Modification of mutant bestrophin-1 processing to prevent retinal degeneration

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2015

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Faculty of Medical and Human Sciences
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<tbody>
<tr>
<td>A</td>
<td>ampere (SI unit of electrical current)</td>
</tr>
<tr>
<td>ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ad</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>ADVIRC</td>
<td>autosomal dominant vitreoretinoc choroidopathy</td>
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<td>AMD</td>
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<td>adult onset vitelliform dystrophy</td>
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<td>A2E</td>
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<tr>
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<td>bovine serum albumin</td>
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<td>cel</td>
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<td>ciliary neurotrophic factor</td>
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<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
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<td>OMIM</td>
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<td>OST</td>
<td>Oligosaccharyl transferase</td>
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<td>platelet-derived growth factor</td>
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<td>pigment epithelium-derived factor</td>
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<td>Proteasome inhibitor II</td>
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<td>protein phosphatase 2A</td>
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<td>Quality control</td>
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<td>ribonuclease</td>
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<td>retinitis pigmentosa</td>
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<td>RPE</td>
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<td>RT-PCR</td>
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<td>SDM</td>
<td>site directed mutagenesis</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SOCE</td>
<td>Store-Operated Calcium Entry</td>
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<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>$T_k$</td>
<td>absolute temperature (Kelvin)</td>
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<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
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<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
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<tr>
<td>TEMED</td>
<td>N,N,N’N’-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloprotease</td>
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<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide</td>
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<tr>
<td>TMD</td>
<td>transmembrane domain</td>
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<td>TUDCA</td>
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<td>WB</td>
<td>western blot</td>
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<tr>
<td>U</td>
<td>unit of enzyme</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
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<td>UTR</td>
<td>untranslated region</td>
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<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>v/v</td>
<td>volume in volume</td>
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<td>VDCC</td>
<td>voltage-dependant calcium channel</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>W</td>
<td>watts (SI unit of power)</td>
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<td>world health organisation</td>
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<td>WT</td>
<td>wildtype</td>
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<td>w/v</td>
<td>weight in volume</td>
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<td>ZO</td>
<td>zonula occludens</td>
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<td>17-AAG</td>
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**Magnitudes**

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<td>μ</td>
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<td>m</td>
<td>milli</td>
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<tr>
<td>K</td>
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Abstract

Name of the University: The University of Manchester
Candidate’s full name: Carolina Uggenti
Degree Title: PhD in Medicine
Thesis Title:
Modification of mutant bestrophin-1 processing to prevent retinal degeneration
Date: 2015

Bestrophin-1 is a homopentameric Ca\(^{2+}\)-gated anion channel which localises to the basolateral plasma membrane of retinal pigment epithelium (RPE) cells. Homozygous and compound heterozygous mutations in the \(BEST1\) gene are associated with autosomal recessive bestrophinopathy (ARB), a retinopathy characterised by altered electrooculogram (EOG), deposits in the retina, and is often associated with the risk of developing angle-closure glaucoma. The mechanism by which mutations in bestrophin-1 cause disease remains unknown.

Expression of four ARB-causing bestrophin-1 proteins in polarised MDCKII cells, a cell model for RPE, results in mutant proteins mislocalisation and degradation. Furthermore, when the ability of the mutant proteins to conduct Cl\(^-\) ions was investigated in HEK293 cells by whole-cell patch-clamp, a reduction in the Cl\(^-\) current was observed in all mutants compared to the WT.

The use of a combination of the small molecules bortezomib and 4-phenylbutyrate (4PBA) successfully restored the expression and trafficking of all four ARB-causing bestrophin-1 proteins. Importantly, 4PBA was also able to restore the ability of the mutant channel to conduct Cl\(^-\) ions. Biotinylation of cell surface proteins shows that the number of active channels at the plasma membrane of HEK293 cells increases following 4PBA treatment. The functional rescue achieved with 4PBA supports the hypothesis that ARB-associated missense mutations reduce the number of functional channels that reach the cell membrane rather than altering other aspects of channel function.

The results presented in this thesis suggest that 4PBA may be a promising therapy for the treatment of ARB and the other bestrophinopathies resulting from missense mutations in \(BEST1\), particularly as 4PBA is already approved for long-term use in infants and adults. These finding also pave the way for the use of small molecule therapies to treat conformational diseases caused by mutation in other protein expressed in the RPE.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Carolina Marseglia and Angelo Raffaele Profilo

who never failed to give me the strength to take on new challenges and to face overwhelming obstacles; the wisdom to value and treasure the little things and the bravery to look at the future with “the scepticism of the scientist and the optimism of the creative”. 
1 Introduction

Human bestrophin-1 is a transmembrane protein expressed in the retinal pigment epithelium (RPE) in the eye and it contributes to the RPE functions by maintaining ion homeostasis. Mutations in the bestrophin-1 gene, BEST1, have been linked to a range of retinal dystrophies and are collectively known as bestrophinopathies, which include autosomal dominant and autosomal recessive disease. There is a large amount of phenotypic heterogeneity associated with the bestrophinopathies although the pathogenic mechanism behind these diseases is not yet fully understood. Moreover there is currently no effective treatment for these disorders. In nearly all cases, mutations lead to a loss of anion channel conductance that, as a consequence, affects several functions of the RPE. An altered electrooculogram (EOG) and the accumulation of a fatty yellow pigment, lipofuscin, are common to all bestrophinopathies. With the RPE no longer able to support the photoreceptor layer, the RPE/photoreceptor structure and this could have major consequences on central vision in late adolescence or adulthood. Despite the controversy that surrounds the functional role of bestrophin-1 in the RPE, and although the pathogenic mechanism of bestrophinopathies is still unclear, recent work has proved that mutations in BEST1 affect trafficking and localization of bestrophin-1 (Davidson et al. 2011; Milenkovic et al. 2011; Johnson et al. 2014; Doumanov et al. 2013). Davidson et al. demonstrated that different bestrophin-1 mutations have different varying degrees of mislocalisation (Davidson, Millar et al. 2011). By using polarized MDCKII cells as a model system they observed that some mutants were retained in the ER and were degraded by the proteasome. However others showed a punctuate pattern of localisation in the cytoplasmic suggesting that some mutant bestrophin-1 proteins might localise to the lysosome and perhaps be degraded by a lysosomal-dependent route. Interestingly some mutants seemed to be correctly trafficked to the cell basolateral membrane. All mutants however had reduced chloride conductance (Davidson et al. 2011). These findings suggest that the disease might be caused by a loss of function due to either mutations that alter the gating property of the
channel (Dickson et al. 2014) or by mutations that interfere with the proper folding and/or trafficking of the channel, thus causing the activation of the cell protein quality control system (QC) which firstly attempts to promote correct folding and then targets misfolded proteins for degradation when rescue is not possible.

Mutant proteins that are retained and/or degraded by QC are often associated with loss-of-function or gain-of-function conditions. A well-known example of a loss-of-function phenotype is cystic fibrosis (CF). Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause the protein to misfold and to be retained in the endoplasmic reticulum (ER) (Yu et al. 2011) where it is unable to fulfil its role as a chloride ion transporter in the epithelial cell membranes of several organs, in particular the lungs and pancreas. The dysregulation of epithelial fluid transport results in an accumulation of thickened mucus in the lung, infections and pancreatic insufficiency characteristic of cystic fibrosis. However defects in QC may also lead to gain-of-function conditions. If disposal is not efficient, the cell may initiate a stress signalling network known as the unfolded protein response (UPR) in an attempt to overcome the stress caused by the accumulation of aberrant proteins. If the stressful condition persists for too long, it will eventually lead to cell death thus triggering severe damage to tissues and organs. An interesting case is the pathology caused by the mutation of α1-antitrypsin (A1AT). A1AT is produced in the liver and delivered to the lungs where it is needed to balance protease function and prevent lung damage. Mutant A1AT is not secreted from liver cells and accumulates thus forming intracellular deposits. The loss-of-function phenotype observed at the level of a patient’s lungs (emphysema) can, therefore, also be accompanied by a gain-of-toxic-function phenotype at the level of the liver (liver cirrhosis) due to its accumulation.

It is highly likely that a reduced level of bestrophin-1 due to its mislocalisation/degradation is unable to sustain its normal physiological function, thus explaining the loss of function phenotype found in some bestrophinopathies such as autosomal recessive bestrophinopathy (ARB). What is less clear is which system is involved in the recognition of the mutant protein and if there is a way to promote proper folding, thus restoring correct trafficking and function, of these disease-causing bestrophin-1 mutants.
In the last few years, novel strategies that use small molecules to promote the correct folding of mutant proteins have been proposed to treat loss- or gain-of-function diseases as alternative to, or as support of, gene therapy. Small molecules can be classed into separate groups, based on their activity: chemical and pharmacological chaperones and proteostasis regulators. Chemical chaperones, such as glycerol and trehalose, are non-specific molecules that establish non-covalent interactions with nascent proteins to stabilise them, reduce aggregation and facilitate their folding and trafficking. Pharmacological chaperons, such as 11- and 9- cis-retinal, are a subset of chemical chaperones but their action is more specific as they only interact with their targets. An additional class of small molecules are the proteostasis regulators that modify the innate biology of the cell altering, for example, the calcium level (Silva et al. 2013) or interfering with the proteasome protein degradation pathway (Wang and Perlmutter 2014). Small molecules have been reported to promote protein folding in ocular diseases such as Leber congenital amaurosis and retinitis pigmentosa. Tauroursodeoxycholic acid (TUDCA), a chemical chaperone, that, in addition to reducing ER stress and inflammatory responses associated with diabetes and liver diseases, preserves the photoreceptor structure and function in a mouse model of Leber congenital amaurosis (Zhang et al. 2012). Similarly the inhibition of the molecular chaperone Hsp90 with the proteostasis regulator 17-AAG in a rat model of retinitis pigmentosa (RP) results in a restoration of the mutant rod opsin localisation to that of the wild type (WT) protein (Mendes et al. 2010; Mendes and Cheetham 2008) and reduced rhodopsin aggregation thus protecting against retinal degeneration in vivo (Aguila et al. 2014). Similar strategies may have the potential for treating diseases caused by mutations in bestrophin-1.

The aim of this PhD project was, firstly, to achieve a better understanding of the pathways involved in the QC of mutant bestrophin-1 and which degradation routes were used for its disposal. Secondly to investigate the therapeutic potential of selected small molecules to promote the correct folding, trafficking and function of mutant bestrophin-1 proteins. To achieve these aims, stable MDCKII cells expressing either WT or ARB-associated bestrophin-1 proteins were generated.
1. Introduction

1.1 The human eye

The eye is a highly specialised organ that converts light into electro-chemical impulses that are transmitted to the brain for decoding and analysis (Forrester et al. 2008). It is divided into 3 basic layers: the outer fibrous layer; the intermediate uveal layer and the inner retinal layer (Fig.1.1). The outermost layer that supports and protects the eye is composed of the sclera, the “white part” at the back of the eye made of densely packed fibres, and the cornea at the front. The cornea is transparent and is the primary structure for focusing light, which passes through the pupil, onto the retina. This is possible due to the cornea’s smooth regular epithelium, the highly regular arrangement of the extracellular and cellular components of the middle stromal layer, and the absence of blood vessels. The cornea draws nourishment directly from the aqueous humor, the clear fluid in the anterior chamber, which is secreted by the ciliary body. The intermediate layer, or uveal tract, is highly vascularised and has three major components: the choroid, located between the sclera and the retina and that provides trophic support; the iris, a muscular structure made of circular and radial muscles that controls the amount of light that enter the eye through the pupil; and, the ciliary body which is a continuation of the choroid and is positioned between the retina and the iris, that is responsible for the production of the aqueous that fills both the anterior chamber, behind the cornea and in front of the iris, and the posterior chamber, behind the iris and in front of the lens and zonule (Forrester et al. 2008). The retina is where the light is converted into nervous impulses (Kolb 1995-Updated 2012 Feb 28). All species from insect to higher vertebrates have developed two types of cells for detecting light: light sensitive rod and cone photoreceptors (Lamb et al. 2007) which form, together with the RPE, two primary layers of the retina. Although the human neurosensory retina consists of several layers, the main is the outermost stratum where the light is detected by the rod and cone photoreceptors. Rods are predominantly distributed at the periphery of the retina. They are responsible for sensing contrast, brightness and motion and they play a crucial role in night vision. On the contrary, cones contribute to the fine and the spatial resolution and to the colour vision. The density of cones increases in the centre of the retina, the macula, and in particular the fovea, where most of the
light is directed (Forrester et al. 2008). Interactions between the inner neurosensory retina and RPE start at beginning of the eye development.

