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INVITED REVIEW

Bestrophins and retinopathies

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Abstract Best vitelliform macular dystrophy (BVMD, also called Best's disease) is a dominantly inherited, juvenile-onset form of macular degeneration, which is characterized by abnormal accumulation of yellow pigment in the outer retina and a depressed electro-oculogram light peak (LP). Over 100 disease-causing mutations in human bestrophin-1 (hBest1) are closely linked to BVMD and several other retinopathies. However, the physiological role of hBest1 and the mechanisms of retinal pathology remain obscure partly because hBest1 has been described as a protein with multiple functions including a Ca^{2+}-activated Cl^- channel, a Ca^{2+} channel regulator, a volume-regulated Cl^- channel, and a HCO_3^- channel. This review focuses on how dysfunction of hBest1 is related to the accumulation of yellow pigment and a decreased LP. The dysfunction of hBest1 as a HCO_3^- channel or a volume-regulated Cl^- channel may be associated with defective regulation of the subretinal fluid or phagocytosis of photoreceptor outer segments by retinal pigment epithelium cells, which may lead to fluid and pigment accumulation.

Keywords Bestrophin · Best vitelliform macular dystrophy · Chloride channel · Retinopathies

Introduction

Best vitelliform macular dystrophy (BVMD), also termed Best's disease, is a dominantly inherited, juvenile-onset form of macular degeneration. Human bestrophin-1 (hBest1) has been identified as the gene responsible for BVMD [44, 55]. So far, over 100 disease-causing mutations in hBest1 have been linked to BVMD and other retinopathies [31]. A key clinical feature of BVMD is a decreased electro-oculogram (EOG) light peak (LP) [2, 14], which is believed to be caused by a defective Ca^{2+}-activated Cl^- channel in the basolateral membrane of the retinal pigment epithelium (RPE) [22, 23, 33] where hBest1 is expressed [25, 41, 44, 55]. Since hBest1 is clearly an anion channel [31, 59, 70, 72, 80, 81, 85, 86] whose function is affected by disease-causing mutations, hBest1 is very likely to be the CaCC responsible for the LP. Thus, it is logical to conclude that loss of anion channel function caused by disease-causing mutations is responsible for the depressed LP in BVMD patients [31, 70]. However, this idea has been seriously challenged by the finding that the LP and CaCCs are normal in mBest1 knockout mice [43]. This challenge is dependent on the presumption that human and mouse retina are the same and that hBest1 and mBest1 function similarly in vivo. However, human and mouse retina have different structures [11, 71], and the identification of the CaCCs responsible for the LP in human and mouse remains ambiguous.

BVMD is a type of retinal degeneration characterized by an abnormal accumulation of yellow pigment in the retinal pigment epithelium [24, 30, 40, 48, 56]. It is mysterious how yellow pigment accumulation occurs in BVMD, although a similar yellow pigment accumulates in age-related macular degeneration (ARMD) [6, 16]. A key component of the yellow pigment in ARMD is a pyridinium bis-retinoid called A2E, which originates from photoreceptor visual pigment. Marmorstein has provided some evidence that A2E is also a component of the yellow pigment in BVMD [3]. The presumed photoreceptor origin

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of the pigment has led to the suggestion that there may be a problem with turnover of photoreceptor outer segments (POS) [17, 30, 31]. Under normal conditions, POS are phagocytosed by the RPE and degraded in the phagolysosome. However, since hBest1 is not located on the apical side of the cell where phagocytosis of POS occurs [41], nor in the phagolysosome [37], it is unlikely that hBest1 is directly involved in POS phagocytosis. It may be that a more general disruption of fluid transport in RPE is linked to the development of BVMD [31]. Recent studies show that hBest1 has a high permeability to HCO_3^- [62], suggesting a potential role of pH regulation by hBest1 in BVMD. Furthermore, the regulation of pH and Ca^{2+} homeostasis by hBest1 is hypothesized to be involved in the regulation of phagocytosis and lysosomal function in RPE [40].

Mutations in Best1 have also been linked to several other forms of retinopathies including adult-onset macular dystrophy [67], autosomal dominant vitreochoidopathy [84], autosomal recessive bestrophinopathy [9], and canine multifocal retinopathy [26]. In addition, recently, it has been suggested that Best1 mutations are also responsible for a subset of retinitis pigmentosa [15]. Thus, the phenotype resulting from Best1 mutations may depend on multiple genetic and environmental factors. In this article, we review the recent studies on hBest1 functioning as a Ca^{2+}-activated Cl^- channel, a Ca^{2+} channel regulator, a volume-regulated Cl^- channel, and a HCO_3^- channel (summarized in Fig. 1).

