Disease-associated missense mutations in bestrophin-1 affect cellular trafficking and anion conductance

Vladimir M. Milenkovic 1, Elena Röhrlich 2, Bernhard H. F. Weber 2,* and Olaf Strauss 1,*

1Experimental Ophthalmology, Eye Hospital, University Medical Center Regensburg, 93054 Regensburg, Germany
2Institute of Human Genetics, University of Regensburg, 93054 Regensburg, Germany
*Authors for correspondence (bweb@klinik.uni-regensburg.de; strauss@eye-regensburg.de)

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Summary
Bestrophin-1, an integral membrane protein encoded by the BEST1 gene, is localized predominantly to the basolateral membrane of the retinal pigment epithelium. Mutations in the BEST1 gene have been associated with Best vitelliform macular dystrophy (BMD), a central retinopathy with autosomal dominant inheritance and variable penetrance. Over 120 disease-causing mutations are known, the majority of which result in amino acid substitutions within four mutational hot-spot regions in the highly conserved N-terminal half of the protein. Although initially thought to impair Cl– channel function, the molecular pathology of BEST1 mutations is still controversial. We have analyzed the subcellular localization of 13 disease-associated BEST1 mutant proteins in polarized MDCK II cells, an established model of apical to basolateral protein sorting. Immunostaining demonstrated that none of the 13 mutant proteins failed to integrate into the cell membrane. The defective proteins were predominantly retained in the cytoplasm, whereas wild-type bestrophin-1 revealed cell membrane localization. Functional analysis of I– fluxes in HEK-293 cells showed that all mutants exhibited a significant reduction in anion conductance. Our data indicate that defective intracellular trafficking could be a common cause of BMD accompanied by impaired anion conductance, representing a loss of anion channel function that is probably due to mistargeting of mutant protein.

Key words: Best vitelliforme macular dystrophy, Bestrophin-1, Iodide flux, YFP-I152L, Missense mutation, Protein trafficking

Introduction
Mutations in the BEST1 gene (MIM number 607854) have been shown to result in several types of retinal degeneration, such as Best vitelliform macular dystrophy (BMD), autosomal dominant vitreoretinopathies (ADVR), adult-onset macular dystrophy (AVMD), autosomal recessive bestrophinopathies (ARB) and retinitis pigmentosa (Boon et al., 2009; Davidson et al., 2009; Xiao et al., 2010). Thus far, over 120 distinct mutations have been identified and associated with the respective disorder (see http://www-huge.uni-regensburg.de/BEST1_database/). Nearly all the disease-associated changes are missense mutations and predominantly affect the N-terminal half of the BEST1 protein, termed bestrophin-1 (Boon et al., 2009; Stohr et al., 2002).