Figure 1.1 Cartoon of the human eye

The outermost layer of the eye is composed by the sclera and the cornea and provides mechanical support and protection to the eye. The middle part comprises the choroid, which provides trophic support; the iris, which regulates the amount of light that enter the eye through the pupil and the ciliary body, whose function is to accommodate the lens to be able to focus on near or far objects. The innermost part, the retina, is comprises the inner neurosensory retina and the RPE (B). The neurosensory retina is made up of ganglion cells which receive visual information from the photoreceptors through the bipolar cells (pale blue) or, indirectly, through interneurons (horizontal and amacrine cells in pale yellow and pale green respectively) Ganglion cells axons will form the optic nerve fibres that transmit the visual information to the brain. Yellow arrows represent the direction of the light entering the eye.
1.2 The Retinal Pigment Epithelium structure and functions

The RPE is a continuous monolayer of hexagonal pigmented epithelial cells that interdigitate with the outer segments of the photoreceptors (Forrester et al. 2008). Like other epithelial cells, RPE cells have an apical-basal polarity depending on which ion channels, transporters, receptors, etc. are differentially distributed on the plasma membrane (Strauss 2005). This polarisation event is thought to be regulated by three different factors: suppression of the basolateral sorting mechanism; suppression of basolateral sorting adaptor expression; the formation of tight junction between RPE cells (Strauss 2005). The basal surface of the RPE is in contact with Bruch’s membrane which provides trophic support. The RPE apical surface is rich in melanin granules (Frost et al. 2014) and makes intimate contact with the photoreceptor outer segments through microvilli (Bonilha et al. 2006). Although the microvilli structure has not been extensively studied their core is mainly composed of densely packed actin filaments whilst the base of the apical processes is rich in Myosin VIIa. The microvilli are involved in highly specialized functions essential for the homeostasis of the neural retina such as phagocytosis of shed photoreceptor outer segments, directional transport of nutrients, removal of waste products from photoreceptor cells and visual pigment transport and regeneration. Thus any disruption of the relationship between cone and rod photoreceptors and the RPE results in pathology. When a separation of the photoreceptor outer segments from its apical RPE microvilli occurs, for example in a retinal detachment, return of normal vision is possible only if there is a restoration of a functional relationship between proteins present in the RPE apical surface and the photoreceptors outer segments (Bonilha et al. 2006).

The RPE has a low regenerative capacity in normal eyes and cell loss is accommodated by hyperplasia of adjacent cells. Thus, in older eyes, a mixture of cell size and shape is evident and the regular hexagonal structure is lost.

Although light absorption is the most obvious function of the RPE, these pigmented cells have an important role in many other functions such as epithelial transport, spatial ion buffering, visual cycle, phagocytosis, secretion and immune modulation.
1. Introduction

1.2 The RPE structure and functions

1.2.1 Absorption of light and photo-oxidative stress protection

In the human eye light is focused onto macular cells causing photo-oxidative stress, a process by which reactive oxygen species such as singlet oxygen, superoxide anion and hydroxyl radicals are produced and mitochondrial DNA damage is triggered (Beatty et al. 2000; Winkler et al. 1999). Furthermore, although the choroid has a higher specific blood perfusion than the kidney (Alm and Bill 1970), the rate of oxygen extraction is relatively low. Thus the RPE is exposed on the retinal side to a strong photo-oxidative environment and from the blood side by a relatively poor supply of oxygen. Since the RPE is involved in the renewal process of the damaged photoreceptors (Kevany and Palczewski 2010), this adds a significant free radical load to the high photo-oxidative environment. To maintain the structural integrity of the retina against free radicals, photo-oxidative exposure and light energy, RPE cells possess different types of pigments. The pigment that gives the RPE its name is melanin, which is present within cytoplasmic granules called melanosomes. In older age, melanin granules often fuse with lysosomes, membrane-enclosed organelles located in the cytoplasm that contain digestive enzymes for the degradation and removal of cellular waste and biological polymer. The removal of melanosomes through lysosomes degradation results therefore in an RPE that looks less pigmented in old people. Melanin absorbs stray light, minimizing scatter within the eye so giving optical benefits, and serves as a free radical stabilizer. Since the light absorption by the melanosomes causes a temperature increase above 40°C (Parver 1991), it is possible that the high perfusion of the choroid serves to transport heat away from the retina. The other major RPE pigment is lipofuscin, but its role in the eye is still unclear. Lipofuscin is found throughout the nervous system and in the retina it is thought to be derived from the degradation of photoreceptor outer segment lipids that have been damaged by light or oxidation. Aged RPE cells shows an accumulation of lipofuscin (Boyer et al. 2012; Delori et al. 2001) that could adversely affect its function (Beatty et al. 1999; Boulton and Dayhaw-Barker 2001; Delori et al. 2001). It is likely that a reduced capability to absorb light and to compensate for oxidative damage could be the initial factors that lead to the onset of age-related macular degeneration in older people.
1.2.2 Epithelial transport

The tight-junctions between the lateral surfaces of RPE cells form a part of the blood/retina barrier and provide an efficient isolation of the inner retina from systemic influences at the choroidal side for nutrients, ion homeostasis and the removal of water and metabolites from retinal tissue. As a result photoreceptors rely on trans-RPE transport. To meet its metabolic needs, the RPE expresses the glucose transporters GLUT1 and GLUT3 in both the apical and basolateral membrane (Ban and Rizzolo 2000; Sugasawa et al. 1994); retinol is taken up from the blood and transported to the photoreceptors through the retinol-binding protein receptors. There is also a significant transport from the subretinal space (the space between the photoreceptor and the RPE) to the choroidal side of the RPE. Photoreceptors have a high metabolic rate that leads to a high production of water, ions, metabolic end products and lactic acid in the subretinal space that would be toxic if allowed to accumulate (Hamann 2002; Miller and Steinberg 1977; Steinberg 1985). The net rate of fluid transport across the RPE is about 4-6µl per cm² per hour. Unlike astrocytes and Muller cells, RPE cells do not express the water transporters aquaporin 3-5, but do express aquaporin-1 (Hamann et al. 1998; Stamer et al. 2003). Water is eliminated from the subretinal space by an active Cl⁻-driven transport linked to the transport of HCO₃⁻ and lactate. Interestingly knock-out mice for the CIC-2 Cl⁻ channel become blind because of a progressive retinal degeneration (Bosl et al. 2001), showing a phenotype comparable to that of the retinitis pigmentosa thus underlying the importance of this channel. Another putative Ca²⁺-dependent Cl⁻ channel that plays an important role in RPE function is bestrophin-1 (see section 1.3.2) (Hartzell et al. 2008).

1.2.3 Spatial buffering of ions

Although the RPE maintains the ion homeostasis of the subretinal space, this is subjected to rapid changes due to light-dependent photoreceptor activity and the activity of the second order neurons. To cope with these changes the RPE has a capacitive compensation (Steinberg et al. 1983) that is faster than the one normally provided by the trans-epithelial transport of ions. To compensate for changes in subretinal K⁺ concentration and retinal volume, the RPE also uses other mechanisms based on the activity of voltage-dependent ion channels.
1.2.4 **Light Cycle**

The visual pigment rhodopsin, a carotene derivative composed of 11-cis retinal and scotopsin, undergoes cycling between active an inactive states in response to light. When the 11-cis retinal opsin in rhodopsin absorbs a photon it is converted into all-trans retinal that initiates the photo transduction cascade that leads to the hydroxylation of cyclic GMP. In order for rhodopsin to be able to respond to additional photons it has to be reconstituted. However photoreceptors are unable to regenerate the all-trans retinal to 11-cis retinal as they do not express an isomerase and so all-trans retinal is processed by the RPE to regenerate 11-cis retinal which is transported back to the rod photoreceptors by IRBP (Baehr et al. 2003; Thompson and Gal 2003).

1.2.5 **Phagocytosis of photoreceptor outer segments**

As photo-oxidative damage causes a constant destruction of the light-sensitive photoreceptor outer segments (POS), those need to be constantly renewed to maintain vision. The RPE phagocytes the shed POS tips which are subsequently replaced by new discs added at the base of the outer segments leaving oldest discs at the further end in contact with RPE microvilli (Kevany and Palczewski 2010). POS phagocytosis and production of new discs follows a circadian rhythm occurring just between the onset of light each day and appears to be regulated by L-type calcium channel and bestrophin-1 (Muller et al. 2014). The phagocytosis process can be divided in four major steps: recognition of shed POS, binding, internalization and digestion. Four RPE plasma membrane receptor proteins have been reported to be involved in POS uptake: a mannose receptor, the lipid scavenger receptor CD36, the integrin alpha v beta-3/5 and the receptor tyrosine kinase (RTK) Mer. In *in vitro* assays, integrin αvβ3 and αvβ5 mediate POS binding to the RPE surface, but they do not bind substrate directly. Rather they bind a milk fat globule-EGF-factor (MFG-E8) that recognises the eat-me-signal on the POS membrane. Binding alone is not sufficient to stimulate the phagocytosis of shed POS (Chaitin and Hall 1983). Indeed both CD36 and MertK, which is activated by a non-receptor tyrosine kinase (FAK) (Finnemann 2003) are required for the internalization of the shed POS (Kevany and Palczewski 2010). Significant cytoskeletal reorganization, resulting in an actin-mediated extension of
pseudopods, is also needed to engulf the old disks. Once the POS are internalized phagosomes associate with microtubule-associated protein 1 light chain 3, a process mediated by the intra-cellular cargo sorting protein melanoregulin, and target them to the lysosome for degradation (Frost et al. 2014).

1.2.6 **Secretion**

In order to interact with the neighbouring tissues and to help to maintain their structural integrity, the RPE is able to secrete a large variety of factors and signalling molecules such as: ATP, fas-ligand (fas-L), fibroblast growth factors (FGF-1, FGF-2, and FGF-5), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), lens epithelium-derived growth factor (LEDGF), members of the interleukin family, tissue inhibitor of matrix metalloprotease (TIMP) and pigment epithelium-derived factor (PEDF) (Strauss 2005).
1.3 Human Bestrophin-1

Human bestrophin-1 is a transmembrane protein primarily expressed in the RPE and is a member of a relatively new family of calcium activated chloride channels, the bestrophins. Although the function of the protein is still subject to debate, it is generally accepted that bestrophins-1 plays and important role in maintaining RPE cell homeostasis and function. Mutations in the BEST1 gene are associated with a range of retinal diseases called bestrophinopathies.