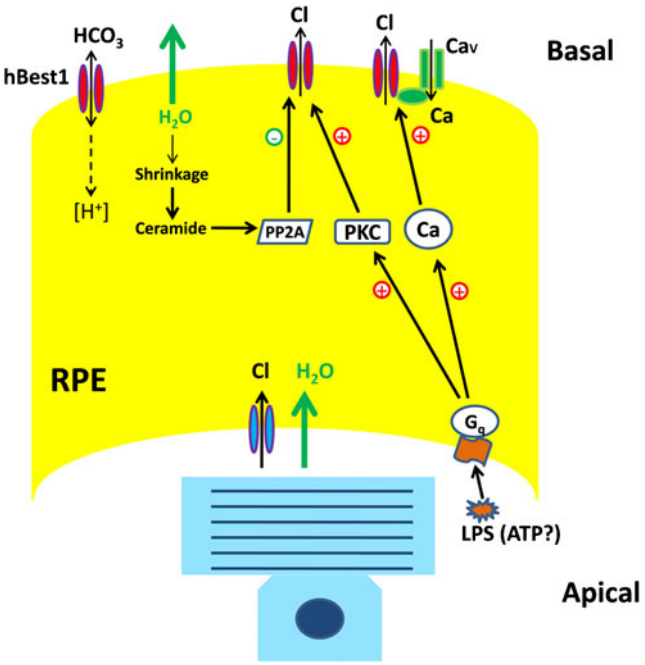

Fig. 1 Summary of hBest1 functions. hBest1 is regulated directly by Ca^{2+}, either through the Ca^{2+} channel or Gq protein-coupled receptors. hBest1 can be phosphorylated by PKC to regulate channel rundown and dephosphorylated by PP2A activated by ceramide in response to hypertonic stress. hBest1 also inhibits Ca^{2+} channel through an SH3 binding domain. The intracellular pH can be regulated by hBest1 through its high permeability of HCO_3^-

Disease-causing mutations of hBest1 are associated with Ca^{2+}-activated Cl^- channel dysfunction and decreased light peak

hBest1 functions as a Ca^{2+}-activated Cl^- channel

hBest1 was first identified as a Ca^{2+}-activated Cl^- channel in 2002 by Sun et al. [70], who found that expression of hBest1 in HEK cells induced Cl^- currents that were regulated by Ca^{2+}. The Ca^{2+} sensitivity of hBest1 is in the physiological range with a K_d of about 200 nM [31, 80]. A Ca^{2+} binding site in hBest1 has been identified at the C-terminus immediately after the last transmembrane domain [36, 80]. A central piece of evidence, that bestrophin is a Cl^- channel, is shown by the findings that the gating and conductance of bestrophins are altered by mutagenesis of critical amino acids [59–61]. For example, the rectification of mBest2 can be altered in opposite directions by substitution of phenylalanine at position 80 with a positively charged arginine or a negatively charged glutamate [59]. Furthermore, mutation in the corresponding phenylalanine in dBest1 alters cation/anion selectivity [13], supporting the idea that bestrophin is an integral part of the channel pore. Using mutagenesis and cysteine accessibility analysis, Qu et al. have shown that the second transmembrane domain (AA69-99) forms the pore and determines ion selectivity [59].

Mutations disrupt hBest1 functional domains

Many of the disease-causing mutations in hBest1 cause Cl^- channel dysfunction [31, 70, 80, 85, 86]. The mutations are clustered in several functional regions of the protein. The highest density of mutations is located in a highly acidic region in the C-terminus immediately after the last transmembrane domain. Disease-causing mutations in this region render hBest1 nonfunctional as a Cl^- channel and many of the mutations are dominant negative [58, 70, 80, 86]. This region is important for Ca^{2+} sensing [80]. In a structure–function analysis of hBest1, all but two (F298W and T307S) of 35 mutations introduced into this region (293–308) caused the channel to be incapable of being activated by Ca^{2+} [80]. Since these mutations do not affect cell surface expression [58, 80], they likely alter Ca^{2+} binding. Qu et al. [58] have shown that the G299E, D301N, and D302N mutations disrupt interaction between the N- and C-termini, which may be important for hBest1 functioning as a multimer in the plasma membrane. This

suggests that the acidic region may participate in multiple important channel functions (Ca^{2+} sensing and subunit interaction).

Another hotspot for disease-causing mutations resides in the second transmembrane domain (TM2), which has been identified as the bestrophin channel pore [59, 61, 72]. Mutations in TM2 likely disrupt the pore structure, which results in a loss of channel function. Another hotspot is in the N-terminus, where some mutations (R19C, R25C, and K30C) have been suggested to alter the interaction between N- and C- termini [58]. The remaining disease-causing mutations are located between TM2 and TM5, but it is not clear how these mutations affect channel function, partly because the topology of bestrophin is controversial in this region. The effects of disease-causing mutations on Cl^- channel function were reviewed in detail in Hartzell et al. [31] and Boon et al. [7].

Association of hBest1 with LP generation

The characteristic feature of BVMD is the diminished LP of the EOG [2, 14]. The LP is believed to be produced by the activation of a Ca^{2+}-activated Cl^- channel in the basolateral membrane of the RPE by a "light peak substance" (LPS), which is released from photoreceptors stimulated by light [21, 39] (Fig. 1). hBest1 is located in the basolateral membrane of the RPE [25, 41, 44, 55], suggesting that hBest1 may be the CaCC responsible for the LP. The diminished LP in BVMD patients is consistent with a defective Cl^- channel function caused by these disease-causing mutations [70, 80, 85, 86].

If hBest1 is the CaCC responsible for the LP, how does the LPS activate hBest1? The identity of the LPS is not known, but ATP is a favorite candidate because extracellular ATP, when applied to the RPE cells, induces an increase in Cl^- conductance across the RPE basal membrane, which is similar to the effect of LPS, and LP- and ATP-evoked responses are blocked by a Cl^- channel blocker, DIDS [31, 54, 69]. It is hypothesized that ATP activates the Gq protein-coupled P2Y receptor, which activates PLC, subsequently producing two second messengers: IP_3 which increases intracellular Ca^{2+} and DAG which activates PKC. Evidence supporting this hypothesis includes the observations that ATP induces an increase in intracellular Ca^{2+}, and ATP-induced response is blocked by P2Y receptor blocker suramin, by ER Ca^{2+}–ATPase inhibitor cyclopiazonic acid, and by Ca^{2+} buffer BAPTA [54].