Bestrophin-1 is strongly expressed in the retinal pigment epithelium (RPE), where it mainly localizes to the basolateral membrane (Gouras et al., 2009; Guziewicz et al., 2007; Marmorstein et al., 2000; Mullins et al., 2005; Neussert et al., 2010). The RPE fulfills a multitude of functions that are essential for excitability and the structural integrity of the photoreceptors (Strauss, 2005). Mutations in bestrophin-1 therefore probably result in altered RPE function, subsequently contributing to disease. This sequence of events has been demonstrated by ocular coherence tomography in BMD patients (Planta et al., 2003), post mortem eyes of BMD patients (Bakall et al., 2007; Mullins et al., 2007; Weingeist et al., 1982) and in canine (Guziewicz et al., 2007) and murine (Zhang et al., 2010) animal models, which reveal alterations of the RPE such as hypertrophy (Bakall et al., 2007; Guziewicz et al., 2007), lipofuscin accumulation (Bakall et al., 2007; Guziewicz et al., 2007; Mullins et al., 2005; Weingeist et al., 1982) and focal loss of RPE cells (Bakall et al., 2007; Guziewicz et al., 2007; Mullins et al., 2005). Patients affected with BMD characteristically have an absent or reduced light-peak in the electro-oculogram (Arden and Constable, 2006; Boon et al., 2009; Cross and Bard, 1974; Ponjavic et al., 1999; Renner et al., 2005; Wabbel et al., 2004; Weleber, 1989). Functionally, bestrophin-1 has been suggested to play a dual role as an anion channel (Fischmeister and Hartzell, 2005; Hartzell et al., 2005; Hartzell et al., 2008; Jentsch et al., 2002; Milenkovic et al., 2009; O’Driscoll et al., 2009; Qu et al., 2004; Sun et al., 2002) and also as a regulator of intracellular free Ca2+ homeostasis (Barro-Soria et al., 2010; Barro Soria et al., 2009; Burgess et al., 2008; Hartzell et al., 2008; Marmorstein et al., 2009; Neussert et al., 2010; Rosenthal et al., 2006; Yu et al., 2008; Zhang et al., 2010). Subsequent reports have suggested that bestrophin-1 also acts as HCO3– channel (Qu and Hartzell, 2008), and it might have a function as an ion channel in the membranes of cell organelles (Barro-Soria et al., 2010; Neussert et al., 2010). Although a plethora of disease-associated mutations in BEST1 are known, little information about the molecular mechanisms by which these mutations affect bestrophin-1 function is available. For example, most mutations appear to disrupt Cl– channel function, although experimental evidence purely relies on cellular overexpression of mutant protein, possibly reflecting spurious artifacts well known for this type of in vitro approach (Clapham, 1998; Jentsch et al., 2002). Topological analysis of mutant bestrophin-1 has suggested that a minor proportion of mutations located in the transmembrane domains (TMDs) of bestrophin-1...
affect membrane topology by destabilizing TMD structure (Milenkovic et al., 2007). Finally, a recent study has demonstrated a mislocalized mutant bestrophin-1 associated with retinitis pigmentosa (Davidson et al., 2009).

Numerous missense mutations in genes encoding integral membrane proteins are associated with defective cellular trafficking (Aridor and Hannan, 2000; Cheng et al., 1990; Hume et al., 2009; Kausalya et al., 2006; Liu et al., 2001; Zhou et al., 1998). Most integral membrane proteins are co-translationally inserted into the membrane of the endoplasmic reticulum (ER), from where they subsequently traffic through the Golgi to the plasma membrane. This protein processing, however, appears to be rather inefficient, even for wild-type proteins. For instance, the cystic fibrosis transmembrane conductance regulator (CFTR) assembles with <50% efficiency. In the case of mutant CFTR protein, the efficiency of cellular membrane integration declines to <25% that of the wild-type protein (Kopito, 1999). Similar observations have been made for a number of other integral membrane proteins, such as epithelial sodium channel (ENaC), band 3 anion exchanger (SLC4A1) and nicotinic acetylcholine receptor (CHRmA) (Paulson et al., 1991; Rajan et al., 2001; Valentijn et al., 1998).

Protein folding and stability studies have demonstrated that a single amino acid change is sufficient to reduce the folding efficiency in a protein from 50% at body temperature to below 10% (Guerois et al., 2002). Because the most common mechanism by which a single amino acid change leads to a disease phenotype is disturbance of protein trafficking or folding (Sanders and Myers, 2004), we examined the trafficking of BMD-related mutants in polarized cells. Use of such a cell system is particularly important for trafficking studies of bestrophin-1, given that this protein localizes to the basolateral membrane of the RPE, a highly polarized cellular monolayer (Marmorstein et al., 2000).

Here, we have analyzed 13 disease-associated missense mutations by studying the subcellular localization and anion conductance of the encoded proteins. Anion permeability was assessed by monitoring the fluorescence quench of YFP I152L by I− ions, whereas the subcellular localization of mutant bestrophin-1 was assessed by monitoring the fluorescence quench of YFP I152L by conductance of the encoded proteins. Anion permeability was assessed by monitoring the fluorescence quench of YFP I152L by I− ions, whereas the subcellular localization of mutant bestrophin-1 was determined in polarized MDCK II cells, a standard model to investigate cellular trafficking (Rodriguez-Boulan et al., 2005). Our findings show that the majority of the mutant proteins are retained in different intracellular compartments and are not targeted to the plasma membrane. All mutants have a reduced anion permeability, suggesting that defective intracellular trafficking is the most common molecular basis for the BMD phenotype.

