeling with anti-MCT1 (A, B), and anti-EZRIN (C, D). In sharp contrast to the young, yet fully matured WT canine retina (A), the age-matched mutant one exhibited loss of the longer MCT1-positive RPE apical microvilli, even before the onset of clinical disease (B, close-up); note the absence of BESTROPHIN 1 labeling (B) compared to the robust Best1 basolateral signals detected in the control RPE (A, red). (C, D) Contrary to the specific anti-EZRIN immunostaining in the WT (C), evaluation of the mutant retina revealed largely diminished fluorescent signals at the RPE apical surface with only a few EZRIN-positive RPE processes (D, arrows). Note the dramatic increase of EZRIN labeling associated with microvilli of Müller cells (D, close-up). (E, F) Although not obvious on H&E images, the cone outer segments of cBest-affected retinae already lack the uniform regularity when immunolabeled with hCAR (F, green) and compared to the WT control (E). Scale bars: 20 μ m (A–F), and 10 μ m (magnified images in A–D). Photomicrographs D–F: modified from Guziewicz et al. *Adv. Exp. Med. Biol.* (in press).

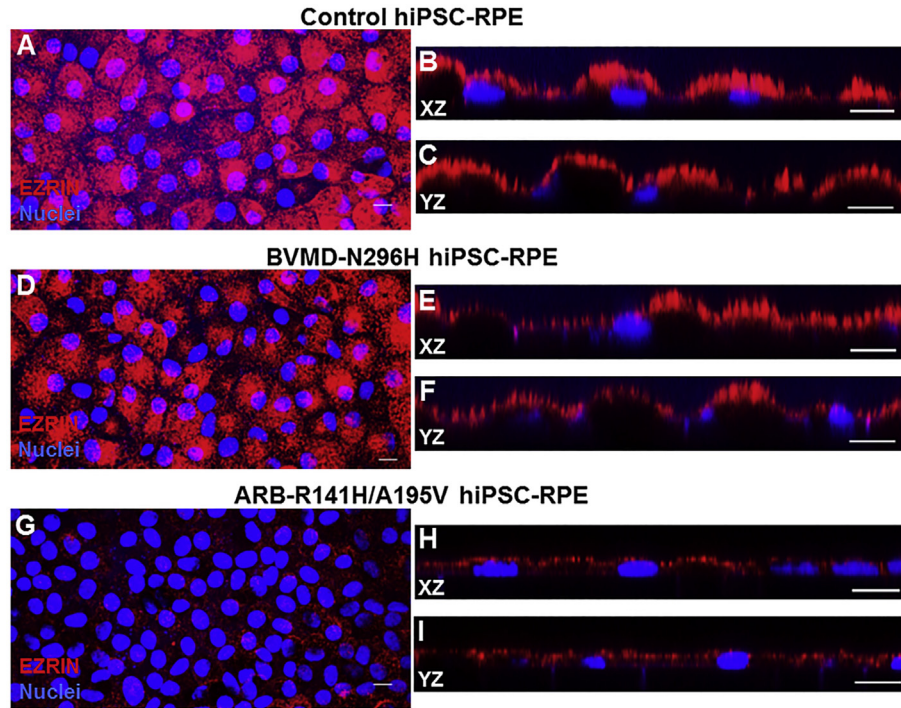


Fig. 8. EZRIN immunolabeling in WT, BVMD, and ARB hiPSC-RPE. (A–C) Immunohistochemical analysis of WT hiPSC-RPE showed strong expression of EZRIN with vertically oriented, apical localization. (D–F) Similar expression and localization of EZRIN was observed in BVMD hiPSC-RPE. (G–I) In the ARB hiPSC-RPE, harboring compound heterozygous mutation in *BEST1*, MV are smaller in height compared to the WT hiPSC-RPE, and express EZRIN in a punctate fashion within a thin apical band. XZ and YZ labeled images are representative images for X-Z and Y-Z optical sections. Scale bar: 10 μ m applies to all panels.

at this early time point cBest1-R25X/P463fs-affected retinae appeared histologically intact, the hCAR-labeled cone OS seemed more fragile and susceptible to destabilization following the natural course of disease.