1.3.1 The BEST1 gene

The human BEST1 gene (previously called VMD2) was located on the long arm of chromosome 11, at 11q13 as a result of a linkage analysis of a 5-generation family with 29 affected members with vitelliform macular dystrophy (VMD) (Stone et al. 1992). The BEST1 gene spans ~15 kilo base (Kb) of genomic DNA and has 11 exons, however only exons 2-11 encode for the protein (Marquardt et al. 1998). There are three paralogs: BEST2, BEST3, and BEST4 (Stohr et al. 2002; Tsunenari et al. 2003). Whilst BEST1 is predominantly expressed in the retinal pigmented epithelium and weakly in the brain, BEST2 expression appears to be confined to the retina non-pigmented ciliary epithelium and colon. BEST3 transcript is found in colon and weakly in fetal brain, spinal cord, retina, lung, trachea, testis and placenta and BEST4 is mainly expressed in skeletal muscle and weaker in the brain, spinal cord, bone marrow, retina, thymus and testis (Stohr et al. 2002).

1.3.2 Bestrophin-1 structure

The BEST1 gene encodes for an integral membrane protein of 585 amino acids (68 kDa) that localises to the basolateral membrane of the RPE (Marmorstein et al. 2000). Based on hydrophobicity profiling, two topological models were suggested for bestrophin-1. The first one by Tsunenari and colleagues hypothesised that, of the six predicted hydrophobic domains, TMD1, TMD2, TMD4, and TMD6 traversed the membrane and TMD5 was embedded in the membrane facing the extracellular space (Tsunenari et al. 2003). The second
model by Milenkovic and colleagues, however, suggested that bestrophin-1 has four transmembrane domains and a large cytoplasmic loop containing TMD3 and TMD4 (Milenkovic et al. 2007). Both models showed that N- and C- terminal were located in the cytoplasm, whilst the major difference was that amino acids ~199-233 were situated in an extracellular loop in the Tsunenari’s model, whereas in the Milenkovic’s model they were integrated in the intracellular loop. The recent resolution of a crystal structure for bestrophin-1 by two independent groups (Dickson et al. 2014; Yang et al. 2014) reveals that that bestrophin-1 has a large N-terminal region that is divided into four subunits (S1-S4) that each includes a transmembrane domain, and a C-terminal tail. Bestrophin1 proteins assemble around a central axis to form a channel pore with their S2 and S3 subunits. The electrostatic surface potential of the channel results in a negatively-charged extracellular surface at the outer entryway; a neutral transmembrane region, and a positively charged inner cavity. Subunit S2 is the most important region of the protein as it contains residue F80 that confers anion selectivity (Yang et al. 2014). Yang and colleagues also demonstrated that a valine in position 205 is responsible for the channel gating (open probability), but does not contribute to the anion selectivity. A coiled coil region at the S2 C-terminus (aa 207-227) is responsible for the self-assembly of bestrophin-1 to form pentamers. Bharill and colleagues demonstrated that bestrophin-1 preferentially assembles with itself rather than with bestrophin-3, bestrophin-4 or with TMEM16 subunits, a member of the calcium activated chloride channel (CaCC) family (Bharill et al. 2014). A small percentage of bestrophin-1 assembled with bestrophin-2 in vitro; however this is not reproducible in vivo due to the different expression pattern of the two bestrophins. Calcium binding is coordinated by the helix loop helix (HLH) domain in the S1 N-terminal and the acidic cluster (Glu300, Asp301-304) in the S4 subunit that work together to form an unconventional EF hand-like domain, an helix-loop-helix domain involved in calcium binding ((Bharill et al. 2014;Dickson et al. 2014). It is interesting that mutations associated with disease are mostly prevalent in the region around the calcium binding site or around the neck that confers anion selectivity.
1.3.3 Bestrophin-1 function in the RPE

Although the role of bestrophin-1 in RPE cells is not entirely clear, in vitro evidence suggests that bestrophin-1 is a calcium activated anion channel (CAAC) and that it is involved in the modulation of calcium signalling by the L-type calcium channel CaV1.3 or by contributing to store operated calcium entry (SOCE) at the ER and plasma membrane. Recently has also been proposed a role of bestrophin-1 in regulating cell volume.

The first evidence of bestrophin-1 being an anion channel came from exogenous expression studies that showed that bestrophin-1 is permeable to chloride ions (Qu et al. 2003; Sun et al. 2002; Tsunenari et al. 2003). Sun and colleague demonstrated that bestrophin-1 Cl⁻ currents were dependent on intracellular Ca²⁺. Indeed when HEK293 cells were transfected with the WT bestrophin-1 construct it was possible to detect a large novel current only when the intracellular solution contained micro molar amount of free Ca²⁺ (Sun et al. 2002). Xiao and colleagues hypothesised the presence of a Ca²⁺ EF-hand like binding site and identified a protein kinase (PKC) phosphorylation site (p.S358) at the C-terminal domain, which appeared to be important in preventing the channel rundown (Xiao et al. 2008). These findings supported the hypothesis that bestrophin-1 had a putative role as a calcium-activated chloride channel (CaCC).

In addition to its role as a CaCC channel, the high permeability of bestrophin-1 to bicarbonate (HCO₃⁻), suggested that bestrophin-1 could play a role as a HCO₃⁻ channel as well (Casey 2006; Qu et al. 2003). Interestingly both Cl⁻ and HCO₃⁻ conductance were abolished by a mutation in the pore residue p.V78C and Ca²⁺ was required to activate both currents (Qu and Hartzell 2008). Dickinson and collaborators resolved the structure of the chicken homolog of bestrophin-1 and also investigated the channel ion permeability in vitro. They showed that the bestrophin-1 pore is selective for monovalent anions and that the channel gating was directly dependent on Ca²⁺, thus confirming bestrophin-1 role as CAAC (Dickson et al. 2014). As mentioned before, the resolution of bestrophin-1 crystal structure confirmed the hypothesis that bestrophin-1 is an anion channel (Yang et al. 2014).
Another putative role of human bestrophin1 is as a regulator of L-type voltage-gated Ca\(^{2+}\) channel, but there is controversy about the mechanism underlying this function. Two independent groups demonstrated that bestrophin-1 accelerates the activation of voltage dependent calcium currents through the CaV1.3 subunit (Rosenthal et al. 2006; Burgess et al. 2008). However a third group did not observe significant changes in the current kinetics, on the contrary, it reported that bestrophin-1 expression significantly reduced the amplitude of CaV1.3 currents (Yu et al. 2008). The negative regulatory effect was hypothesised to be caused by the SH3 binding domain that lies between the calcium binding site and the EF-hand like domain (Xiao et al. 2008). Reichhart and colleagues demonstrated that bestrophin-1 can bind to the β-subunit of L-type channels through a proline-rich motif (Reichhart et al. 2010). This interaction resulted in the decrease of L-type channel currents. Bestrophin-1 residues p.P330 and p.P334 appear critical for this regulation as when they were mutated the effect was abolished (Yu et al. 2008).

The proportion of bestrophin-1 that localise intracellularly is suggested to be involved in the store operated Ca\(^{2+}\) entry (SOCE). However, bestrophin-1 role in SOCE is yet to be defined (Strauss et al. 2014; Barro-Soria et al. 2010; Han et al. 2013). When calcium is depleted from ER stores the calcium intraluminal sensor Stim-1 (stromal-interacting molecule-1) dissociates from calcium and interacts with the calcium release-activated-calcium channel protein-1 (Orai-1). This interaction resulted in the decrease of L-type channel currents. Bestrophin-1 residues p.P330 and p.P334 appear critical for this regulation as when they were mutated the effect was abolished (Yu et al. 2008).

Calcium is a critical regulator of many RPE function such as epithelial transport, secretion of growth factors and phagocytosis of photoreceptor outer segments. Recent studies showed that knock down of bestrophin-1 enhance phagocytosis in porcine RPE cells (Muller et al. 2014). Bestrophin-1 mutations that results in a defective SOCE could interfere with the phagocytosis process.
leading to the accumulation of lipofuscin which is known to be one of the characteristic common among all bestrophinopathies (Wimmers et al. 2007; Frost et al. 2014; Strauss et al. 2016; Marmorstein et al. 2015; Strauss et al. 2014).
1. Introduction

1.3 Human Bestrophin-1

Figure 1.2 Bestrophin-1 localisation, structure and functions in the RPE

Bestrophin-1 (green) mainly localise to the basolateral plasma membrane of RPE cells (1) where it acts as calcium activated anion channel (CAAC), it is involved in the modulation of calcium signalling by the L-type calcium channel CaV1.3. Furthermore bestrophin-1 contributes to store operated calcium entry (SOCE) at the ER (2). A role in regulating cell volume was also proposed. Recent resolution of the structure of two bestrophin-1 homologues reveals that bestrophin-1 associate in homopentamers to form anion channels that open following calcium binding (Dickson et al. 2014; Yang et al. 2014). Figures A-F were taken from Yang and colleagues’ paper and show a ribbon diagram of bestrophin-1 pentamer with each protomer colored differently (A abd B). (A) shows the view from outside the membrane and (B) as viewed from the side. The electrostatic potential at the molecular surface viewed are represented in C and D and in particular red indicates negative potential and blue indicates positive potential (Yang et al. 2014).
1.4 **Bestrophinopathies**

Bestrophinopathies are a collection of retinal diseases caused by mutations in *BEST1* that are characterised by an abnormal electrooculogram and elevated lesion in the central region of the neurosensory retina, the macula, possibly due to the accumulation of a fatty yellow pigment (lipofuscin) in the subretinal space (Mullins et al. 2007; Kay et al. 2012) with consequent loss of central vision. Lipofuscin originates from the phagocytosis of photoreceptor outer segments by RPE cells and it is made up of auto fluorescent materials, oxidized proteins and lipids (Grey et al. 2011; Murdaugh et al. 2011). Although the accumulation of lipofuscin in lysosomes of RPE cells normally occurs with age, it is likely to be accelerated in bestrophinopathies (Boyer et al. 2012). The major component of lipofuscin is the fluorophore N-retinylidene-N-retinylethanolamine (A2E) (Boyer et al. 2012; Grey et al. 2011). A2E has several effects when accumulated in cells: it may disrupt membrane integrity by a detergent–like effect and, when photooxidised, its products can activate the complement system and promote apoptosis (Zhou et al. 2009). Although the localisation of lipofuscin has been a subject of debate recent the use of high-definition optical coherence tomography (OCT) made it possible to anatomically examine the vitelliform lesion in vivo. Kay and colleagues observed that the vitelliform lesion is predominantly located in the subretinal space. However they also noted deposits of sub-RPE material in some patients whilst the three patients with a p.N302A BEST1 mutation, compared to twelve patients with other genetic variants, had a dense nodule of material located under the RPE that is likely to be fibrotic scar tissue (Kay et al. 2012). Over time, the abnormal accumulation of lipofuscin leads to aberrant interaction between photoreceptors and the RPE resulting in the disruption of the outer retina and consequentially affecting the central vision (Kay et al. 2012).
1.4 Bestrophinopathies

Figure 1.3 Example of mutation in *BEST-1* that cause bestrophinopathies.