**Results**

**Expression and cellular localization of BMD-associated mutant bestrophin-1 proteins**

To investigate whether BMD-associated missense mutations affect plasma membrane targeting, wild-type bestrophin-1 and 13 missense mutations, located in four mutational hot-spots of bestrophin-1 (White et al., 2000), were generated by site-directed mutagenesis and stably expressed in polarized MDCK II cells. The additional pore-affecting mutation S79C (Qu et al., 2004) was also examined (Fig. 1A). A mean transepithelial resistance (TER) of 194 ± 46 Ω cm², after correction for background resistance, verified the integrity of the MDCK II cell monolayers, which were subsequently used for immunostaining. All constructs were also transiently expressed in HEK-293 cells. The wild-type and all mutant bestrophin constructs had comparable expression levels (Fig. 1B; supplementary material Fig. S1).

Polarized MDCK II cells expressing wild-type bestrophin-1 and the different bestrophin-1 mutants were co-stained for the integral membrane protein monocarboxylate transporter 1 (MCT1). As expected, wild-type bestrophin-1 was targeted to the basolateral membrane and showed strong [Pearson’s correlation coefficient (PCC) 0.92] colocalization with the plasma membrane marker MCT1 (Fig. 2A; Table 1). By contrast, nine out of the 13 bestrophin-1 mutants predominantly accumulated in the cytoplasm and revealed no colocalization with the MCT1 membrane protein (PCC 0.09–0.17) (Fig. 2B–J; Table 1). Several of the mutants (S79C, F80L, L82V and A243T) did show predominant localization to the plasma membrane (Fig. 2B–F; Table 1). However, compared with wild-type bestrophin-1, the efficiency of this localization was reduced. A Student’s t-test analysis of the PCCs for the BMD-associated mutants and wild-type bestrophin-1 demonstrated a significant

![Diagram](image-url)
Fig. 2. Colocalization of bestrophin-1 mutants with the plasma membrane marker MCT-1 in MDCK II cells. (A–J) Monolayers of MDCK II cells stably expressing wild-type and various mutated bestrophin-1 constructs were stained with antibodies for human bestrophin-1 (left-hand panel, red) and MCT-1 (middle panel, green). Merged images (right-hand panel) show the extent of colocalization of bestrophin-1 mutants and MCT-1. As shown, bestrophin-1 mutants are predominantly targeted to the cytoplasm. The results of quantitative colocalization analysis are summarized in Table 1. Scale bars: 20 μm.

Table 1. Summary of steady-state localization of wild-type and mutant bestrophin-1 in MDCK II cells

<table>
<thead>
<tr>
<th>Bestrophin-1 form</th>
<th>Surface expression</th>
<th>Average PCC with MCT1</th>
<th>ER</th>
<th>Golgi</th>
<th>EEA1</th>
<th>LAMP1</th>
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<tr>
<td>Wild-type</td>
<td>+</td>
<td>0.92</td>
<td>+</td>
<td>*+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>T6P</td>
<td>–</td>
<td>0.13</td>
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<td>–</td>
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<td>L21V</td>
<td>–</td>
<td>0.17</td>
<td>–</td>
<td>*+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>W24C</td>
<td>–</td>
<td>0.11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S79C</td>
<td>+</td>
<td>0.71</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>F80L</td>
<td>+</td>
<td>0.75</td>
<td>–</td>
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<tr>
<td>L82V</td>
<td>+</td>
<td>0.61</td>
<td>–</td>
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<tr>
<td>R92S</td>
<td>–</td>
<td>0.14</td>
<td>–</td>
<td>*+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>R218C</td>
<td>+</td>
<td>0.88</td>
<td>–</td>
<td>*+</td>
<td>–</td>
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<tr>
<td>L224M</td>
<td>–</td>
<td>0.11</td>
<td>+</td>
<td>*+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Y227N</td>
<td>–</td>
<td>0.11</td>
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<td>–</td>
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<td>T237R</td>
<td>–</td>
<td>0.15</td>
<td>–</td>
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<tr>
<td>A243T</td>
<td>+</td>
<td>0.56</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*+</td>
</tr>
<tr>
<td>F305S</td>
<td>–</td>
<td>0.09</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V311G</td>
<td>–</td>
<td>0.12</td>
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Quantitative colocalization with the plasma membrane marker MCT1 was used to differentiate between surface and intracellular expression of bestrophin-1 (n≥9). Values in bold indicate significant colocalization with MCT1; +, colocalization; –, no colocalization; *, predominant steady-state localization.
difference (P<0.0001) between the subcellular localization of all the mutants examined with the exception of mutant protein R218C, which had an intracellular distribution highly similar to wild-type bestrophin-1 (P>0.05) (Fig. 3E; Table 1).