Although Best1 has been a leading candidate for mediating the LP, there are several other Cl^- channels in the basolateral membrane that should be considered. An increase in intracellular cAMP induces an activation of Cl^- conductance, resulting in a decrease in basolateral membrane resistance, and an increase in transepithelial potentials [29, 47, 75]. CFTP is expressed in RPE cells [29, 47, 75] and can be activated by cAMP-dependent activation of PKA. The LP is reduced in both CFTR knockout mice and mice with ΔF508 mutations, suggesting that CFTR contributes to the generation of LP [78]. Since other RPE-related components of ERG are also reduced, CFTR mutations likely cause a general disruption of RPE cells [78]. However, there are no reports in human CF patients with abnormal LPs. The functions of CFTR in retinopathies have not been well studied yet.

CLC-2 has also been reported to be expressed in RPE cells [29, 75, 76]. Knockout of CLC-2 leads to retinal degeneration, which might result from abnormal transepithelial ion and fluid transport, and subsequent photoreceptor impairment [8]. Furthermore, CLC-2-like currents have been recorded from RPE cells [29], and RPE short-circuit currents are reduced in CLC-2 knockout mice [8]. However, the precise role of CLC-2 in RPE ion and fluid transports largely remains unknown.

The newly discovered anoctamin family provides another candidate channel that might be responsible for the LP [10, 32, 66, 83]. There are ten anoctamins and Ano1 and 2 have been shown to be Ca^{2+}-activated Cl^- channels. The CaCC currents that have been recorded in RPE cells resemble Ano1 and Ano2 currents expressed in HEK cells [89], but the expression of Anos in RPE are unexplored and their role as CaCCs responsible for LP is only speculative. Therefore, the identification and function of CaCCs in RPE need to be further explored.

The conclusion that the LP is generated by the hBest1 Cl^- channel is disfavored by the finding that patients with certain disease-causing mutations have normal or near-normal LPs. These mutations include T216T, A243V, ΔI295, D312N, and L567F [86]. The two mutations T216I and L567F exhibit wild-type-like Cl^- currents, and may have been incorrectly identified as disease-causing mutations because no family history of BVMD was shown [86]. The A243V, ΔI295, and D312N mutations clearly show a defect in hBest1 Cl^- channel function [85, 86], and most patients, except a few, have depressed LPs. There are several possible explanations why some patients with these mutations have normal LPs. Although it is often said that these mutations exhibit reduced penetrance, the possibility exists that these mutations are actually null mutations that sometimes are disease causing in the heterozygous state. This is supported by the finding that the D312N mutation in a compound homogygous condition with another recessive mutation (M325T) produces retinopathy associated with a severely diminished LP [9]. Alternatively, the patients with hBest1 mutations who have normal LPs could be explained if these individuals have some type of suppressor mutation or compensatory response.

The most challenging argument against hBest1 as the CaCC responsible for LP generation comes from mBest1 knockout mice, which have normal CaCCs in the RPE [43]. The LP in these animals is altered, but in a manner opposite to that predicted if mBest1 was generating the LP; namely, the relationship between the amplitude of the LP and light intensity is shifted to the left. Furthermore, mBest1 knockout mice do not exhibit an ocular disease [43], further suggesting that mBest1 is not the CaCC responsible for the LP in the mouse RPE. However, recently, it has been shown that a mouse harboring the W93C mutation does have an eye disease that resembles BVMD [89]. The LP in W93C animals is enhanced at low light intensities and reduced in the middle of the intensity range, but the CaCC currents in the W93C RPE cells are normal. This suggests that, although mBest1 is not responsible for the RPE CaCC, it somehow regulates the channel that is responsible for the LP. The difference in phenotype between the Best1 knockout and the W93C mutant mouse suggests that BVMD is caused by gain-of-function mutations in Best1. However, this conclusion is hard to reconcile with the clear loss of Cl^- channel function associated with these mutations [70, 86] and the loss of the LP in humans with autosomal recessive bestrophinopathy (ARB) which is thought to be caused by null mutations in hBest1. It is possible that hBest1 and mBest1 have different functions, but this explanation is disfavored because mBest1 functions as a CaCC when expressed heterologously [51] (but see below) and shares high homology with hBest1 including the Ca^{2+} binding region and the pore. However, it should be pointed out that disease-causing mutations have been evaluated only in hBest1 and not in mBest1.

It has been suggested that Best1 plays a role in the regulation of intracellular Ca^{2+}, which is proposed to be important in the pathogenesis of BVMD [40, 88]. This hypothesis is supported by the finding that hBest1 can inhibit voltage-gated Ca^{2+} channels [64, 87]. However, for reasons discussed below, it seems unlikely that mutations in hBest1 result in changes in intracellular Ca^{2+} solely through regulation of $Ca_V1.3$. Furthermore, unlike hBest1, mBest1 does not inhibit $Ca_V1.3$ [87], further suggesting that mBest1 may not function like hBest1. The finding that hBest1 is highly permeant to HCO_3^- [62] has raised the possibility that some of the effects of Best1 mutations may be related to changes in pH, but this has not yet been experimentally tested.