Locations of missense mutations associated with bestrophinopathies mapped on the structure. See text for more details.
Another characteristic common to all bestrophinopathies is an altered electrooculogram (EOG). The EOG is used clinically to assess the RPE health and function. It measures the potential difference between the cornea and the RPE by using two skin electrodes placed on the right and left temples and one reference electrode placed in the middle of the forehead. The normal ratio between the light peak and the dark trough, known as Arden ratio, is generally >2.0. However patients affected by bestrophinopathies show an Arden ratio <1.5, whereas Arden ratios between 1.5 and 2.0 are considered borderline (Ramsden et al. 2012). Thus variation in the EOG is considered a prerequisite for clinical diagnosis of bestrophinopathies. However, some BEST1 mutation carriers show a normal EOG, even in the latest stage of the disease (Low et al. 2011) which means than only mutation analyses of the BEST1 gene can provide an effective diagnosis for the disease.

To date more than 200 mutations in the BEST1 gene have been reported to cause disease of different phenotype (Burgess et al. 2009; Davidson et al. 2011; Davidson et al. 2009; Boon et al. 2009; Schulz) and they have been associated either with the juvenile form (Apushkin et al. 2006; Atchaneeyasakul et al. 2008; Caldwell et al. 1999; Eksandh et al. 2001) or the adult-onset of the disease (Kramer et al. 2000) (Fig. 1.3). However it is possible to group bestrophinopathies in four distinctive classes: Best vitelliform macular dystrophy (Best disease), autosomal dominant vitreoretinochoroidopathy (ADVIRC), autosomal dominant retinitis pigmentosa (RP)-like retinal degeneration and autosomal recessive bestrophinopathies (ARB).

1.4.1 Best vitelliform macular dystrophy (BVMD, Best-disease)

BVMD is an autosomal dominant disorder with a variable age of onset. First described by Friederich Best in 1905, BVMD is characterized by a macular lesion due to an accumulation of lipofuscin in the RPE. The age onset is highly variable ranging from the first decade to mostly the 2nd decade of life (Marquardt et al. 1998; Petrukhin et al. 1998). Most BVMD patients present with decreased visual acuity although photophobia, metamorphobia and night blindness may also be noted (Renner et al. 2005). The patients’ age correlates significantly with visual
acuity although each eye can be affected in a different way and can show different
degree of BVMD lesion (Clemett 1991; Lin et al. 2015; Wittstrom et al. 2011; Lacassagne et al. 2011).

Based on the ophthalmoscopic aspects of lesions, Mohler and Fine defined the
major clinical stage of the disease (Mohler and Fine 1981). The first stage, known
as the carrier or previtelliform stages, shows a normal fovea or discrete RPE
alteration together with an abnormal EOG. In the following stages a vitelliform
“egg yolk” macular lesion develops in the macular region of the retina due to the
accumulation of lipofuscin above the RPE and below the outer segment tips (Kay
et al. 2012). The vitelliform material could then break up (vitelliruptive stage)
causing a reduction in visual acuity and, in quite rare cases, retinal detachment.
The final stage of the disease is associated with severe visual loss (vision may
range from 20/30 – 20/200) sometimes complicated by subretinal
neovascularisation (Ozdek et al. 2011; Miller et al. 1976).

1.4.2 Autosomal dominant vitreoretinochoroidopathy (ADVIRC)

ADVIRC is a rare autosomal dominant disease first described by Kaufman in
1982. It is characterized by peripheral circumferential retinal hyperpigmentation,
midperipheral and peripapillary chorioretinal atrophy, punctate retinal and
vitreous deposits and condensations (Kaufman et al. 1982). Yardley and
colleagues identified BEST1 as the gene mutated in ADVIRC in 2004. They used
five families with autosomal dominant developmental eye abnormalities for
genetic analysis and established linkage of an ADVIRC phenotype to the
pericentromeric region of chromosome 11. BEST1 was noted to be within the
critical region and an abnormal EOG was common to both Best disease and
ADVIRC, BEST1 was sequenced and pathogenic mutations were found in all five
families (Yardley et al. 2004). ADVIRC patients with BEST1 mutations can
develop congenital or early-onset cataract, nanophthalmos, microcornea and
closed-angle glaucoma. This suggests that BEST1 could also be involved in eye
development. Furthermore the EOG is severely abnormal in most BEST1-related
ADVIRC patients, though it may be normal in exceptional cases. The age of onset
for ADVIRC can be as early as six years of age and the disease progression is
variable (Vincent et al. 2011; Yardley et al. 2004). Visual acuity is quite good throughout life even if, in quite rare circumstances, patients develop macular oedema, chorioretinal atrophy, retinal detachment and vitreous haemorrhage. Interestingly ADVIRC is the only bestrophinopathy that is thought to be caused by altered pre-mRNA splicing of BEST1 (Burgess et al. 2009). It has been shown, in fact, that missense mutations might give rise to three forms of bestrophin-1 such as a WT and two missense isoforms, one containing a missense substitution and the other a deletion of an internal, in-frame, exon (Yardley et al. 2004).

1.4.3 Autosomal dominant RP-like retinal degeneration

RP is an inherited, degenerative eye disease characterised by progressive degeneration of photoreceptors and of the RPE (Berson 1993; Rattner et al. 1999; PHelan and Bok 2000; Shintani et al. 2009) with subsequent severe vision impairment and blindness. RP age of onset is highly variable ranging from infancy to adulthood (Shintani et al. 2009) and this disease can be associated with various diagnostic features such as night blindness due to primary rod photoreceptor dysfunction; loss of peripheral vision (tunnel vision); loss of central vision (in advanced cases); slow adjustment from dark to light environments and vice versa. However progression of the disease, in the vast majority of cases, leads to visual loss (Berson 1993; Rattner et al. 1999; PHelan and Bok 2000; Shintani et al. 2009). RP fundus images are typically associated with pigmentary deposits resembling bone spicules, attenuation of retinal vessels, waxy pallor of the optic disc and retinal atrophy. RP could be either autosomal recessive (20-30% of RP cases (Shintani et al. 2009)) or autosomal dominant (15-20% of cases (Shintani et al. 2009)). However less common X-linked (6-10% of cases (Shu et al. 2007; Shintani et al. 2009)), digenic and mitochondrial inheritance patterns have also been associated with RP (Kajiwara et al. 1994; Goldberg et al. 1995; Dryja et al. 1997; Hartong et al. 2008). Around 50% of RP cases occur sporadically (Shu et al. 2007). As a particular form of RP, concentric RP, resembled ADVIRC phenotype, patients diagnosed with concentric RP was screened for mutations in BEST1 (Urquhart 2005). In 2009 Davidson and colleagues identified three new
missense mutations in \textit{BEST1} in patients diagnosed with RP suggesting that \textit{BEST1} is involved in the disease aetiology.

1.4.4 \textbf{Autosomal recessive bestrophinopathy (ARB)}

ARB was first described in 2008 by Burgess and colleagues (Burgess et al. 2008) and represents the null bestrophin-1 phenotype in humans. ARB has an age of onset ranging from 4 to 40 years. Affected individuals present central vision loss, hypertrophy and shallowed anterior chamber. There is also high incidence of sub-acute angle-closure glaucoma (Crowley et al. 2014; Burgess et al. 2008). Ophthalmoscopic examinations show irregular RPE alteration and deposit of yellow-white material, however vitelliform lesions are not observed. Interestingly the altered EOG phenotype is accompanied by a markedly abnormal electroretinogram (ERG), indicating an abnormal function of the retina, which is usually un-altered in bestrophinopathies. The full-field ERG shows a progressive reduction in rods and cones responses, which indicates that photoreceptors function is compromised (Burgess et al. 2008).

In order to study the ability of small molecules to rescue bestrophin-1 localisation, stability and function, four missense mutation causing ARB were chosen based on their position in the bestrophin-1 structure (Fig 1.4): p.L41P (transmembrane domain); p.R141H and p.M325T (cytoplasm and in the calcium binding region); p.R202W (cytoplasm and close to the gating site of the channel).
Figure 1.4 Location of ARB causing mutations chosen for this study

The human bestrophin-1 crystal structure was modelled on that empirically determined for chicken bestrophin-1 (Dickson et al. 2014) by Prof Simon Lovell (FLS, UoM). The structure was visualised using KiNG (http://kinemage.biochem.duke.edu)
1.5 Protein quality control in the ER

The native conformation of a protein is, by definition, given by the achievement of the lowest energy state possible (Anfinsen et al. 1954). The free energy (Gibbs free energy, $\Delta G$) is defined by the change in the enthalpy ($\Delta H$) and the entropy ($\Delta S$) of the system, or rather, the amount of heat used or released in a reaction and the measure of the disorder of the system. The relationship between free energy, enthalpy and entropy at constant temperature (T) and pressure (P) can be described by the following equation

$$
\Delta G = \Delta H - T\Delta S
$$

As protein folding proceeds, the polypeptide structure becomes more organised thus resulting in a decrease of the entropy. According to the equation, the reduction of $\Delta S$ would generate a positive $\Delta G$ which is incompatible with spontaneous protein folding. However, the contribution of the entropy is balanced by the disruption of hydrogen bonding, ionic salt bridges, and Van der Waals forces which generate a negative $\Delta H$. Therefore, the resulting free energy is negative thus allowing protein folding to occur spontaneously.

Around 1/3 of the nascent proteins in the cell are targeted to the ER where folding and sorting occur together with translation. However protein folding is an intrinsically error-prone process and proteins can fail to fold correctly due, for example, to genetic mutations or adverse environmental conditions (Hebert and Molinari 2007). Such non-native proteins are potentially harmful to the cell as they can interfere with the cell homeostasis and functions. Thus cells possess quality control systems (QC) at several stages of the secretory pathway to identify proteins that are misfolded in order to assist proper folding or, when correct folding is not achieved, to degrade them.

A number of disease-associated bestrophin-1 proteins fail to localise to the basolateral plasma membrane of cultured RPE cells or MDCKII cells transiently transfected with mutant bestrophin-1 constructs (Davidson et al. 2011; Milenkovic et al. 2011). Mutant bestrophin-1 proteins have been localised either to the cytoplasm, to the Golgi, to the ER or to early endosomes (Milenkovic et al. 2011) suggesting that disease-causing bestrophin-1 proteins are recognised along the
secretory pathway by QC systems and retained in the cell. Nonetheless very little is known about the identity of the QC pathway involved in mutant bestrophin-1 recognition or about the mechanism of its retention.

1.5.1 The endoplasmic reticulum quality control (ERQC)

The ER is an organelle that takes up 10% of cell volume. It probably originates from the invagination of the plasma membrane, in fact the ER lumen composition resemble that of the extracellular space (Schroder and Kaufman 2005). The ER has several functions: it is a Ca$^{2+}$ storage, the site of lipid and sterol biosynthesis and plays an important role in protein biosynthesis such as many membrane proteins, including bestrophin-1, and those destined for secretion.