These intriguing insights open a new avenue toward understanding the pathogenesis of bestrophinopathies and warrant further studies on RPE-PRs complex before disease onset. A number of research studies provided strong evidence that EZRIN is a major determinant in the maturation of surface differentiations of RPE, promoting morphogenesis of apical microvilli (Bonilha et al., 1999; Saotome et al., 2004; Miura et al., 2015). Our findings in the canine model support these results and strongly suggest that underdevelopment of cone-associated apical projections along with abnormalities of the insoluble cone extracellular matrix domain constitutes a key factor that underlies formation of the macular-selective subretinal lesions in bestrophinopathies. The fact that these prominent structural alterations associated with cone ensheathment are observed in cBest dogs as early as 6 weeks of age would also explain the early onset of ARB and BVMD along with the predisposition of the macular region to its primary detachment in affected dogs and patients.

5.3. RPE microvilli in ARB- and BVMD patient-specific hiPSC-RPE

In vitro, BVMD patient-specific human induced pluripotent stem cell-derived RPE (hiPSC-RPE) have been shown to successfully recapitulate several key cellular features of Best disease pathophysiology, including delayed POS degradation, increased accumulation of autofluorescent material after POS feeding, changes in endoplasmic reticulum-dependent Ca^{2+} homeostasis, and impaired fluid flux in response to physiological stimuli (Singh et al., 2013a, 2015). Both hiPSC-RPE and cBest models have complemented each other not only in gaining insights into BVMD

pathophysiology, but also in testing potential therapeutic strategies (Singh et al., 2015). Using this hiPSC model system, we have followed up on observations from the cBest model to look for differences in the apical processes of WT, BVMD and ARB hiPSC-RPE cultures. Human iPSC-RPE from all hiPSC sources were immunolabeled for EZRIN (Singh et al., 2013b, 2015), which was present in a thick, vertically oriented band along the apical region of WT hiPSC-RPE, consistent with its localization within apical processes (Fig. 8A–C). BVMD (hBEST1-N296H) hiPSC-RPE also demonstrated a vertically oriented apical band of EZRIN immunolabeling (Fig. 8D–F); however, in ARB hiPSC-RPE (hBEST1-R141H/A195V) EZRIN immunolabeling was less prevalent, punctate, and localized within a much thinner apical band (Fig. 8G–I), akin to observations made in the cBest model.

Given that EZRIN immunostaining appeared relatively similar between WT and BVMD hiPSC-RPE, transmission electron microscopy (TEM) was performed on ultra-thin sections of RPE to further assess whether differences exist between the apical microvilli of WT and BVMD hiPSC-RPE. TEM analysis showed decreased length and density of microvilli in BEST1-N296H vs WT hiPSC-RPE (Fig. 9). Given that the hiPSC-RPE was cultured in isolation as homogenous monolayers, these differences in apical RPE microvilli between WT, BVMD, and ARB hiPSCs are independent of interactions with cone photoreceptors.

6. Pathogenesis of early vitelliform lesions: insights from canine model

The structural and metabolic defects identified in the cBest model certainly contribute to, if not directly trigger, the separation of the neuroretina from the underlying RPE, the salient structural abnormality of bestrophinopathies. Further studies on molecular pathology of early focal and multifocal lesions confirmed the range