It should be noted that the mouse LP is considerably smaller than the cat, which has served as a model for the LP (Fig. 2). The LPs in human and cat are much larger than the C-wave [39], whereas the LP in mouse is usually smaller than the C-wave [43]. In rats, the LP is very small, if not completely absent [40]. This suggests that the ionic mechanism underlying the LP in human and rodents may

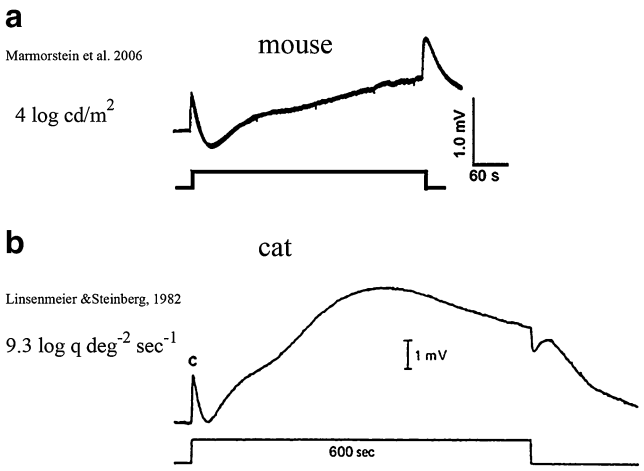

Fig. 2 The difference in LP in mice and cats. The dc-ERG was recorded **a** in mice to a 7-min light stimulus [43] or **b** in cats to a 10-min light stimulus [39]. The LP is smaller than the C-wave in mice, while the LP in cats is much larger than the C-wave

be different. Furthermore, in our hands, transient expression of mBest1 in HEK cells does not induce Ca^{2+}-activated Cl^- currents (Fig. 3). This result is surprising, given the fact that two other laboratories have reported that mBest1 does induce currents [51, 53]. Although we consistently observe robust expression of currents with hBest1 and mBest2, we have never seen currents with mBest1. The differences

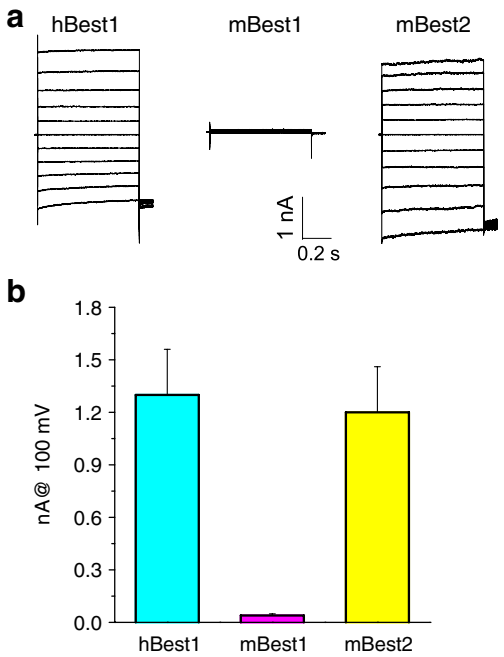

Fig. 3 Expression of hBest1, mBest2 and mBest1 in HEK cells. **a** Representative whole-cell current traces recorded from cells transfected with hBest1, mBest1, and mBest2 in the presence of 10 μM Ca^{2+}. **b** Current amplitudes at 100 mV for hBest1 (n=13), mBest1 (n=10), and mBest2 (n=9)

between our results and those of other labs may come from the different cell lines which may express different levels of mBest1 regulatory molecules, such as kinases and regulatory subunits. These molecules may affect mBest1 functional expression and regulation. However, this is only speculative and has not been explored.

Although there is evidence arguing against bestrophin as the CaCC responsible for LP generation, hBest1 remains a candidate since a majority of BVMD-causing mutations cause defects in Cl^- channel function [31, 70, 80, 85, 86]. Furthermore, like BVMD patients, patients of autosomal recessive bestrophinopathy also show depressed LPs, which is consistent with ARB-associated mutations causing defective Cl^- channel function [9]. Unlike BVMD mutations, ARB mutations are not dominant negative, and people with heterozygous mutations in hBest1 have normal LPs [9]. This suggests that the LP is correlated with Cl^- channel function of hBest1. Mutations in Best1 cause another recessive retinopathy in dog called canine mutifocal retinopathy (CMR) [26]. However, there is no report about LPs in CMR dogs. Mutations in hBest1 cause both dominant and recessive diseases, which are similar to myotonia-causing mutations in CLC-1. In recessive myotonia, some truncation mutations are unable to produce dominant negative effects due to their inability to form a dimer with wild-type subunits [34]. Similarly, a C73X stop mutation in CMR [26] and a R200X stop mutation in ABR [9] could also result from their inability to associate with wild-type Best1. Other recessive mutations may also disrupt formation of multimers with wild-type subunits. The D312N, V317M, and M325T mutations that cause ABR are located in the EF hand-like structure responsible for Ca^{2+} binding. Other mutations in this region have been shown to disrupt interaction between N- and C-termini and suggest that this region may also be important in channel multimerization.

In summary, the fact that mBest1 knockout mice have normal LPs clearly show that mBest1 is not the CaCC responsible for mouse LPs [43]. However, this finding does not exclude the possibility that hBest1 is the CaCC responsible for the human LP since mBest1 does not mimic the function of hBestl.

hBest1 regulation by phosphorylation

The hBest1 C-terminus contains an EF hand-like structure that binds Ca^{2+} [80] and a PKC phosphorylation site (S358) that is believed to regulate channel function [81]. Thus, increases in intracellular Ca^{2+} probably activate hBest1 by binding to the EF hand-like structure. Following Ca^{2+} activation, hBest1 undergoes a time-dependent decrease in current amplitude, a process called rundown. The rundown can be inhibited by phosphorylation of the PKC phosphorylation site (S358) [81] (Fig. 1). Therefore, hBest1 currents are activated by Ca^{2+}, but PKC-dependent phosphorylation may be necessary to maintain the current. Although rundown has so far been studied only with hBest1 heterologously expressed in HEK cells, it is possible that the mechanism responsible for rundown also operates in native RPE cells. We hypothesize that the processes underlying rundown modulates the timing of current turn-on and turn-off.