The ER represents the entry point of the secretory pathway and the start point for the maturation of secretory proteins, proteins destined for the plasma membrane and for the endocytic and exocytic compartments. Proteins that need to enter the ER secretory pathway are characterised by a short N-terminal hydrophobic polypeptide sequence known as the signal sequence. As soon as protein synthesis begins at the ribosome, the signal sequence is recognised by an RNA-protein complex, the signal recognition particle (SRP), which binds the signal sequence and causes protein translation to stop. The ribosome-protein complex is then transferred to the SRP receptor at the ER membrane (Schwartz and Blobel 2003) and the nascent protein is inserted into the Sec61 translocation complex, known as the translocon (Nakatsukasa and Brodsky 2008). SRP is then released in the cytoplasm where it is free to bind to another nascent polypeptide. Protein synthesis starts again, with the polypeptide entering the ER lumen as an extended chain of amino acids. The rapid targeting of the nascent polypeptide chain to the ER ensures that translocation occurs co-translationally, meaning that the folding of the polypeptide chain will take place within the ER lumen (Nakatsukasa and Brodsky 2008). At the ER, the nascent protein may undergo posttranslational modifications such as disulphide bond formation between cysteine residues and asparagine (N)-linked glycosylation. The folding process is facilitated by ER-resident chaperone proteins and folding factors. The categories of folding factors are based upon whether they catalyse specific steps or are able to bind to intermediates in the folding pathway.
One of the best characterised chaperone systems in the mammalian ER is represented by the heat shock proteins (HSPs), which have been classified by family according to their size (Kampinga et al. 2009): HSP40 (DNAJ), HSP60, HSP70 (HSPA), HSP90 (HSPC) and HSP110 (HSPH) and small HSPs (HSPB) (Brandvold and Morimoto 2015; Kampinga et al. 2009).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Old names</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPA1A</td>
<td>HSPA1A</td>
<td>HSP70-1; HSP72; HSPA1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>HSPA1B</td>
<td>HSPA1B</td>
<td>HSP70-2</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>HSPA1L</td>
<td>HSPA1L</td>
<td>hum70t; hum70t; Hsp-hom</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>HSPA2</td>
<td>HSPA2</td>
<td>Heat-shock 70kD protein-2</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>HSPA5</td>
<td>HSPA5</td>
<td>BIP; GRP78; MIF2</td>
<td>ER</td>
</tr>
</tbody>
</table>

Furthermore, based on the type of interaction with the substrate, they can also be classified as foldases, holdases and disaggregases. Foldase are ATP-dependent and are directly involved in protein folding. Holdases are ATP-independent and can stabilise the partially folded proteins, prevent their aggregation and pass them to the foldases. Disaggregates are ATP-dependent and, as the name suggests, they recognised protein aggregates and disaggregate them so that partially folded proteins are able to interact with either a foldases or a holdase (Diaz-Villanueva et al. 2015). The immunoglobulin heavy-chain binding protein BiP, also known as GRP78, is a molecular chaperone part of the Hsp70 family and resides in ER (Diaz-Villanueva et al. 2015; Behnke et al. 2015; Otero et al. 2010; Braakman and Hebert 2013). The ability of BiP, as for the majority of HSPs, to bind the substrate is tightly regulated by a cycle of ATP binding, hydrolysis, and nucleotide exchange which can be regulated by a number of co-chaperones (Diaz-Villanueva et al. 2015; Braakman and Hebert 2013). BiP has two functional domains: a carboxy (C)-terminal substrate-binding domain and a highly-conserved amino (N)-terminal nucleotide-binding domain. When ATP occupies the cleft in the N-terminal domain, the lid located above the cleft at the C-terminal domain is open. In this conformation the partially folded protein binds to BiP with low affinity. The hydrolysis of ATP in ADP is facilitated by a class of Hsp40, ERdj, which
function ad holdase and therefore referred to as co-chaperones (Melnyk et al. 2015). ATP hydrolysis induces a structural change in the C-terminal domain. As a consequence, the lid closes onto the substrate, which now binds the C-terminal domain with high affinity (Diaz-Villanueva et al. 2015; Braakman and Hebert 2013). In this conformation premature folding or aggregation of the substrate is prevented. The polypeptide is released when ADP is exchanged for ATP, a reaction that is catalysed by the nucleotide exchange factors BAP and GRP170 (Melnyk et al. 2015). Once the client protein is released, it has the opportunity to fold.

The disposal of proteins that fail to achieve a native conformation requires that terminally misfolded proteins are recognized and targeted to the ER associated degradation machinery. Although this transition for glycoproteins is quite well known and involves the calnexin/calreticulin complex, for BiP substrates, that may not have N-linked glycans, this transition is less clear. Recent studies suggest that BiP function in protein folding, retention in the ER and degradation could be achieved by the interaction with different co-chaperones. In particular ERdj3/6 might be involved in promoting protein folding whereas switching to ERdj4/5 might promote protein degradation (Lai et al. 2012; Otero et al. 2010). How this switching occurs is unclear. Studies with ERdj3/6 revealed that ERdj3s can release the partially folded protein once BiP has been successfully bound to it. It is likely that this provides the opportunity for another ERdj family member to bind to the client protein and target it for degradation (Otero et al. 2010).

Once the correct conformation has been achieved, newly synthesised proteins are concentrated into transport vesicles and they enter to the next stage in the secretory pathway, the Golgi apparatus (Braakman and Bulleid 2011). Here, proteins may undergo further modification, such as O-glycosylation and processing of N-glycans, before being sorted to their final destination by the trans-Golgi network.

Although bestrophin-1 is synthesised in the ER, there is no evidence that it undergoes N-glycosylation (Forbes Manson personal communication) and no N-glycosylation consensus sequence has been found in its amino acid sequence. The quality control mechanisms that determine the fate of non-glycosylated proteins
that enter the ER is much less clear compared to those that operate for \textit{N}-glycosylated proteins (Kleizen and Braakman 2004). Current models suggest that folding-defective glycoproteins are subjected to two phases of retention-based ER quality control, the first in the calnexin chaperone system and the second in the BiP chaperone system. As recognition of proteins by the calnexin chaperone system is usually mediated by the presence of \textit{N}-glycans, it is highly likely that bestrophin-1 folding and QC depend more on the BiP chaperone system.

1.5.2 \textbf{ER associated degradation (ERAD)}

Following recognition of misfolded or damaged proteins by the protein QC systems, the next crucial step in the acute response to protein misfolding is the decision between damage elimination by repair or by degradation. In the ER, terminally misfolded proteins are processed by the ERAD (ER associated degradation) (Olzmann et al. 2013;Smith et al. 2011). This multistep pathway involves recognition of the misfolded protein, movement of the misfolded protein back across the ER membrane into the cytosol (process known as retrotranslocation/dislocation) and degradation by the ubiquitin proteasome system in order to be destroyed by proteolysis (Olzmann et al. 2013;Smith et al. 2011).

Targeting factors such as EDEM, OS9, grp94 and possibly BiP, are responsible to recognise and deliver the non-native protein to the dislocation and ubiquitination machinery (Nakatsukasa and Brodsky 2008). Molecular chaperones, in particular holdase, can also play a role in the recognition of misfolded proteins which are then transferred to one of the targeting factor. The identity of the dislocon, the complex through which ERAD substrates move into the cytosol, is not clear. However various candidates have been proposed including the Sec61 complex that mediates import of proteins into the ER, the Derlin family of proteins and the ubiquitin ligase enzymes that catalyse polyubiquitination of the misfolded protein (Nakatsukasa and Brodsky 2008). Polyubiquitination is required for degradation of most ERAD substrates and occurs concomitantly with retrotranslocation. Modification with ubiquitin occurs by the covalent bonding of the C-terminal glycine of ubiquitin to a lysine residue on a substrate protein. Polyubiquitin chains are formed by the attachment of
additional ubiquitin molecules to lysine residues within the ubiquitin polypeptide itself. The outcome of ubiquitin addition to proteins is not always the degradation of the protein but depends both on the residue on which ubiquitin is added and on the number of ubiquitin molecules attached. Mono-ubiquitination and oligo-ubiquitination may target proteins for endocytosis (Hicke 1999) whilst the poly-ubiquitination targets them to degradation (Soltes et al. 2011). Addition of ubiquitin to a substrate protein is catalysed by the sequential action of three enzymes: an E1-activating enzyme, which prepares the C-terminal Gly of ubiquitin for binding, an E2-conjugating enzyme, which receives the activated ubiquitin molecule from E1, and an E3 ubiquitin ligase enzyme, which transfers the activated ubiquitin molecule from the E2 enzyme to the protein substrate (Soltes et al. 2011). The substrate recognition step which leads to protein ubiquitination is carried out by the E3 ligase enzyme, which identifies distinct ‘ubiquitination’ signals on the substrate protein. Therefore, it is believed that these enzymes provide specificity to the ubiquitination process.

Polyubiquitination recruits a number of ubiquitin-binding proteins, such as the p97/Ufd/Npl4 complex, which help to extract the ERAD substrate from the ER and guide it to the proteasome for degradation (Soltes et al. 2011). The mammalian proteasome is a 26S multimeric complex that consists of a 20S core particle (CP) and a 19S regulatory particle (RP) conserved among eukaryotes (Sledz et al. 2013; Bedford et al. 2010). The RP contains an ubiquitin-binding subunit and functions in priming the CP, which contains the catalytic protease subunits, for degradation. The RP also contains six distinct ATPase domains which provide energy for the degradation process and possibly for unfolding of degradation substrates prior to their entry into the CP (Ciechanover and Stanhill 2014). Proteasomal degradation does not produce single free amino acids but rather short peptide chains, 3–23-amino acid long, which are then further degraded by cellular hydrolases and aminopeptidases (Kisselev et al., 1999; Nussbaum et al., 1998; Osmulski and Gaczynska, 1998).

Degradation through the proteasome often requires dislocation of the non-native protein from the ER. Misfolded proteins, however, can form insoluble protein aggregates that are unable to dislocate from the ER (Engelender
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1.6 Diseases associated with protein misfolding/mistrafficking

2012; Ebrahimi-Fakhari et al. 2011). These proteasome resistant conformers are then degraded by autophagy, a lysosomal degradation pathway that delivers damage organelles and protein aggregates to the lysosomes for degradation (Wang et al. 2015). Furthermore it has been recently showed that misfolded, ERAD-resistant but non-aggregated ER membrane proteins can be recognised by QC and eliminated by autophagy ((Buchberger 2014; Houck et al. 2014), a process that has been termed ERQC autophagy (Houck et al. 2014).

1.5.3 The Unfolded Protein Response (UPR)

Despite these mechanisms that help to repair or degrade acutely damaged proteins, misfolded or unfolded proteins can build up in the ER. This situation is known as ER stress. The accumulation of misfolded proteins can be damaging because they alter the normal homeostasis of the cell and can interfere with its function. Therefore, cells possess an adaptive response known as the unfolded protein response (UPR) that aims to reduce levels of misfolded proteins in the ER and restore homeostasis (Wek and Cavener 2007). The major effects of the UPR are a decrease in global protein translation, and an upregulation of molecular chaperones and proteins involved in the ERAD pathway. Together, these outcomes reduce the input of nascent proteins into the ER, enhance the folding capacity, and increase the rate of degradation of misfolded proteins. If homeostasis not restored, prolonged or severe UPR signalling can initiate apoptosis (Jager et al. 2012). Three ER membrane proteins are involved in detection of misfolded proteins in the lumen and activation of the UPR. Two of these are protein kinases: IRE1 (Inositol Required Enzyme I) and PERK (PKR Related ER Kinase). The third factor is ATF6, an ER transmembrane transcription factor (Wek and Cavener 2007).

1.6 Diseases associated with protein misfolding/mistrafficking

A large number of human diseases are caused by misfolding of particular proteins. Genetic mutations that alter the primary amino acid sequence (point mutation, deletion, frame shift) can inhibit the ability of the polypeptide to fold into the correct three-dimensional structure. As described above, such misfolded proteins will be recognised by QC checkpoints at the ER and later stages in the
secretory pathway, preventing proper localization of the protein and leading to degradation by ERAD or by autophagy/lysosomes. Protein misfolding can cause to loss-of-function or a gain-of-function diseases. In the case of loss of function diseases, the mutant gene product lacks function due to the effect of the mutation itself, or as a consequence of misfolding, mislocalisation and/or degradation of the mutant protein. The phenotype associated with loss of function diseases is often recessive. An example of this is the cystic fibrosis (CF), an autosomal recessive genetic disorder that mostly affects the lungs (OMIM:219700). Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel cause the protein to misfold and to be retained in the ER and degraded by ERAD (Yu et al. 2011), leading to loss of functional CFTR at the cell surface, thus preventing the normal transport of chloride and sodium across the epithelium.