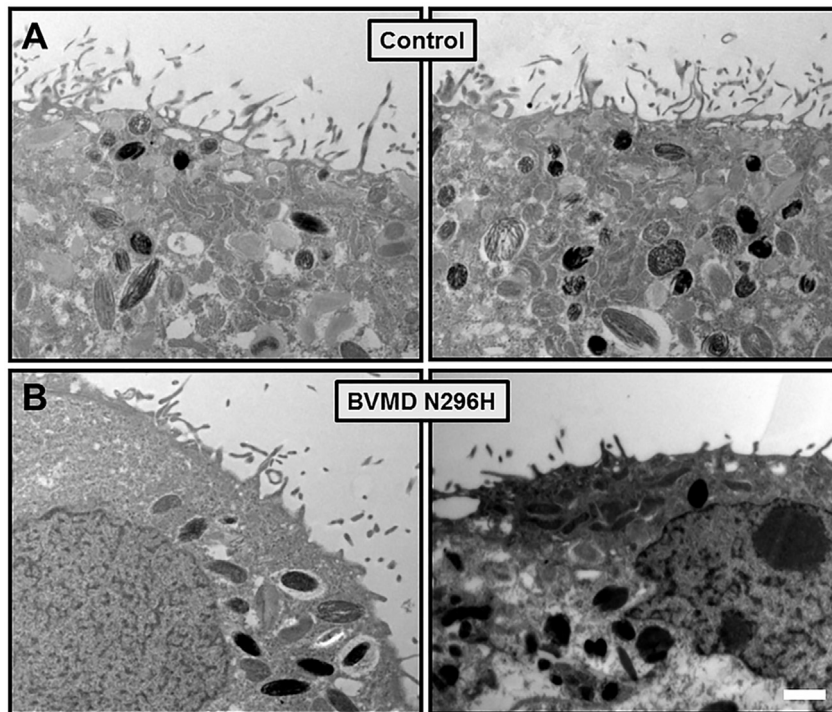


Fig. 9. TEM of WT and BVMD hiPSC-RPE. TEM analysis of WT hiPSC-RPE (A) and BVMD (BEST1-N296H) hiPSC-RPE (B) reveal shorter and more sparse microvilli in BEST1-N296H versus WT control hiPSC-RPE. Scale bar: 1 μ m.

of abnormalities as directly associated with subretinal detachment in cBest mutant retinæ (Fig. 10). We found an abnormal accumulation of lipofuscin material within RPE cells consistently associated with both the early and more advanced cBest lesions, remarkably aggregating not only at the detachment site, but also in its close vicinity (Fig. 10A–E). The more advanced cBest lesions represent longer standing retinal disassociations, where the autofluorescent debris starts to occupy subretinal space in transition to the pseudohypopyon stage (Fig. 10D,E). A noticeable increase in EC-BODIPY 493/503 was found to be associated with the photoreceptor layer in all early lesions examined, evident at the site of retinal separations and within still intact areas adjoining to the lesions (Fig. 10C). A series of IHC evaluations confirmed absence of microvillar elongations accompanied by a strikingly undulated RPE apical surface at the sites of detachments mapped *in vivo* by cSLO/SD-OCT (Fig. 10D,E). The primary lesion has a tendency to expand and secondary small satellite lesion(s) can be observed in its immediate vicinity often with a subtle retinal elevation between the individual separations (Fig. 10F,G). Eventually, after a prolonged serous detachment, the cellular debris accumulating in the subretinal space will constitute the solid phase of the gravitating pseudohypopyon material in the advanced disease. The rate of disease progression from early vitelliform to pseudohypopyon stage varies greatly between individuals and factors responsible for this mechanism are currently under investigation.

Best1 is a pentameric channel protein with defined roles in mediating anion transport and regulating calcium signaling in RPE cells (Kane Dickson et al., 2014; Yang et al., 2014; Strauss et al., 2014). Since intracellular calcium has been implicated to play important role in microvilli formation (Miura et al., 2015), the altered calcium homeostasis caused by Best1 dysfunction could underlie the defective villus architecture detected in cBest (Figs. 5–7, and 10) and hiPSC-RPE (Figs. 8 and 9) models, and reported in BVMD (Mullins et al., 2007) (Fig. 11). While the studies on

ARB- and BVMD patient-specific hiPSC-RPE will further explore Best1 interactors as well as specific cellular pathways affected by dysregulated calcium signaling (Singh et al., 2013a, 2015), the studies on the natural canine model will continue to provide vital insights into the mechanism and kinetics of retinal detachment, the salient clinical structural abnormality of bestrophinopathies. Based on the studies to date, the proposed mechanism of lesion formation in canine and human bestrophinopathies is summarized in Fig. 11.