Some of the mutants were studied in more detail by confocal imaging to obtain vertical optical sections across MDCK II cell monolayers. MDCK II monolayers were properly polarized as demonstrated by co-staining with the tight-junction protein zonula occludens-1 (ZO-1) and MCT-1 (Fig. 4A). In the polarized monolayers, wild-type bestrophin-1 colocalized with MCT1 to the basolateral part of the cell (Fig. 4B). By contrast, the F305S mutant protein, which is mistargeted to the cytoplasm (Fig. 2I), showed diffuse intracellular staining throughout the cell (Fig. 4C). However, the R218C mutant protein, which seems not to affect plasma membrane targeting (Fig. 3E), R218C almost completely colocalized to the basolateral membranes with MCT1 (Fig. 4D).

**Bestrophin-1 mutants with defective membrane targeting localize to the Golgi complex, the ER or to early endosomes**

Next, we aimed to identify intracellular compartments to which bestrophin-1 mutants with predominant intracellular distribution could localize. In order to clearly conclude about a possible mechanism we concentrated on mutant bestrophin-1 with predominant cytosolic localization. MDCK II cells stably expressing the bestrophin-1 mutants L21V, R92S, L224M or F305S, each one representing a mutational hot-spot of BEST1, were co-stained with anti-bestrophin-1 antibody and antibodies against the early endosome marker EEA1 (Fig. 5A), the Golgi complex marker GM130 (Fig. 5B), the ER marker BIP (Fig. 5C) and the lysosomal marker LAMP1 (Fig. 5D). All four mutants showed no noticeable overlap with the lysosomal marker. By contrast, mutants L21V, R92S and L224M extensively colocalized with the Golgi marker. Mutants L21V, L224M and F305S also colocalized with ER and early endosome markers. Mutant F305S had the least colocalization with all the subcellular markers tested. In order to more clearly identify the subcellular compartments in which mutant bestrophin-1 proteins are trapped, colocalization experiments were performed in subconfluent MDCK II cells (supplementary material Fig. S2). These results suggest that bestrophin-1 mutants mainly accumulate in the Golgi complex and the ER.

**BMD-associated mutant bestrophin-1 proteins show decreased anion permeability of the cell membrane**

To investigate the impact of missense mutations on the ability of bestrophin-1 to increase anion permeability of the cell membrane, I⁻ fluxes were measured by halide-sensitive YFP I152L fluorescence quench. As MDCK II cells are known to endogenously express a large variety of ion channels including Ca²⁺-dependent Cl⁻ channels, these cells are largely unsuitable for use in expression systems to study ion channel function (Lang and Paulmichl, 1995; Rothstein and Mack, 1990; Shen et al., 2002). Accordingly, HEK-293 cells were triple-transfected with different bestrophin-1 constructs, YFP I152L and human P2Y purinoceptor 2 receptor (P2Y2). In a first step, extracellular Cl⁻ ions were replaced by I⁻, which led to no quenching of the YFP I152L fluorescence. By contrast, cells additionally transfected with wild-type bestrophin-1 showed a strong quench in YFP I152L fluorescence when ATP was applied indicating an influx of I⁻ ions into the fluorescence. Thus, the presence of bestrophin-1 increased the membrane permeability for anions. The mutant bestrophin-1 constructs T6P, L21V,
L82V, Y227N and F305S showed a strongly reduced ability to increase the anion permeability of transfected HEK-293 cells (Fig. 6A,B) despite a expression level comparable to the wild-type protein (Fig. 1B; supplementary material Fig. S1). By contrast, expression of the other nine mutant bestrophin-1 constructs (W24C, S79C, F80L, R92S, R218C, L224M, T237R, A243T and V311G) led to an anion permeability that was significantly higher than that of cells transfected with YFP I152L alone (P<0.05) (Fig. 6B).