Gain of function mutations, on the contrary, may result in a gene product that has an abnormal and harmful function or activity. Such diseases may be caused by inefficient degradation of the misfolded mutant protein, leading to accumulation in the ER or elsewhere in the secretory/endocytic pathway. Misfolded proteins are potentially toxic, since they have a high risk of aggregating and abnormal interactions with other proteins and/or membranes, and therefore when accumulated can disrupt cellular function. In addition, the build-up of misfolded proteins in the ER can cause severe and/or long term activation of the UPR, disrupting cell function and/or inducing apoptosis, thus leading to a gain of function disease. UPR will eventually lead to cell death causing severe damages to tissues and organs. This is the case for most of neurodegenerative diseases such as Parkinson or Alzheimer disease. Accumulation of misfolded protein can also be observed in several eye diseases. In retinitis pigmentosa (RP) (OMIM: 268000), for example, mutations in the rhodopsin gene can affect rhodopsin structure, function and localization resulting in a gain of function and dominant negative effect (Saliba et al. 2002; Kaushal and Khorana 1994). Mutant rhodopsin proteins accumulate in the ER where they can induce prolonged activation of the UPR which leads to photoreceptor cells death (Chapple and Cheetham 2003; Saliba et al. 2002).
The situation is more complicated than this since, a combination of loss and gain of function effects may contribute to disease pathology (as in the α1-antitrypsin deficiency mentioned in the preface). Furthermore, different mutations in the same gene can perturb function and folding of the mutant protein in distinct ways, leading to different disease phenotypes as observed in bestrophinopathies.

In the last decade different therapeutic strategies have been proposed for the treatment of protein misfolding disease: gene therapy, small molecule rescue and combination therapy (Liu et al. 2011), the latter being a combination of the gene therapy and the use of small molecules. The aim of the gene therapy is to compensate for the loss of function of the mutant protein. This approach might prove beneficial for the treatment of many ocular diseases, as shown by the promising clinical trial for the treatment of Leber congenital amaurosis (Stein et al. 2011). Nonetheless the delivery of a replacement WT protein by gene therapy can be a high risk, take a long time, and be extremely expensive. For these reasons alternative approaches have been investigated over the years. The use of small molecules such as chemical and pharmacological chaperones or proteostasis modulators to rescue the phenotype caused by protein misfolding or aggregation became a valid alternative to gene therapy. Chemical chaperones are small compounds with a low molecular weight that can stabilise the native conformation of a protein for example by temporary interacting with the nascent polypeptide in order to prevent aggregation through non-specific hydrophobic interactions. The action of chemical chaperones is not specific as opposed to the one of pharmacological chaperones or proteostasis regulator which target particular protein or pathway respectively in order to overcome the protein defect caused by mutations and restore the cell homeostasis. Examples of small molecules that have been used to rescue misfolding defects are reported in table 2. Later in this thesis the mechanism of action of some of these small molecules will be discussed.
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1.6 Diseases associated with protein misfolding/mistrafficking

<table>
<thead>
<tr>
<th>Small molecule used</th>
<th>Protein target</th>
<th>Associated disease</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-phenylbutyrate (4-PBA)</td>
<td>CFTR RPE65</td>
<td>Cystic fibrosis Retinitis pigmentosa; Leber congenital amaurosis</td>
<td>(Zeitlin et al. 2002) (Jin et al. 2016)</td>
</tr>
<tr>
<td>TUDCA</td>
<td>Rhodopsin RPE65</td>
<td>Retinitis pigmentosa; Leber congenital amaurosis</td>
<td>(de Almeida et al. 2007) (Zhang et al. 2012)</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>α-Antitrypsin</td>
<td>α-Antitrypsin deficiency</td>
<td>(Hidvegi et al. 2010)</td>
</tr>
<tr>
<td>Celastrol</td>
<td>Huntingtin</td>
<td>Huntington disease</td>
<td>(Wang et al. 2005)</td>
</tr>
<tr>
<td>11-cis-7-ring retinal opsins</td>
<td>opsins</td>
<td>Retinitis pigmentosa</td>
<td>(Noorwez et al. 2003)</td>
</tr>
</tbody>
</table>

1.7 **Aims**

The aims of this PhD project were:

- To generate cell base system for expression of WT and mutant forms of bestrophin-1 associated with ARB.

- To define the localization of mutant estrophin-1 and to identify the QC system involved in the recognition of different mutants.

- To examine the ability of a range of small molecules to promote folding, trafficking and function of mutant bestrophin-1 in vitro that can be used as potential therapeutic leads in ARB and generally bestrophinopathies.
2 Materials and methods

2.1 Methods

2.1.1 Tissue culture procedures

2.1.1.1 Cell culture and maintenance

In order to study bestrophin-1 localisation, stabilisation and function, Madin-Darby canine kidney (MDCK) II cells, HeLa cells and Human Embryonic Kidney (HEK) cells were used.

MDCKII cell lines were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) (Gibco) supplemented with 2 mM L-Glutamine (Sigma), 0.1 mM MEM non-essential amino acids (Sigma) and 10% v/v heat-inactivated foetal bovine serum (Sigma). After reaching 80-90% confluency, cells were split using 1x trypsin-EDTA solution (Sigma) into fresh cell culture dishes/flasks (15-10- 6 cm dishes; T75 flasks; 6 well dishes and 12 well dishes (Corning)). Cells were incubated at 37 °C, 8% CO₂.

HeLa cell lines were maintained in DMEM (Sigma) supplemented with 2 mM L-Glutamine (Sigma), 0.1 mM MEM non-essential amino acids (Sigma) and 10% v/v heat-inactivated foetal bovine serum (Sigma). Cell were split after reaching 90-100% confluency and incubated at 37 °C, 8% CO₂.

HEK293 cells were cultured in DMEM without sodium pyruvate (Sigma) supplemented with 10% v/v heat-inactivated foetal bovine serum (Sigma). Cells were split after reaching a confluency of 70-80% and incubated at 37 °C, 5% CO₂.

2.1.1.2 Transient transfections

For biochemical assays such (as immunoprecipitation) MDCKII cells were split 24 hours prior to transfection and seeded in 10-3.5cm plates/6-12 well plates. Unless otherwise indicated, transfection reagents were used according to manufacturer instruction (see 2.7).
For biotinylation studies HEK293 cells were used. HEK cells cultured into 6/10cm dishes were co-transfected with either WT or mutant bestrophin-1 constructs in pAdLox and GFP with a ratio of 4:1 (BEST1:GFP). The transfection mixture was prepared in OptiMEM and cells were incubated for 6-8h at 37°C before replacing transfection medium with complete DMEM plus treatments. Cells were then incubated at 37°C for at least 24h to allow good expression of transfected genes and to test the effect of the treatments.

To study the functionality of bestrophin-1 channel HEK293 cells transiently transfected with WT or mutant bestrophin-1 construct were used to perform patch clamp studies. HEK cells were transfected as described above with the only difference being the amount of total DNA used (1µg for biotinylation compared to 0.6µg for patch clamp analysis).

### Establishment of stable cell lines

In order to generate MDCKII cells and HeLa cells expressing WT and mutant bestrophin-1 proteins under the control of a tetracycline operator, the Flp-In™ TREx™ system was used.

MDCKII cells were split 24 hours prior to transfection and seeded at a density of 5x10^5 cells/well in a six well plate. Cells were transfected with a total of 4 µg of plasmid DNA (9:1 pOG44/pcDNA5/FRT/TO+insert ratio) using Lipofectamine™ LTX according to the manufacturer’s protocol to generate stable cell lines. Flp-In™ TREx™ parental cells stably expressing the tetracycline repressor protein...
and with a Flp-In target site encoded in the genome were provided by Dr. Joris Robben, Post-doctoral researcher, Radboud University Nijmegen Medical Centre, the Netherlands (MDCKII) and by Professor Stephen Taylor, University of Manchester (HeLa). Expression of the protein of interest was induced with 1 \( \mu g/ml \) tetracycline.

2.1.1.4 **Cell viability (MTT) assay**

The MTT assay is a colorimetric assay used to assess cell viability and metabolic activity. In this study the MTT assay was used to determine the amount of hygromycin required for selection of MDCKII cells that incorporated WT or mutant bestrophin-1 constructs. In order to assess hygromycin toxicity parental MDCKII cells were seeded in 24 well plates and grown in the presence of the treatment (antibiotic/compound) for 24-72 hours. Two hours prior to collection cells were incubated with a 0.5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in DMEM. Formazan crystals were dissolved with DMSO. The absorbance at 570 nm was determined using a *Synergy H1 Hybrid Multi-Mode Microplate Reader*. Triplicate wells were assayed for each condition.

2.1.1.5 **Treatment with inhibitors and small molecules**

In order to study bestrophin-1 stability proteasome inhibitors or lysosomal proteases inhibitors were used as follow.

Stable MDCKII cells or HeLa cells expressing WT or mutant bestrophin-1 proteins were incubated with either 10 \( \mu M \) proteasomal inhibitor, benzyloxy carbonyl-Leu-Leu-phenylalaninal (Z-LLF-CHO) also known as proteasome inhibitor II (Merck Chemicals Ltd) or a combination of lysosomal protease inhibitors, 100 \( \mu M \) leupetin (Enzo Life sciences) and 1 \( \mu g/ml \) of pepstatin A (Sigma) for 5-6 hours. When 25nM Bortezomib was used as proteasome inhibitor, the incubation time was of at least 16-24 hours.

In order to test the ability of small molecules (see *Error! Reference source not found.*.6) to restore bestrophin-1 stability and function, chemical chaperones or
proteostasis regulators were added to the cultured media of MDCKII cells or HEK cells 24 h prior to harvesting, fixation or patch clamp analysis.

2.1.2 Nucleic acid procedures

2.1.2.1 RNA extraction from mammalian cells

In order to study bestrophin-1 mRNA expression level in stable MDCKII and HeLa cell lines, total RNA was extracted using TRIzol® Reagent (AMBIION, Life Technologies) according to manufacturer instructions. Briefly cells were harvested in TRIzol® Reagent by using cell scrapers to help cells lysis. Chloroform was then added to allow separation into three phases: a clear upper aqueous layer (containing RNA), a white interphase (containing DNA) and a red lower organic layer (containing proteins). After isolation of the aqueous phase, isopropanol was added in order to precipitate RNA. The precipitated RNA was washed to remove impurities, dried, resuspended in DNase/RNase free water and DNase treated with TURBO DNA-free™ kit (AMBIION), before use in downstream applications. First-strand cDNA was generated form 2 µg of RNA by using the High capacity RNA-to-cDNA kit (Applied Biosystems) according to manufactures instructions.

2.1.2.2 RNA/DNA concentration measurements

The measurement of RNA/DNA concentration was performed by using a NanoDrop spectrophotometer (Nanodrop Technologies, USA).

2.1.2.3 PCR

In this study the polymerase chain reaction (PCR) was used to investigate bestrophin-1 expression or to sub-clone bestrophin-1 constructs from the pAdlox vector to pcDNA5/FRT/TO.