7. Concluding remarks and future directions

The pathological mechanisms of pediatric retinal disorders, such as juvenile *BEST1*-associated maculopathies, are often challenging to explore; this can be attributed to the shortage of quality bio-specimens harvested at the disease-relevant stages and, until now, to the dearth of *bona fide* animal models to carry out mechanistic studies. Genetic disorders caused by mutations in a single locus, yet resulting in a variable clinical expression, pose perhaps the greatest challenge, which can be extremely difficult to address if the laboratory-induced animal models fail to recapitulate the human disease (Marmorstein et al., 2004; Marmorstein and Marmorstein, 2007; Zhang et al., 2010; Xiao et al., 2010). It is now possible, however, to undertake such studies with the canine *BEST1* disease model as it shares all the fundamental aspects with human bestrophinopathy, including the salient predilection of subretinal lesions to the canine macular region (Guziewicz et al., 2007, 2011, 2012; Beltran et al., 2014; Singh et al., 2015).

Here, we provide key insights into the complex molecular pathology of *BEST1*-related disorders, revealing factors predisposing macula to its primary detachment in *BEST1*-linked maculopathies, which may also account for the abnormal EOG and Arden ratio in BVMD and ARB patients. In view of the fact that RPE monolayer exhibits a considerable mosaicism (Burke and Hjelmeland, 2005), including not only topographic variations and regional