Discussion
To gain further insight into the molecular mechanisms leading to BMD pathology, we examined intracellular trafficking of mutant bestrophin-1 proteins in polarized MDCK II cells. We analyzed 13 known disease-associated mutations and one mutation (S79C) that has been predicted to change the properties of the anion channel pore (Qu et al., 2004). Eight of these mutations (L21V, W24C, F80L, L82V, L224M, T237R, F305S and V311G) were functionally investigated for the first time, whereas none of the known BMD mutations had been examined in polarized epithelial cells previously. As a general theme, we found that the mutants analyzed either failed to traffic to the cell membrane or showed reduced efficiency to incorporate into the cell membrane, with exception of mutation R218C, which was targeted to the plasma membrane in a manner similar to the wild-type protein. Interestingly, colocalization of wild-type bestrophin-1 and MCT-1, a typical marker for the basolateral membrane, was not entirely congruent, as a small proportion of bestrophin-1 appeared to be associated with the lateral membrane, but it also localized within the cytoplasm. Similar observations have been made previously in other studies and were thought to implicate bestrophin-1 in Ca\(^{2+}\) signaling by the ER (Barro-Soria et al., 2010; Neussert et al., 2010). Furthermore, by co-staining of bestrophin-1 and \(\beta\)-catenin or pan-cadherin in native porcine RPE, we identified a proportion of bestrophin-1 close to the basolateral membrane but not integrated into the membrane (Neussert et al., 2010).

A refined colocalization analysis with various subcellular markers revealed that the mutants L21V, R92S, L224M and F305S were partially retained in the Golgi complex, the endoplasmic reticulum and in the early endosomes. This suggests that mutant bestrophin-1 proteins are synthesized in the ER and move to the Golgi complex where they fail to undergo further processing. Proteins with missense mutations located on regions of bestrophin-1 that reside in the cytoplasm (T6P, L21V, W24C, L82V and V311G) showed a tendency to a more severe trafficking defect than mutations located in the transmembranal areas of bestrophin-1 (S79C, F80L, T237R and A243T). Thus, regions that localize to cytoplasm seem to be important for proper processing.

To further investigate the properties of the disease-associated bestrophin-1 mutations, anion conductance was measured by halide-sensitive YFP I152L assays in transfected HEK-293 cells.
HEK-293 cells are an established model for studying heterologously expressed ion channels (Varghese et al., 2006), and most data on bestrophin-1 ion channel function have been obtained with measurements in this cell line (Hartzell et al., 2008; Pusch, 2004). To increase cytosolic free Ca\(^{2+}\), the cells were additionally transfected with the human P2Y2 receptor and stimulated with ATP. This is more physiological than the alternative approach using Ca\(^{2+}\) ionophores because stimulation of P2Y2 receptors involves activation of a physiological signal transduction cascade including generation of either Ins(1,4,5)P\(_3\).

Analysis of activation mechanisms of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels in the RPE showed marked differences between the application of either Ins(1,4,5)P\(_3\) or a Ca\(^{2+}\) ionophore (Strauss et al., 1996). As a main observation we found that all mutant bestrophin-1 led to a strongly reduced increase in Ca\(^{2+}\)-dependent anion permeability; the observed I\(^{-}\) quenches were significantly smaller than those seen with wild-type bestrophin-1. This observation is in accordance with patch-clamp data with the same or similar mutations in bestrophin-1 (Qu et al., 2009; Sun et al., 2002; Yu et al., 2006). Interestingly, nine of the mutant bestrophin-1 proteins (W24C, S79C, F80L, R92S, R218C, L224M, T237R, A243T and V311G) had significantly increased Ca\(^{2+}\)-dependent anion permeability compared with those from cells transfected with YFP I152L plus hP2Y2 alone. Thus, although activity was still much lower than with normal bestrophin-1, these mutations still have residual ion-conductance function.