The PKC phosphorylation site (S358) is dephosphorylated by PP2A, which increases hBest1 channel rundown. This suggests that PP2A could switch off the LP signal, especially in the dark when less LPS is released and thus less PKC is activated. Therefore, regulation of hBest1 phosphorylation by PKC and PP2A may underlie the time course of the LP. Though this hypothesis is based on the observations from heterologous expression of hBest1 in HEK cells, it may be physiologically relevant since hBest1 can be coimmunoprecipitated with PP2A from human RPE cells [42].

The PKC phosphorylation site (S358) appears not to be a PKA phosporylation site since both cAMP and forskolin show no effects on hBest1 [81]. However, S358 is likely a substrate for p21-activated kinase PAK2 since S358A mutation eliminates the phosphorylation in vitro by PAK2 [5]. Barro-Soria et al. [5] suggest that hBest1 facilitates Ca^{2+} release from ER by acting as a counterion pathway. PAK2 phosphorylation of hBest1 further promotes Ca^{2+} release from ER and subsequently enhances Ca^{2+}-activated Cl^- and K^+ channels. Since PAK2 shares the same phosphorylation site (S358) in hBest1 as PKC, PAK2 increases hBest1 function likely through inhibition of hBest1channel rundown.

Since hBest1 functions can be regulated by kinases such as PKC and PAK2, it is likely that hBest1 function can be coupled to many types of receptors. However, until now, most studies show that bestrophins are activated by Ca^{2+} in the patch pipettes in whole-cell recordings and not by GPCRs. Further experiments should be done to test whether bestrophins can be activated by GPCR through agonists in either overexpressing cells or native RPE cells. Furthermore, hBest1 can be dephosphorylated by PP2A through ceramide, which is coupled to several stress stimuli such as hyperosmotic stress [81].

Regulator of voltage-gated Ca^{2+} channels

In addition to functioning as a Cl^- channel, Best1 has been shown to influence intracellular Ca^{2+} signaling by several different mechanisms, but results are often contradictory and do not simply explain the pathogenesis of BVMD. hBest1 can regulate voltage-gated Ca^{2+} channels [9, 64, 87] (Fig. 1). Both Rosenthal et al. [64] and Burgess et al. [9]

reported that hBest1 accelerated the activation of $Ca_V1.3$ currents, but Yu et al. [87] did not observe significant changes in current kinetics. Rather, Yu et al. [87] reported that hBest1 expression significantly reduced the amplitude of $Ca_V1.3$ currents. This effect is explained by an SH3 binding domain (330–350) that binds Ca_V β subunits to regulate $Ca_V1.3$. The hBest1 residues P330 and P334 are critical for the regulation since mutation of P330 and P334 abolishes this effect [87]. This SH3 binding domain lies between the Ca^{2+} binding site and a regulatory domain critical for channel rundown [80]. Some disease-causing mutations, G299R, G222E, and A146K, partly eliminate the inhibition of $Ca_V1.3$ by hBest1 [87]. However, other disease-causing mutants, D312N and R92S, have the same effect on $Ca_V1.3$ as wild type [87]. The effects of hBest1 disease-causing mutants on the activation and inactivation of Ca_V currents also seem inconsistent or at least difficult to reconcile with a scheme involving regulation of Ca_V channels as a primary mechanism of Best1 action. Although wild-type hBest1 accelerates Ca_V activation, the W93C mutation slows $Ca_V1.3$ activation and inactivation, the R213C mutation accelerates inactivation [64], and the R141H mutation eliminates the accelerating effect of hBest1 on $Ca_V1.3$ activation [9]. It is not obvious how these changes in the regulation of Ca_V channels caused by mutations in hBest1 may be related to the disease phenotype. The inconsistent effects of different mutations on $Ca_V1.3$ suggest that BVMD is unlikely to be caused by changes in $Ca_V1.3$ function.

If hBest1 regulates Ca_V function through interaction of Ca_V β subunits, the question arises whether hBest1 function is affected by Ca_V β binding. This possibility is supported by the finding that the nonfunctional hBest1 350X mutant, which has the C-terminus deleted beyond amino acid 350 but contains the Ca^{2+} binding site and the SH3 binding domain, can be activated by Ca^{2+} when coexpressed with Ca^{2+} channels in HEK cells (Fig. 4). This suggests that the ligation of the SH3 domain could alter the conformation of hBest1.

Regardless of the role of Best1, voltage-gated Ca^{2+} channels clearly play a role in mouse LP generation [43]. The LP is reduced by the Ca^{2+} channel blocker nimodipine [43], and deletion of the genes for $Ca_V1.3$ [43] or β4 eliminates the light peak [79]. Whether Ca^{2+} influx through the Ca_V channel is involved in CaCC activation and generation of the LP is unknown. Alternatively, interaction of $Ca_V1.3$ with Best1 may alter Best1 function. However, unlike hBest1, mBest1 does not inhibit $Ca_V1.3$ [87], suggesting mBest1 does not directly regulate the Ca^{2+} channel to modulate intracellular Ca^{2+} concentration.