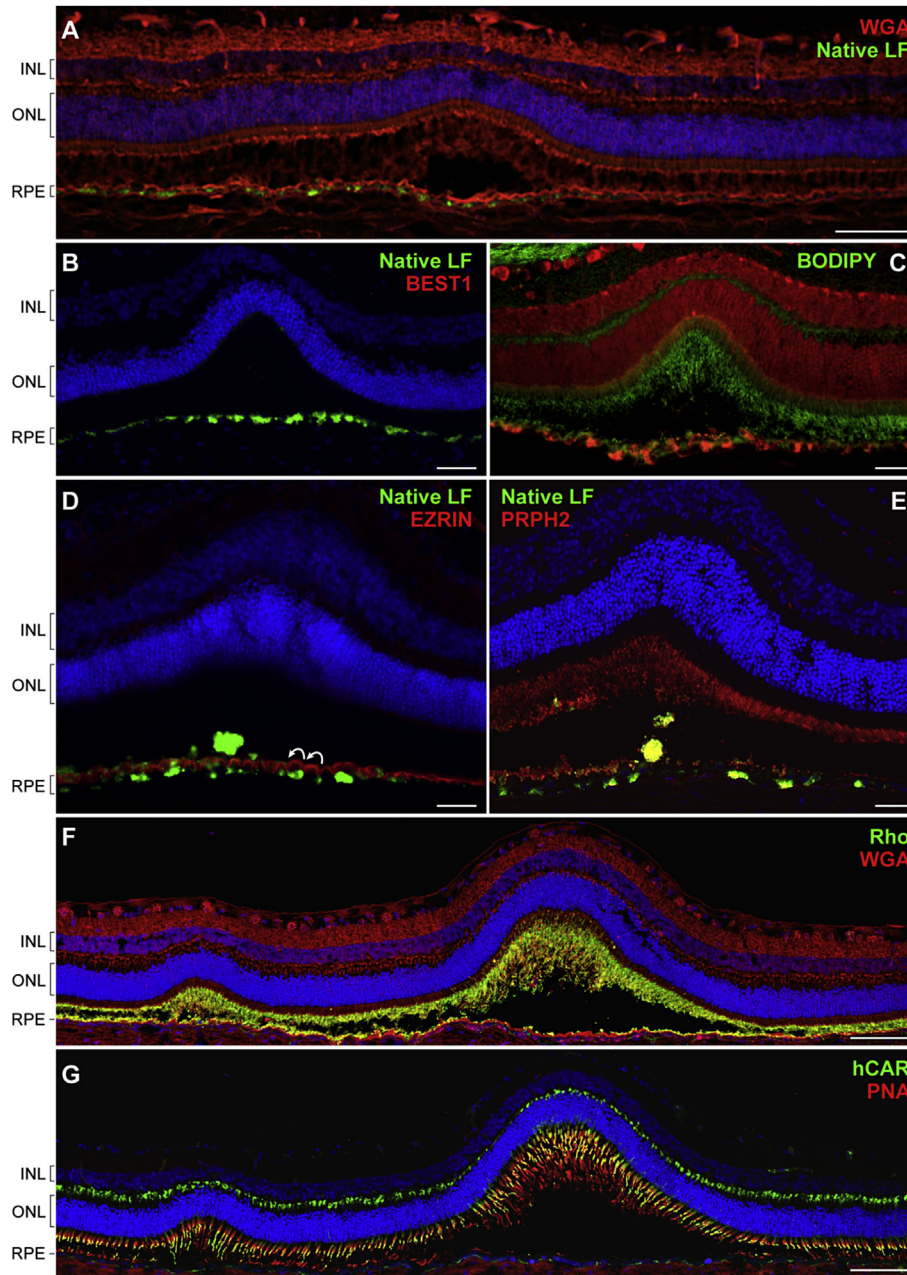


Fig. 10. Canine *BEST1*-associated maculopathy is a disease of an RPE-photoreceptor interface. (A) Confocal photomicrograph of an early pre-vitelliform lesion. The section was stained with wheat germ agglutinin (WGA, red) and DAPI (blue). Bright green fluorescent signals of lipofuscin granules (native LF) were found to be associated with RPE cells directly underlying the primary neuroretinal disruption as well as its immediate surrounding areas. (B) Cryosection from a 30-month-old cBest1-R25X-affected dog with more advanced subretinal lesions comparable to stage II (vitelliform) in BVMD. *BEST1*-deficient RPE cells accumulate fluorescent debris subjacent the serous retinal detachment. (C) Retinal cross-section through an early vitelliform lesion from a 25-month-old dog harboring premature stop mutation (R25X) in the canine *BEST1*. The section was pretreated with ethanol before applying BODIPY 493/503 (green) and propidium iodide (red) for nuclear counterstaining. An evident increase of EC-BODIPY was recorded within the detached and more intact areas adjoining to the lesion. (D) Advanced vitelliform lesion immunolabeled with anti-EZRIN (red) and stained with DAPI (blue). Note the striking undulation of the RPE apical membrane (white curved arrows), absence of EZRIN-positive RPE apical projections, and beginning of a buildup of autofluorescent debris in the subretinal space (native LF fluorescence). (E) Similar cBest lesion in a 25-month-old cBest1-R25X-affected dog labeled with anti-PRPH2 (red). Peripherin-stained photoreceptor debris occupy RPE apical surface delimiting its scalloped nature and RPE cell hypertrophy after the serous neuroretinal detachment. Cellular debris appear as yellowish subretinal aggregates. (F, G) Montage confocal photomicrographs of a large subretinal lesion and a satellite microlesion in vitelliform stage labeled with WGA (red) and anti-Rhodopsin (Rho, green) (F) or a combination of peanut agglutinin lectin (PNA, red) and anti-cone arrestin (hCAR, green) (G). Both the Rho- and hCAR-positive cellular debris were detected in the subretinal space of the mutant retinae. Nuclei were counterstained with propidium iodide (C) or DAPI, respectively. INL: inner nuclear layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium. Scale bars: 100 μm (A, F, and G), and 40 μm applies to (B–E) panels.

specializations, but also cell-cell heterogeneity in gene expression (Mullins et al., 2007) and individual ability to support adjacent photoreceptors, single cell profiling may be required for elucidating the multifocal form of bestrophinopathy, in order to gain a full understanding of the spectrum of changes that each RPE cell must

withstand before losing its direct interacting apposition.