Mutants S79C, F80L and R92S showed strongly reduced ability to increase Ca\(^{2+}\)-dependent anion permeability compared with wild-type bestrophin-1, and mutant L82V failed to increase

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**Fig. 5. Subcellular localization of representative bestrophin-1 mutants.** Bestrophin-1 constructs, with missense mutations selected from each mutational hot-spot, were stably expressed in MDCK II cells and immunostained for bestrophin-1 (red), ZO-1 (blue) and the early endosome marker EEA1 (A), the Golgi marker GM130 (B), the ER marker BIP (C) or the lysosomal marker LAMP1 (D) (green). Merged images show the extent of colocalization. The mutants L21V, R92S and L224M extensively colocalized with GM130. The mutants L21V, L224M and F305S also colocalized with BIP and EEA1. F305S was least colocalized with all the subcellular markers. Scale bars: 20 \(\mu\)m.
Fig. 6. Anion permeability of HEK-293 cells expressing mutant and wild-type bestrophin-1. (A) Purinergic activation of wild-type and selected mutated bestrophin-1 constructs in HEK-293 cells. Activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{–} channels was measured by I\textsuperscript{–} influx measurements using the I\textsuperscript{–} sensitive protein YFP I152L (YFP\textsubscript{S152L}). The figure shows examples of single measurements from cells which either express YFP I152L with human P2Y2 (hP2Y2) alone or YFP I152L with P2Y2 together with either wild-type bestrophin-1 or the mutant forms. (B) A summary of the effects of ATP stimulation on I\textsuperscript{–} uptake in HEK-293 cells expressing various bestrophin-1 constructs. All mutant bestrophin-1 showed significantly decreased I\textsuperscript{–} permeability compared with that of cells expressing wild-type bestrophin-1. However, the mutants W24C, S79C, F80L, R92S, R218C, L224M, T237R, A243T and V311G showed a significantly larger I\textsuperscript{–} permeability compared with cells which express YFP I152L with P2Y2 alone. The number of cells is given in the bar. *P < 0.05.

the anion permeability. These mutations are located in the putative pore region that localizes around amino acid positions 70–90. Interestingly, S79C, a mutant not known to be associated with BMD, was utilized previously to demonstrate a putative pore region in the homologue bestrophin-2 (Qu et al., 2004; Xiao et al., 2010) but failed to increase the Ca\textsuperscript{2+}-dependent anion conductance for Cl\textsuperscript{–} by itself (Barro-Soria et al., 2010). Thus, our observations on I\textsuperscript{–} flux changes by bestrophin-1 and its mutant forms could also reflect altered Ca\textsuperscript{2+} signaling in response to P2Y2 receptor stimulation. Because the spatial distribution of Ca\textsuperscript{2+} signals is of importance for their regulative effects (Berridge et al., 2000; Petersen et al., 2001), it might well be that an altered subcellular distribution of bestrophin-1 leads to altered spatial distribution of Ca\textsuperscript{2+} signals and a subsequently altered activation of anion conductance.

Our data provide new insight into the molecular disease pathology of bestrophin-1 mutations. It appears that mutations located in the N-terminal region of bestrophin-1 result in a loss of function by impaired trafficking to the cell membrane, by a loss of pore function or by a combination of both. A loss of function in the disease-associated mutant proteins could explain the observed reduction of the light-peak in the electro-oculogram of BMD patients (Hartzell et al., 2008; Marmorstein et al., 2009; Xiao et al., 2010). The decreased anion conductance in the RPE probably influences transepithelial transport of Cl\textsuperscript{–} ions and water and might also impair volume regulation. Both properties are essential to RPE function and might cause disturbances in the interaction of photoreceptor outer segments and the RPE (Strauss, 2005).

Materials and Methods

Cell culture

HEK-293 (ATCC, cat# CRL-1573) and MDCK II (ATCC, cat# CCL-34) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing L-glutamine, 4500 mg/l glucose and 110 mg/l sodium pyruvate. All media were supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin (PAA, Colbe, Germany). Cells were cultured at 37°C, under an atmosphere with a relative humidity of 95% and containing 5% CO\textsubscript{2}. HEK-293 cells were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s instructions. MDCK II cells were transfected by electroporation using ECM 830 (BTX Harvard Apparatus, Holliston, MA, USA). Cells stably expressing wild-type and mutant bestrophin-1 were cultured for 2 weeks in a selection medium containing 500 μg/ml G418 (PAA). Pools of at least three independent stable colonies were used for immunofluorescent studies.