In addition, hBest1 also regulates Ca^{2+} release from the ER. RPE cells from mBest1 knockout mice show a greater increase in intracellular Ca^{2+} concentration in response to ATP than wild-type mice [40, 89], suggesting that mBest1 has an inhibitory effect on the release of Ca^{2+} from internal stores. However, Barro-Soria et al. [5] suggest that hBest1 is an ER-resident protein that *facilitates* Ca^{2+} release from ER by acting as a counterion pathway. A possible explanation for this discrepancy might be the differences between mBest1 and hBest1 or the use of knockout mice vs. transfected cells.

It is unclear how mouse models can be extrapolated to the function of human RPE, since mice, unlike human, have no obvious macula, and BVMD is clearly a macular disease. Mouse retina contains predominantly rod photoreceptors with only 2% to 3% of cone photoreceptors [11, 71]. Thus, mouse retina is more similar to human peripheral retina than macula, where cone photoreceptors are concentrated. It is unknown why BVMD primarily affects human macula since hBest1 is expressed more in peripheral retina than in macula [49]. In addition, the high density of cone photoreceptors in the macula may require high phagocytic function of RPE in response to light.

Lipofuscin accumulation in BVMD

Clinical and histopathological analysis of BVMD patients' eyes shows an accumulation of yellow pigment, often referred to as lipofuscin, in the RPE [24, 31, 40, 48, 56]. The major fluorescent component of lipofuscin is A2E (*N*-retinylidene-*N*-retinylethanolamine), which is the metabolite of retinal, a visual pigment from photoreceptors. Accumulation of A2E has been found to promote apoptosis of RPE [68] and to disrupt lysosomal function in the RPE [18]. The photoreceptor origin of A2E suggests that accumulation of lipofuscin may be related to a problem with the phagocytosis of photoreceptor and/or phagolysosome function [17, 30, 31, 40]. However, it is unclear how dysfunction of hBest1 is related to the accumulation of lipofuscin.

Although a large fraction of hBest1 is expressed intracellularly [70, 80, 85, 86], hBest1 is concentrated in the ER [5] and is not coexpressed with lysosomal markers [37]. Furthermore, hBest1 is expressed on the basolateral side of the RPE [41, 44], not the apical side where phagocytosis of POS occurs. Thus, it is unlikely that hBest1 directly participates in the regulation of phagocytosis or phagolysosome function, but it may have an indirect effect. A common feature of diseases caused by Best1 mutations is edema and accumulation of fluid in the outer retina [46, 56, 57, 73]. Disruption of fluid transport in RPE due to the dysfunction of hBest1is hypothesized to be the mechanism of BVMD [31]. Here, we review evidence that hBest1 functions as a volume-regulated Cl^- channel and a HCO_3^- channel.

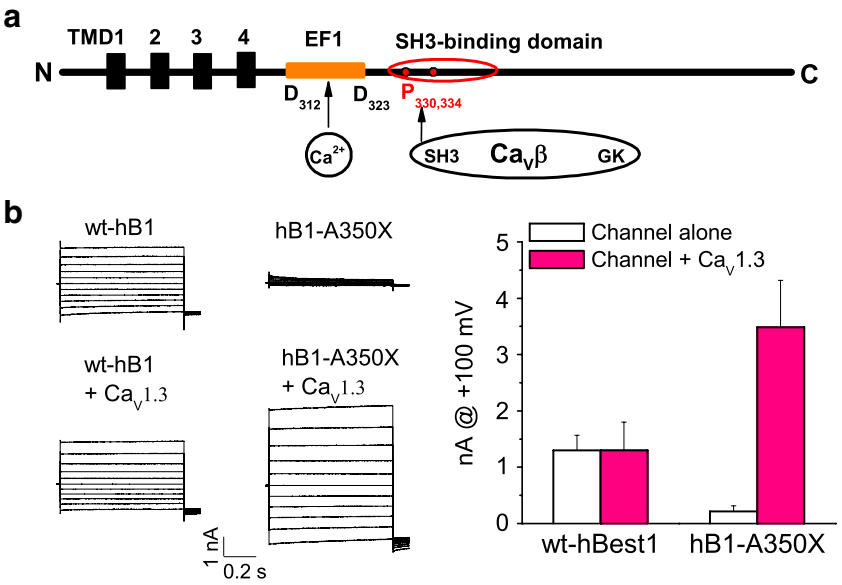

Fig. 4 Regulation of hBest1 by Ca^{2+} channel through an SH3 binding domain. **a** Model of functional domains in hBest1. Orange EF1 is an EF hand-like structure (D312–D323) critical for Ca^{2+} binding. The *red circle* represents an SH3 binding domain, which binds to β subunits of $Ca_V1.3$ and inhibits $Ca_V1.3$. Two prolines (P330 and P334 in *red*) are critical for this regulation. Representative current traces (**b**) and current amplitudes (**c**) recorded from cells transfected with wild-type hBest1 or the 350X mutant with and without $Ca_V1.3$. The 350X mutation was made by introducing a stop codon at position 350, thus deleting C-terminus beyond 350 but containing Ca^{2+} binding site and the SH3 binding domain. The 350X cannot be activated by Ca^{2+}, but this nonfunctional channel was rescued by $Ca_V1.3$

Volume-regulated anion channel

Bestrophins including hBest1, mBest2, and dBest1 are regulated by cell volume [12, 13, 19, 81]. dBest1 has been demonstrated to be a native volume-regulated Cl^- channel in *Drosophila* S2 cells [12, 13]. The dBest1 Cl^- currents are activated by hyposmolarity and inhibited by hyperosmolarity. The osmotically sensitive currents are knocked down by dBest1 RNAi and can be rescued by transfection of both wild-type dBest1 and a mutant dBest1 with altered ion permeability [12, 13]. However, bestrophin is not the classic volume-regulated anion channel (VRAC) in mammals because the VRAC is unaffected in macrophages from mBest1 and mBest2 knockout mice [13]. Furthermore, the classic VRAC currents show outward rectification and inactivation at positive potentials in a time-dependent manner, while bestrophins exhibit little outward rectification and no inactivation at positive potentials. Thus, bestrophins may be one of the several Cl^- channels that play a role in the regulation of cell volume.