Considering the rapid progress in hiPSC research, the key insights from a reliable animal model and its immediate verification in patient-specific hiPSC-RPE generate an exceptional momentum that will accelerate progress to the clinical applications (Guzewicz

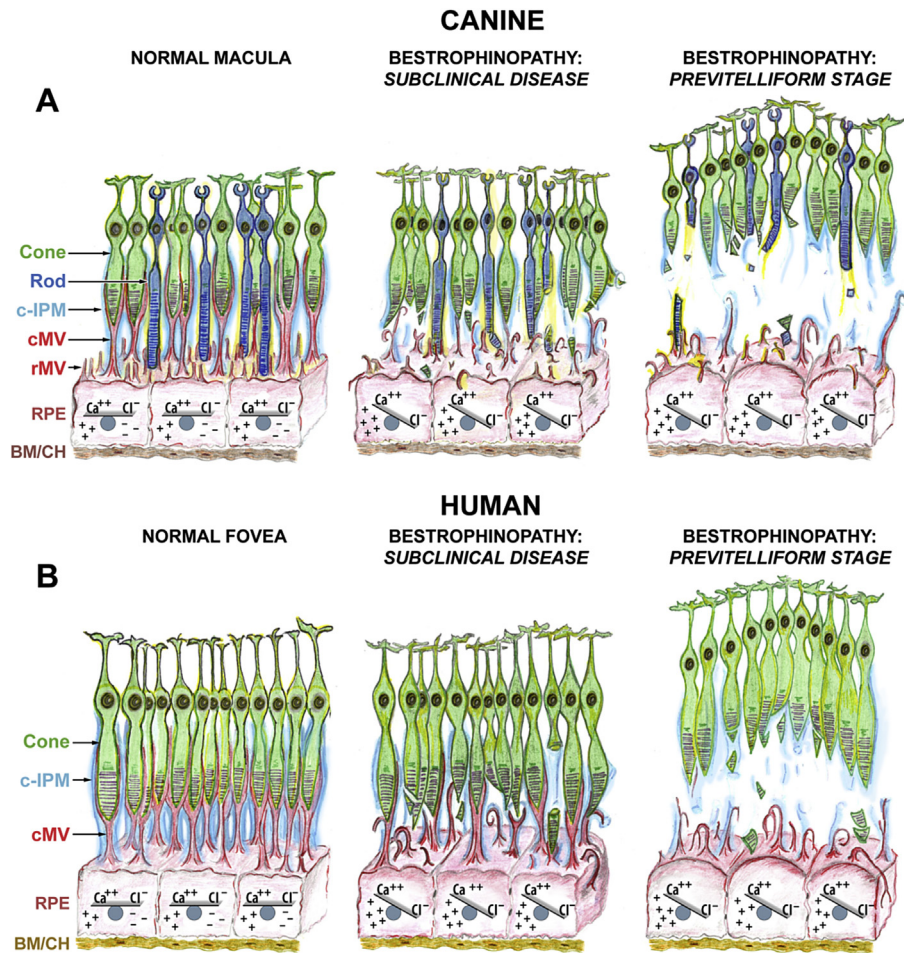


Fig. 11. Proposed mechanism of lesion formation in canine and human bestrophinopathies.