PCRs were performed using the ReddyMix™ custom PCR Master Mix (Abgene). PCRs were (Abgene). PCRs were prepared in 0.2 thin-walled PCR tubes. The standard PCR reaction included:
reaction included: 1x ReddyMix\textsuperscript{TM} master mix; 0.5\,\mu\text{M} forward and reverse primers; 20 ng of primers; 20 ng of DNA; MQ water. A G-STORM\textsuperscript{TM} GS2 multi block Thermal Cycler was used to perform the reactions. The cycling parameters are indicated in Table 2.2.

<table>
<thead>
<tr>
<th>Duration</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1</td>
</tr>
<tr>
<td>30 seconds</td>
<td>94°C</td>
<td></td>
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<tr>
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<td>60°C</td>
<td>15</td>
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<tr>
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<td>72°C</td>
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<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>store</td>
<td>4°C</td>
<td>infinite</td>
</tr>
</tbody>
</table>

2.1.2.4 \textbf{TA cloning}

The pcDNA5/FRT/TO\textsuperscript{©} TOPO\textsuperscript{®} TA Expression Kit (Invitrogen) was used to clone PCR products into TA vectors, according to the manufacturer’s instruction. Analytical restriction digest with KpnI restriction enzyme (Roche) was performed to test for the orientation of the insert in the pcDNA5 FRT/TO plasmids following TA cloning.

2.1.2.5 \textbf{Side directed mutagenesis (SDM)}

SDM was performed to incorporate desired mutations into target DNA sequences using the KOD Hot start SDM KIT (Toyobo, Novagen) according to manufacturer instructions. Briefly 50 \,\mu\text{l} of reaction mix contained 20 ng pcDNA template, 0.3 \,\mu\text{M} of forward and reverse mutagenic primers (see Error! Reference source not found.), 1U KOD hot start DNA polymerase, 1X KOD buffer, 2 mM dNTPs (each) and 2.5 mM Mg\textsubscript{2}SO\textsubscript{4}. PCRs were performed on a Techne Thermal Cycler TC-312. The cycling parameters are indicated in Table 2.3. Samples were then digested with 10 units of DpnI restriction enzyme to eliminate any residual template. DH5\alpha competent cells were transformed with the newly synthesised pcDNA and colonies were screened for the presence of the desired mutation.
Table 2.3 Thermo cycler settings for SDM

<table>
<thead>
<tr>
<th>Duration</th>
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<th>Cycles</th>
</tr>
</thead>
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<tr>
<td>5 minutes</td>
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<td>1</td>
</tr>
<tr>
<td>PAUSE (add KOD polymerase)</td>
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<tr>
<td>30 seconds</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>1 minute</td>
<td>55°C</td>
<td>15</td>
</tr>
<tr>
<td>7 minutes</td>
<td>70°C</td>
<td></td>
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<tr>
<td>15 minutes</td>
<td>70°C</td>
<td>1</td>
</tr>
<tr>
<td>store</td>
<td>4°C</td>
<td>infinite</td>
</tr>
</tbody>
</table>

2.1.2.6 Transformation of TOP10/ DH5α chemically competent E. Coli

One Shot® TOP10 Chemically Competent E. Coli kit (Invitrogen) was used to transform plasmid generated with the TA cloning into TOP10 chemically competent E. Coli using the manufacturer’s protocol. Briefly, a mixture of fresh PCR products, salt solution, sterile water and the TOPO® vector were added into a vial of One Shot® TOP10 Chemically Competent E. coli. Cells were heat-shocked for 30 seconds at 42 °C and immediately transferred to ice. 250 μl of antibiotic-free SOC medium was added and tubes were incubated at 37 °C (200 rpm) for 1 h. 20-100 μl from each transformation reaction were spread on a pre-warmed selective plate and incubated overnight at 37 °C. At least 20 colonies from each plate were screened for the presence of the desired construct.

DH5α chemically competent E.Coli was used to transform plasmid generated with any other application with the same protocol used for TOP10 cells.

2.1.2.7 Plasmid DNA extraction and purification

In order to extract the plasmid DNA from successfully transformed E. Coli, individual colonies from LB-agar + ampicillin plates were inoculated in LB broth supplemented with 100 μg/ml ampicillin and grown overnight at 37 °C (215 rpm). Plasmid DNA was extracted using plasmid preparation kits according to manufacturer’s instruction. The QIAprep mini kit® and the QIAprep midi kit® were used to extract DNA from 5 ml and 50 ml bacterial cultures respectively.
2.1.2.8 **DNA Sequencing**

Sequence analysis of plasmid DNA using CMV forward or RGH reverse universal primers was performed by GATC biotech (http://www.gatc-biotech.com). Sequencing reactions with customised primers (see **Error! Reference source not found.**) were performed by the DNA sequencing facility at the University of Manchester.

2.1.3 **Protein procedures**

2.1.3.1 **Immunofluorescence microscopy**

Immunofluorescence was used to investigate bestrophin-1 localisation in stable HeLa cells. HeLa cells were grown on 13 mm glass coverslips and either fixed with 3% Paraformaldehyde (PFA) in PBS (20’ at RT). Cells were then washed with PBS, quenched with 1 ml PBS with 4 drops of 1 M glycine, pH 8.5, for 5 min at RT, permeabilised with 0.1% Trion X-100 for 10 min. Following permeabilisation cells were subjected to immunofluorescence staining as described by (Allan 2000). Briefly, coverslips were incubated face down on a 30 µl drop of primary antibody solution in PBS for 30 min at RT. Coverslips were then rinsed in PBS and washed for in PBS. Cells were then incubated as above with fluorescent secondary antibodies and DAPI in PBS for 30min, preventing exposure to light as much as possible, and were mounted on microscope slides with 7 µl mowiol solution.

Samples were analysed with an Olympus BX60 microscope using a X60 or X100 objective, and images were taken on a CoolSNAP EZ camera (Photometrics, USA) using MetaMorph 7.5.6.0 software (MDS Analytical Technologies, USA).

2.1.3.2 **Confocal Immunofluorescence microscopy**

Confocal immunofluorescence microscopy was used to investigate bestrophin-1 localisation in stable polarised MDCKII cells prior to or after treatment with small molecules.
MDCKII cells grown on transwell® polyester membrane inserts (pore size 0.4; Corning) were washed with phosphate-buffered saline (PBS), fixed with 3 % paraformaldehyde (in PBS) for 20 min at RT. Cells were then washed with PBS, quenched with 1 ml PBS with 4 drops of 1 M glycine, pH 8.5, for 5 min at RT, permeabilised with 0.1 % Trion X-100 for 10 min and washed with PBS. Following fixation cells were incubated with the appropriate primary antibody in PBS for 1 h and washed for 5 min with PBS (x3) before being incubated with the appropriate secondary antibody and 100 ng/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 30 min, preventing exposure to light as much as possible. Transwell filters were then cut out from the inserts and mounted onto microscope slides with 7 µl of mowiol solution or Prolong Gold® anti-fade reagent (Life Technologies) and sealed with nail varnish.

Images were collected using a Nikon C1 confocal on an upright 90i microscope with a 60x/1.40 Plan Apo objective and 3x confocal zoom. The confocal settings were as follows: pinhole 30 µm, scan speed 400 Hz unidirectional, format 1024 x 1024. Images for DAPI, FITC and Texas red were excited with the 405 nm, 488 nm and 543 nm laser lines respectively. When it was not possible to eliminate cross-talk between channels, the images were collected sequentially. When acquiring 3D optical stacks the confocal software was used to determine the optimal number of Z sections. Only the maximum intensity projections of these 3D stacks are shown in the results.

2.1.3.3 SDS-PAGE and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In order to investigate bestrophin-1 expression and stability in MDCKII/HeLa/HEK cells, protein samples were extracted either in SDS sample buffer with 100 mM DTT to reduce disulphide linkages or in IP buffer + 0.1 % Trion X-100+ 1 mM PMSF protease inhibitors. Samples were heated for 10 min at 70 °C, loaded onto a 12 % Tris-glycine polyacrylamide gel and run alongside ColorPlus prestained protein marker (broad range 7-175 kDa, New England Biolabs, USA) on 8-10 % acrylamide gel made of stacking and separative
solutions (see 2.2.1) at 30 mA in running buffer (25 mM Tris, 190 mM glycine, 0.08 % (w/v) SDS) until sufficiently resolved.

Proteins were then transferred onto nitrocellulose membranes (LI-COR, USA) by wet transfer at 300 mA for 1 h in transfer buffer. Membranes were blocked in 5 % milk (MARVEL, UK) in TBS for 1 h before incubation with primary antibody in 2 % milk-TBS + 0.01 % NaN solution overnight at 4 °C, with constant mixing. Membranes were washed 3 times for 5 min each in TBS before labelling with fluorescently labelled secondary antibodies in 2 % milk-TBS for 1 h at RT. Membranes were scanned at Odyssey Infrared Imaging System (LI-COR, USA) and quantified using Odyssey Sa software (LI-COR, USA).

2.1.3.4 *Co-Immunoprecipitation*

Co-immunoprecipitation was used to investigate bestrophin-1 binding partners. Cells were rinsed with chilled PBS then incubated with 20 mM N-Ethylmaleimide (NEM) for 5’ on ice to avoid formation of disulphide bond in cytosolic proteins during lysis. Cells were lysated with IP lysis buffer for 30 minutes on ice, then centrifuged for 10 minutes at 10,000 rpm at 4 °C to pellet insoluble material. When interaction with Hsp70 was investigated, 10 U/ml apyrase was added to the lysis buffer to prevent Hsp70 dissociation. 10 % of the supernatant was taken as control of total protein loading. SDS sample buffer was added and samples were heated at 70 °C for 10 minutes. 0.5-1 µl of primary antibody was added to the rest of the lysate (90 %), which was incubated 3.5 h-O/N at 4 °C on a rotator. Lysate were incubated with 10 µl of proteinA/protein G sepharose beads for 2-3 hours at 4 °C to allow primary antibody binding to the beads. Beads were then precipitated by centrifugation at 1000 rpm for 30-60 seconds to isolate the protein of interest and the supernanant was discarded. Pellet was washed with IP lysis buffer for 3 times then 10-20 ul of 2 x SDS sample buffer was added. Samples were heated at 70 °C for 10 minutes to elute the protein and SDS PAGE and western blotting were performed.
2. Materials and methods

2.1.3.5 **Biotinylation of cell surface proteins**

In order to investigate whether bestrophin-1 was trafficked to the plasma membrane of HEK cells, a biotinylation assay was performed as follow.

HEK293 cells cultured into 6/10 cm dishes were co-transfected with either WT or mutant or mutant bestrophin-1 constructs in pAdLox and GFP (see 2.1.1.2 and ). After treatment with compound of interest, cells were pre chille on ice and labelled ith 0.5 mg/ml EZ-Link™ Sulfo-NHS-SS-biotin (Pierce Biotechnology) in PBS/C (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) for 30 minutes. Cells were then washed with PBS/CM and 50 mM NH₄Cl in PBS/CM was used to quench unreacted biotin. Cells were lysed in biotin lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1.25 % triton X-100, 0.25 % SDS, 1 mM PMSF) and cell lysates were centrifuged at 10000 rpm for 15 min to remove non-soluble debris. 10 % of the supernatant was kept for control of total protein loading and the rest of the lysate was incubated with NeutrAvidin beads (Thermo Scientific) for 2.5 h. Beads were washed 3 times in biotin lysis buffer, resuspended in 2X SDS sample buffer and analysed by SDS-PAGE and western blotting.