Bestrophins can be regulated by both Ca^{2+} and cell volume. The two pathways can be independent of but can interact with each other [12]. Ca^{2+} potentiates dBest1 current amplitudes activated by increases in cell volume [12]. hBest1 currents activated by Ca^{2+} can be inhibited by decreases in cell volume [19, 81] (Fig. 1). Furthermore, increases in cell volume often result in increases in intracellular Ca^{2+} [45]. Therefore, hBest1 could be activated by hyposmotic swelling through Ca^{2+}, since hBest1 currents are activated by intracellular Ca^{2+} with an EC_{50} of 140 nM [80], which can be reached during cell swelling in response to hyposmotic treatment. The direct studies of hBest1 activation by hyposmolarity are thwarted by endogenous VRACs, which are ubiquitously expressed in cell lines such as HEK.

The mechanism underlying the regulation of hBest1 by cell volume has been studied in a heterologous expression system [19, 81]. hBest1 currents expressed in HEK, HeLa, and ARPE-19 cell lines are strongly inhibited by small increases in extracellular osmolarity [19, 81]. The inhibition of hBest1 by hypertonic solution is mediated through dephosphorylation of a PKC phosphorylation site (S358) [81] (Fig. 1). Furthermore, ceramide, a PP2A activator, mimics the effects of a hypertonic solution, and a neutral sphingomyelinase inhibitor blocks the effects of hypertonic stress, suggesting that hypertonic stress activates neutral sphingomyelinase to release ceramide, which dephosphorylates S358 to accelerate channel rundown [81].

It is unclear how regulation of hBest1 by hypertonic solution in a heterologous expression system could be extrapolated to function of hBest1 in vivo. However, it is interesting that, like hBest1 currents, the LP is inhibited by a hypertonic condition [50]. In addition, hBest1 has been identified to be coimmunoprecipitated with PP2A in human RPE cells [42], further suggesting that dephosphorylation of hBest1 by PP2A may play an important role in hypertonic inhibition of hBest1 in vivo.

One very speculative idea is presented here. RPE cells play an important role in removing the daily shed

photoreceptor outer segments (POS) through phagocytosis, which is crucial for photoreceptor survival. Just before POS are shed, a large amount of osmolytes such taurine, glutamate, aspartate, and glycine are leaked from POS, accumulated in the extracellular space, and subsequently transported into RPE. RPE will undergo swelling as water follows the uptake of osmolytes into the cells [31]. Cell swelling could activate hBest1 or classical VRACs either directly or through an increase of intracellular Ca^{2+}. Opening of Cl^- channels will cause a Cl^- efflux, which could cause the cells to undergo regulatory volume decrease (RVD) and shrink. The mechanical force following cell swelling and shrinkage could play a role in RPE phagocytosis. Shrinkage will subsequently decrease Cl^- efflux by inhibiting hBest1, possibly through the production of ceramide (see below) [81]. Then, RPE cells will undergo a process of regulatory volume increase (RVI) to resume their original cell volumes [38]. Thus, hBest1 is hypothesized to control the POS phagocytic process through regulating cell volume (summarized in Fig. 5).

Ceramide may play an important role in hBest1 regulation and in the response of RPE to osmotic changes. Ceramide mimics the effect of hypertonic stress, and a neutral sphingomyelinase inhibitor blocks the hypertonic inhibition of hBest1 current, suggesting that hyperosmolarity-induced cell shrinkage could activate sphingomyelinase to release cermide, which mediates the hypertonic inhibition of hBest1[81]. Inhibition of hBest1 Cl^- current will result in the accumulation of Cl^- and water, which causes cells to undergo RVI to recover their initial volume. Therefore, hBest1 plays a key role in cell volume regulation through ceramide. Ceramide, a lipid signaling molecule mediating several cellular responses to stress stimuli such as cytokines and oxidative stress [27, 28, 35, 52, 65], promotes RPE apoptosis [4] and is implicated in photoreceptor apoptosis during several kinds of retinopathies including macular degeneration, diabetic retinopathy, and retinal detachment [1, 20, 63, 82].

HCO_3^- channel function of bestrophins

Bestrophins including hBest1-4 and mBest2 are highly permeable to HCO_3^- [62]. The relative permeability of HCO_3^- to Cl^- is larger than most of other anion channels such CFTR, CLC, and ligand-gated anion channels. It is likely that bestrophins conduct both Cl^- and HCO_3^- through the same pore since the mutation of a pore residue (V78C) changes the relative HCO_3^- permeability [62]. Furthermore, the disease-causing mutations (Y85H, R92C, and W93C) abolish both Cl^- and HCO_3^- currents equally, and Ca^{2+} is required for activation of both Cl^- and HCO_3^- currents. Thus, it is possible that, physiologically, bestrophins function as both Cl^- channels and HCO_3^- channels (Fig. 1).