(A) Schematic representation of canine macula (a.k.a. *area centralis*) illustrating the normal RPE-photoreceptor apposition, sustained by intra- and extracellular ionic equilibria (NORMAL MACULA, left panel). Ionic imbalance caused by mutation(s) in *BEST1* gene initially affects RPE cell homeostasis, including expression of Ca^{++} -dependent molecules (i. a., EZRIN). Impaired expression of EZRIN, the key determinant in the maturation of surface differentiations of RPE, leads to the underdeveloped RPE apical surface with fewer and rather immature microvilli that are not capable of investing and supporting the photoreceptor outer segments (BESTROPHINOPATHY: SUBCLINICAL DISEASE, middle panel). Once the disease progresses, RPE cells gradually hypertrophy and lose their ability to mediate the ionic milieu of subretinal space. The cumulative effect of biochemical insult, accompanied by the structural abnormalities at the RPE apical surface weakens the adhesive forces of RPE-photoreceptor complex, which consequently results in separation of neuroretina from the RPE, and formation of serous lesion (PREVITELLIFORM STAGE, right panel). (B) Schematic representation of human fovea. This rod-free structure is densely packed with cone photoreceptors tightly enveloped by RPE apical microvilli (cMV) and ensheathed in cone-specific interphotoreceptor matrix (c-IPM) (NORMAL FOVEA, left panel). Due to the extremely high density of cones at this site, their outer segments *in vivo* will appear thinner in diameter, resembling rod photoreceptors; however, the cone outer segments are shorter than that of rods, and the RPE apical projections form long pedicles in order to reach them (cMV, red). The downstream effects of *BEST1* mutation(s) manifest as both the ionic disequilibrium within RPE cells (and later within the adjacent subretinal space) and structural disintegration associated with RPE-derived cone ensheathment (BESTROPHINOPATHY: SUBCLINICAL DISEASE, middle panel). As a consequence, the underdeveloped cone-associated RPE apical extensions are not able to physically support cone OS, which together with the reduced RPE functional capacity resulting directly from the decreased apical surface area, leads to the formation of macular-selective subretinal lesions at this highly metabolically active site (BESTROPHINOPATHY: PREVITELLIFORM STAGE, right panel). The compromised cone-associated insoluble IPM (c-IPM, cyan) may result from the chronic metabolic insult perturbing the delicate microenvironment of subretinal space and, secondarily, from the physical disassociation of the RPE-photoreceptor complex. The rate of disintegration of this extracellular matrix may constitute an important factor directly related to the expansion of vitelliform lesion and disease progression. Cone: cone photoreceptor cell; Rod: rod photoreceptor cell; c-IPM: cone-associated interphotoreceptor matrix; cMV: cone-associated microvilli; rMV: rod-associated microvilli; RPE: retinal pigment epithelium; BM/CH: Bruch's membrane/choroid.

et al., 2013; Sinha et al., 2016). As a complementary system, hiPSC-RPE-based *in vitro* modeling of BVMD provides a platform for assessing pharmacological and gene therapy based interventions (e.g., lengthening of apical microvilli as a therapeutic outcome measure). As such, homeostatic restoration of RPE-photoreceptor interface should be considered an important outcome measure in the assessment of experimental therapies for bestrophinopathies that will warrant the sustained disease reversal.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.preteyeres.2017.01.005>.

Abbreviations

RPE: retinal pigment epithelium; **BVMD:** Best Vitelliform Macular Dystrophy; **VMD2:** Vitelliform Macular Dystrophy, 2; **ARB:** Autosomal Recessive Bestrophinopathy; **cBest:** canine bestrophinopathy; **cSLO/SD-OCT:** confocal scanning laser ophthalmoscopy spectral domain optical coherence tomography; **FAF:** fundus autofluorescence; **EKG:** electrooculogram; **hiPSC-RPE:** human induced pluripotent stem cells - derived RPE; **hBest1:** human BESTROPHIN 1 protein; **cBest1:** canine BESTROPHIN 1 protein; **POS:** photoreceptor outer segment; **IPM:** interphotoreceptor matrix; **c-IPM:** cone-associated interphotoreceptor matrix; **MV:** microvilli; **CMV:** cone-associated RPE apical microvilli; **IHC:** immunohistochemistry; **HAI:** Hyperspectral Autofluorescence Imaging; **I_{em}:** emission intensity; **FC:** free cholesterol; **EC:** esterified cholesterol; **7KCh:** 7-ketocholesterol; **HNE:** 4-hydroxy-2-nonenal; **ORO:** Oil Red O; **MCT1:** Monocarboxylate Transporter, Member 1; **RLBP1:** Retinaldehyde Binding Protein 1; **hCAR:** human cone arrestin; **PNA:** peanut agglutinin lectin; **WGA:** wheat germ agglutinin; **IMPG1:** Interphotoreceptor Matrix Proteoglycan 1.

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