Transepithelial electrical resistance

Transepithelial electrical resistance of the MDCK epithelial monolayers was monitored with the Endohm apparatus (World Precision Instruments, Sarasota, USA). MDCK II monolayers were grown on Transwell polycarbonate filters (0.4 μm pore size), which consist of tissue-culture-treated polyester membranes with an insert diameter of 12 mm and an effective growth area of 1 cm\textsuperscript{2}. The upper chamber (apical side) contained 0.5 ml medium and the lower one (basolateral side) contained 1.8 ml medium. MDCK cells were seeded at a density of 10\textsuperscript{4} cells per ml and were grown to reach a stable TER, representing a confluent monolayer, generally 5 days after seeding. The medium was changed every 2 days. The TER was normalized to the area of the filter after removal of the background resistance of a blank filter submerged in medium only. TER was measured in ohms cm\textsuperscript{2} (Ω cm\textsuperscript{2}).

Site-directed mutagenesis of BEST1

Full-length BEST1 was amplified by reverse transcriptase (RT)-PCR from mRNA from human RPE and was directionally cloned into the pcDNA3 vector. Missense mutations were generated on a full-length BEST1 cDNA template (GenBank accession number, NM_004183; 1764 bp in length encoding 585 amino acids), applying the QuikChange site-directed mutagenesis kit (Stratagene). The constructs were made with the primer pair combinations given in supplementary material Table S1. The final BEST1 constructs were fully sequenced to ensure the targeted mutagenesis had occurred correctly and to exclude the presence of
undesired sequence alterations. Sequencing reactions were performed with the Big Dye terminator cycle sequencing kit version 3.1 (Applied Biosystems) according to the manufacturer’s instructions and were analyzed in an ABI Prism Model 3100 or 3730 Analyzer (Applied Biosystems).

**Cellular YFP I152L fluorescence measurement**
HEK-293 cells were triple transfected using equal amount of each plasmid (encoding YFP I152L, rabbit monoclonal anti-bestrophin-I, and rabbit polyclonal anti-myc C-terminal receptor). At 12–16 hours after transfection, cells were seeded onto 18-mm diameter round glass cover slips, and subsequent measurements were performed 36 hours after transfection. The YFP I152L plasmid was kindly provided by J.V. Galietta, Istituto Giannina Gaslini, Genova, Italy. Images were acquired with a Coolsnap HQ CCD camera (VisiRon, Puchheim, Germany) and processed with the Metafluor software (VisiRon). HEK-293 cells were grown on 18-mm diameter round glass cover slips and mounted in an imaging chamber maintained at room temperature. Cells were continuously perfused with 5 ml of K+ free solution, where the solution cells were exposed to 20 mM K+ by replacing NaCl with equimolar NaI and maintaining the total concentration of NaCl plus NaI at 137 mM. Typically, measurements of fluorescence intensity were performed in groups of 5–15 transfected HEK-293 cells and represent the average intensity of all fluorescent cells within the selected area. Initial fluorescence values were similar among cells transfected with different BEST1 cDNA constructs. The decrease of cell fluorescence caused by addition of NaI was analyzed to calculate anion transport. The fluorescence decay phase was fitted with an exponential function to derive the maximal slope. Maximal slopes were converted into rates of YFP fluorescence at a given time compared with the initial fluorescence.

**Quantitative colocalization analysis**
To analyze colocalization quantitatively, MDCK II cells stably expressing wild-type or mutant bestrophin-1 proteins were grown on filters and double labeled with anti-bestrophin-I and anti-MCT-1 antibodies. After secondary incubation with secondary antibodies conjugated with Alexa Fluor 488 and 543 (Invitrogen), filters were mounted in confocal matrix (Micro Tech Lab, Graz, Austria) and examined with a Zeiss LSM 510 laser scanning confocal microscope. Images were acquired with a Coolsnap HQ CCD camera (VisiRon, Puchheim, Germany) and processed with the Metafluor software (VisiRon). Images were acquired with a Coolsnap HQ CCD camera (VisiRon, Puchheim, Germany) and processed with the Metafluor software (VisiRon). Images were acquired with a Coolsnap HQ CCD camera (VisiRon, Puchheim, Germany) and processed with the Metafluor software (VisiRon). Pearson’s correlation coefficient (PCC) was employed to evaluate colocalization according to Abramoff (Abramoff et al., 2004). The PCC is one of the standard techniques applied in pattern recognition for matching one image to another in order to describe the correlation of the intensity distributions between channels. It only takes into consideration the similarity of shapes between two images, and does not depend upon image pixel values. Values of PCC range from −1 to 1, where −1 indicates no overlap and 1 is a complete colocalization.