Since hBest1 disease-causing mutations also abolish the conductance of HCO_3^-, it raises the question whether abnormal HCO_3^- transport in the RPE may contribute to Best's disease. Metabolically active photoreceptors produce large amounts of CO_2 which could lower the pH to inhibit photoreceptor function [74, 77]. Thus, removal of HCO_3^- from the subretinal space is important for photoreceptor function. Furthermore, altered intracellular pH in RPE cells

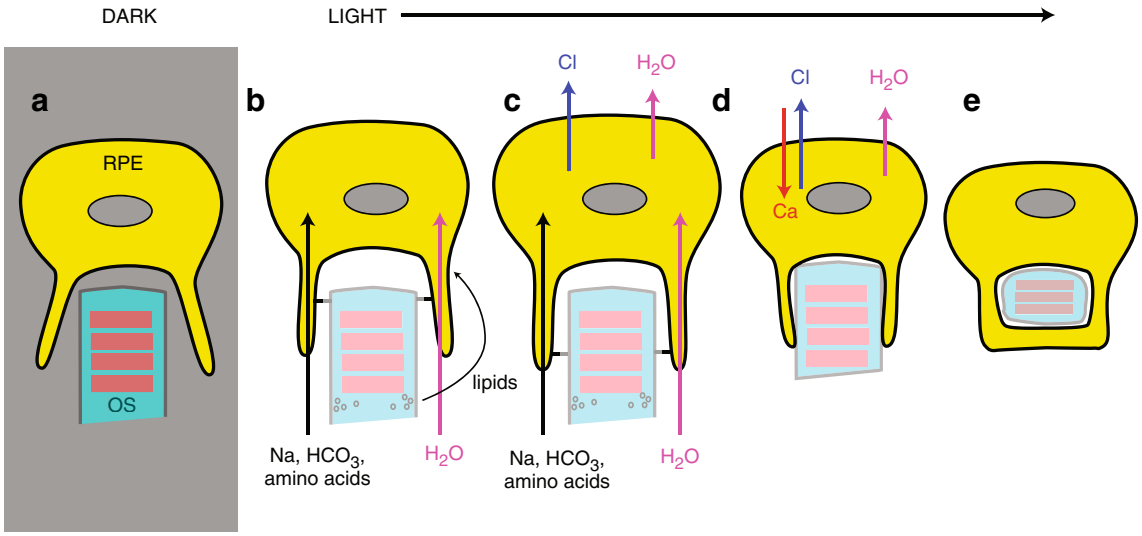

Fig. 5 Model of hBest1 as a volume-regulated Cl^- channel in the regulation of phagocytosis of POS. **a** In the dark, RPE and photoreceptors are not closely attached. **b** After the lights turn on, a large amount of osmolytes such as glutamate, aspartate, and glycine is leaked from POS, accumulates in the extracellular space, and subsequently transported into RPE. Water is passively absorbed into RPE. **c** RPE swells as water enters into the cells. Cell swelling could activate hBest1. **d** Opening of hBest1 Cl^- channels will cause a Cl^- efflux, which could cause the cells to undergo RVD and shrink. Photoreceptors are attached to RPE cells following cell swelling and shrinkage. **e** Phagocytosis of POS into RPE

is hypothesized to be important in engulfment of POS, maturation and acidification of lysosome, and delivery of lysosomal enzyme [40]. Altered phagocytosis and phagolysomal function is proposed to increase the formation of A2E and other lipofuscin substances [40]. Therefore, dysfunction of hBest1 as HCO_3^- channels may result in abnormal accumulation of lipofuscin in BVMD.

Conclusions and significance

Bestrophins have clearly been shown to be CaCCs in overexpressing cells, but evidence in native RPE cells is inconclusive. The CaCC in mouse RPE cells is not changed in Best1 knockout mice and W93C Best1 knock-in mice [43, 89]. Further studies to identify how hBest1 is regulated by these signaling molecules in RPE cell as well as in animal model would be helpful in the understanding of bestrophin-related retinopathies.

Bestrophin has a large HCO_3^- conductance, suggesting that it may have a physiological role as a HCO_3^- channel. mBest2 has been identified as a HCO_3^- channel in the distal colon [88]. Whether bestrophin functions as HCO_3^- channel in RPE cells is to be determined. Direct measurement of HCO_3^- currents as well as pH in RPE cells from wild-type and knockout mice could help to elucidate the physiological function of bestrophins in RPE.

It is still unclear how dysfunction of hBest1 causes the pathology of BVMD such as lipofuscin accumulation. Recently, the BVMD-causing mutation W93C was introduced in mice [89]. These mice show a phenotype resembling BVMD and have a suppressed response to ATP-induced increase in intracellular Ca^{2+} concentration. It is unclear why knock-in of this mutation causes lipofuscin accumulation and how knock-in of this mutation alters Ca^{2+} signaling. Identification of the signaling pathway involving bestrophin is critical for unraveling the pathology of BVMD. In addition, mutations in Best1 have also been linked to several other forms of retinopathies including adult-onset macular dystrophy [67], autosomal dominant vitreochoidopathy [85], autosomal recessive bestrophinopathy [9], and canine multifocal retinopathy [26]. This suggests that dysfunction of hBest1 in different retinopathies can cause different disease phenotypes, and it is likely that multiple genetic and environmental factors may be involved in these retinopathies. Discovery of the genes and/or proteins which interact with bestrophins would be a potential target for therapy of these bestrophin-related retinopathies.

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