**Immunohistochemistry**
For the immunofluorescence experiments, MDCK II cells stably expressing wild-type and mutant bestrophin-1 were grown on permeable supports (Costar Transwells, Fischer Scientific, Schwerte, Germany). Cells were fixed for 10 minutes at room temperature with 4% (w/v) paraformaldehyde. After three washing steps with 1× PBS, cells were permeabilized with blocking–permeabilization solution [10% (v/v) normal goat serum, 0.5% (v/v) Triton X-100 in 1× PBS] for 30 minutes. Cells were then labeled overnight with antibodies against bestrophin-1 (ab2182, mouse monoclonal) (Marmostein et al., 2000), MCT-1 (ab10994, rabbit polyclonal), GM1-30 (alg25269, rabbit polyclonal) (Shaheen et al., 2009), GRP78 BIP (ab21685, rabbit polyclonal) (Dykstra et al., 2010) (all Abcam, Cambridge, UK), LAMP1 and EEA1 (both mouse monoclonal antibodies, BD Biosciences, Heidelberg, Germany) diluted 1:100–1:500 in 2% normal goat serum and 0.1% Triton X-100 in 1× PBS. Antibodies against ZO-1 (rat monoclonal, R26.4C, Developmental Studies Hybridoma Bank, University of Iowa, USA) specifically stain tight junctions. After three additional washing steps, cells were incubated for 1 hour with appropriate secondary antibodies (conjugated with Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 633; Invitrogen). Cells were mounted in confocal matrix (Micro Tech Lab, Graz, Austria) and examined with a confocal microscope LSM510 (Carl Zeiss, Göttingen, Germany).

**Western blotting**
Protein lysates from transiently transfected HEK-293 cells were analyzed by SDS-PAGE and western blotting (Milenkovic et al., 2009) with antibodies against bestrophin-1 diluted 1:5000 (ab2182, Abcam, Cambridge, UK) and β-actin diluted 1:20,000 (Sigma). Quantification of protein expression was achieved by densitometry analysis of western blots with the help of a digital imaging analysis system (Chemimager, Biozym, Oldendorf, Germany).

**Data presentation and statistical analysis**
All experiments were performed independently at least three times. Statistical significance was tested with the unpaired Student’s t-tests, with P<0.05 considered significant.

We thank Elfriede Eckert and Andrea Dannullis for expert technical assistance, Karl Kunzelmann for providing the human P2Y2–His6 expression vector and the rabbit polyclonal antibody against human bestrophin-1, Eugen Kerkhoff for helping with high-resolution microscopy to analyze colocalization with organelles, Miriam Breunig, Pharmazeutische Technologie, Regensburg for providing access to confocal microscopy, Enrique Rodriguez-Boulan for fruitful discussions and helpful suggestions and Myriam Mirza for editing the manuscript. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (STR480/9-2, STR480/10-1, WE1259/16-2 and WE1259/20-1).

**Supplementary material available online at** [jcpsbiologists.org](http://jcps.biologists.org/lookup/suppl/dx.doi.org/10.1242/jcs.085878/D1C1)

**References**
Abraham, M. D., Magelhaes, P. J. and Ram, S. S. (2004). Image processing with imageJ. Biophotonics Int. 11, 36-43.


Fig. S1.
Fig. S2.
Table S1. The missense mutations were generated using pCDNA3-BEST1 as template and the following primers with the changed nucleotide in boldface.

<table>
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<tr>
<th>DNA change</th>
<th>Oligonucleotide sequence (5’-3’)</th>
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<tr>
<td>16A&gt;C</td>
<td>T6P-F: ATGACCATCACCTTACCCCAAGCCAAGTGGGT;</td>
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<td>T6P-R: GCGGCACTTTGCACTTGATGATGCTAT;</td>
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<td>61C&gt;G</td>
<td>L21V-F: CTTTCCGCCCTCTGCTGTGGTGGGCGGG;</td>
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