2.1.3.6 **Concavalin A binding assay**

Concavalin A binding assay was used in this study to test whether bestrophin-1 was subjected to post-translational modification, specifically mannosylation, in MDCKII cells.

In particular stable MDCKII cells expressing WT bestrophin-1 were lysed in 1 ml IP buffer for 30 minutes on ice. Lysates were centrifuged at 10000 rpm at 4 °C for 15 minutes to remove non-soluble debris. 10 % of the supernatant was kept for control of total protein loading. The resulting supernatant was incubated with 120 μl (25 % v/v) concanavalin A sepharose beads (Sigma) for 1 h on a roller at room temperature. The beads were washed 3 times with IP buffer, each time collecting the beads by centrifugation at 1000 rpm for 1 minute. Bounded glycoproteins were eluted from the beads by incubation with 100 μl 0.25 M α-
methylmannosidase (Sigma) in IP buffer for 1 h at 25 °C with shaking. Samples were analysed by SDS-PAGE and western blotting.

2.1.4 **Electrophysiology**

2.1.4.1 **MDCKII cells polarisation and TEER measurement**

The integrity of polarised MDCKII cells monolayers was checked by the measurement of Trans Epithelial Electrical Resistance (TEER). MDCKII were grown on transwell® polyester membrane inserts (pore size 0.4; Corning) for 3-5 days to allow cell polarisation. The medium in the upper and lower chamber was changed daily. EVOM - Epithelial Voltohmmeter (World Precision Instruments Inc, USA) was used to assess trans-epithelial resistance (TEER) according to manufacturer instructions.

2.1.4.2 **Whole cell patch clamp**

Whole cell patch clamp was used to measure the sum activity of bestrophin-1 channels at the plasma membrane of HEK293 cells transfected with WT or mutant bestrophin-1 constructs.

Whole cell voltage-clamp recordings were performed using borosilicate glass capillaries “GC100F-10” (Harvard Apparatus, Edenbridge, UK) which were pulled with a Model P-97 pipette puller (Sutter Instrument CO, Novato, USA) and fire polished to a resistance of 1.3-2 MΩ using a microforge. The electrophysiology setup consisted of a MultiClamp 700 B amplifier controlled by pCLAMP 10.4 (Molecular Devices, Sunnyvale, USA). Recordings were sampled at 20 kHz and filtered online at 20 kHz. The intracellular solution (317.6 osmol) contained: 20 mM CsCl, 10 mM EGTA, 7.2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM Glucose, 110 mM CsAspartate, pH 7.2 with CsOH. The extracellular solution (339 osmol) contained: 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM Glucose, 30 mM Mannitol, pH 7.4 with NaOH. Only cells showing a GFP signal were used for assessment of current magnitudes.
2.1.5 Data analysis

Quantitative data collected from at least three separate experiments were plotted as means with error bars indicating standard error of the mean (±SEM). Statistically significant differences among groups were identified by one-way or two-way ANOVA, followed respectively by Bonferroni or Tukey multi-comparison test, using GraphPad Prism 2D graphing and statistics software (GraphPad, La Jolla, USA).

2.2 Materials

Table 2.4 Constructs used/generated during this study

<table>
<thead>
<tr>
<th>Construct</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA5/FRT/TO BEST-1 WT</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 L41P</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 L140V</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 R141H</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 A195V</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 R202W</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO BEST-1 M325T</td>
<td>Generated during this study</td>
</tr>
<tr>
<td>pAdlox BEST-1 WT</td>
<td>Forbes Manson (UoM)</td>
</tr>
<tr>
<td>pAdlox BEST-1 L41P</td>
<td>Forbes Manson (UoM)</td>
</tr>
<tr>
<td>pAdlox BEST-1 L140P</td>
<td>Forbes Manson (UoM)</td>
</tr>
<tr>
<td>pAdlox BEST-1 R141H</td>
<td>Forbes Manson (UoM)</td>
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<tr>
<td>pAdlox BEST-1 A195V</td>
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<td>pAdlox BEST-1 R202W</td>
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<td>pAdlox BEST-1 M325T</td>
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UoM = the University of Manchester
### Table 2.5 Primers for PCR and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<td>H-BEST-1 Rev ApaI</td>
<td>5’-GGGCCCTTATAGGATGCTTATCCCTGT-3’</td>
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<td>H-BEST-1 Fwd 696bp</td>
<td>5’-CCACCTGTTACATACACAG-3’</td>
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<tr>
<td>H-BEST-1 Rev 1630bp</td>
<td>5’-ATCCGTCAGTTAAACTCC-3’</td>
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<tr>
<td>H-BEST-1 Fwd 279bp</td>
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### Table 2.6 List of primary antibodies

<table>
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<th>Primary antibody</th>
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<th>Dilution (use)</th>
<th>Source</th>
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<td>Bestrophin-1 (E6)</td>
<td>Mouse</td>
<td>1:4000 (WB)</td>
<td>Novus biological</td>
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<td></td>
<td></td>
<td>1:500 (IF)</td>
<td></td>
</tr>
<tr>
<td>MCT-1</td>
<td>Rabbit</td>
<td>1:4000 (WB)</td>
<td>Abcam</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500 (IF)</td>
<td></td>
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<tr>
<td>LAMP1</td>
<td>Rat</td>
<td>1:500 (IF)</td>
<td>DSHB, University of Iowa</td>
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<tr>
<td>ZO1</td>
<td>Rat</td>
<td>1:500 (IF)</td>
<td>DSHB, University of Iowa</td>
</tr>
<tr>
<td>Actin</td>
<td>Mouse</td>
<td>1:3000 (WB)</td>
<td>Abcam</td>
</tr>
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<td>Calreticulin</td>
<td>Rabbit</td>
<td>1:100 (IF)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>HA</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
<td>Sigma</td>
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</table>

**IF** = ImmunoFluorescence microscopy; **WB** = western blotting

### Table 2.7 List of secondary antibodies

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<th>Antibody</th>
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<th>Host Species</th>
<th>Dilution (use)</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>IRDye® 800CW(G)</td>
<td>α-rabbit</td>
<td>Donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye® 680CW(R)</td>
<td>α-mouse</td>
<td>Donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye® 680CW(R)</td>
<td>α-rabbit</td>
<td>Goat</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye® 800CW(G)</td>
<td>α-mouse</td>
<td>Donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>DyLight 488 (G)</td>
<td>α-rabbit</td>
<td>Donkey</td>
<td>1:500 (IF)</td>
<td>Jackson</td>
</tr>
<tr>
<td>DyLight 594 (R)</td>
<td>α-rat</td>
<td>Donkey</td>
<td>1:200 (IF)</td>
<td>Jackson</td>
</tr>
<tr>
<td>DyLight 594 (R)</td>
<td>α-mouse</td>
<td>Donkey</td>
<td>1:200 (IF)</td>
<td>Jackson</td>
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<tr>
<td>Alexa Fluor® 594</td>
<td>α-rabbit</td>
<td>Donkey</td>
<td>1:200 (IF)</td>
<td>Life technology</td>
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<td>Alexa Fluor® 488</td>
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<td>Donkey</td>
<td>1:500 (IF)</td>
<td>Life technology</td>
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</tbody>
</table>

**IF** = ImmunoFluorescence microscopy; **WB** = western blotting
Table 2.8 List of inhibitors

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<tr>
<th>Inhibitor</th>
<th>Stock conc.</th>
<th>Dilution</th>
<th>Final conc</th>
<th>Inhibit</th>
<th>Source</th>
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</thead>
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<tr>
<td>Leupeptin</td>
<td>50 mM</td>
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<td>Enzo Life Sciences</td>
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<tr>
<td>Pepstatin</td>
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<td>1:1000</td>
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<tr>
<td><strong>Proteasome Inhibitor II</strong></td>
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<td>Proteasome</td>
<td>Calbiochem</td>
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<tr>
<td>Bortezomib</td>
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<td>1:400</td>
<td>25 nM</td>
<td>Proteasome</td>
<td>Selleckchem.com</td>
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Table 2.9 List of small molecules and concentrations

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<th>Source</th>
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<td>4-phenylbutyrate (4-PBA)</td>
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<td>Sigma</td>
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<tr>
<td>17-N-Allylamino-17-demethoxygedanamycin (17-AAG)</td>
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<td>PR</td>
<td>Sigma</td>
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<tr>
<td>Trimethylamine N-oxide (TMAO)</td>
<td>50 mM</td>
<td>CC</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tauroursodeoxycholic acid (TUDCA)</td>
<td>1 mM</td>
<td>CC/PR</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

CC chemical chaperone; PR proteostasis regulator
2.2.1 General buffers and solutions

**IP buffer**
140 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4

**IP lysis buffer**
IP buffer, 1 % Triton X-100, 1 mM PMSF

**LB Broth**
172 mM NaCl, 1 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract

**LB Broth Agar**
172 mM NaCl, 1 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract, 1 % (w/v) agar

**Phosphate-buffered saline (PBS) 1X**
137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4 with HCl

**Protein A/Protein G sepharose**
50 % Protein A/Protein G sepharose were prepared in IP buffer/TX-100 and pre-blocked with 20 % BSA in PBS

**Running buffer 1X**
25 mM Tris, 192 mM Glycine, 0.08 % (w/v) SDS

**SDS sample buffer 1X**
0.01 % bromophenol blue, 31.25 mM Tris, 2% (w/v) SDS, 5 % (v/v) Glycerol, 100 mM DTT, pH 7.6

**Tris-acetate-EDTA (TAE) 1X**
40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8

**Transfer buffer 1X**
20 mM Tris, 150 mM Glycine, 20 % (v/v) Methanol

**Tris-buffered saline (TBS) 1X**
20 mM Tris base, 150 mM NaCl, pH 7.4 with HCl
### 2.2.2 Bioinformatics tools

<table>
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<td>Multiple DNA/protein sequences alignment</td>
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<tr>
<td>Oligo Analyser</td>
<td><a href="https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/">https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/</a></td>
<td>Analysis of physical properties of oligo sequences</td>
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3 Generation of a cell model to study bestrophin-1 trafficking

3.1 Introduction

The overall aim of this PhD was to examine the ability of small molecules to rescue mutant bestrophin-1 expression, localisation and function. In order to achieve this, a cellular model that reproduce the correct physiological localisation of bestrophin-1 as observed in human RPE was needed.

Although there are few RPE cell lines available that could be used for this purpose, they have several disadvantages. RPE cells rely on the interaction with photoreceptors for proper development and vice-versa (Amirpour et al. 2013 Marmorstein, 1998 #448 Rizzolo, 1997 #449; Rizzolo 1997; Marmorstein et al. 1998). It is possible to fully differentiate RPE cell in vitro by growing cells at specific condition. However it can take months before the cells acquire most of the characteristics of RPE cells, such as synthesis and accumulation of melanin into melanosomes, or expression of RPE specific proteins which is usually induced by factors secreted by the neural retina (Sun et al. 2008; Ablonczy et al. 2011). Furthermore changes of the culture conditions may induce RPE cells to transdifferentiate, therefore generating a mixture culture of immature and mature RPE phenotypes (Zhao et al. 1997; Ganti et al. 2007; Burke 2008; Pratt et al. 2008). It has been shown that some cell lines, such as ARPE-19, fail to express bestrophin-1 (Marmorstein et al. 2000). Although bestrophin-1 expression can be restored by transient transfection or virus-mediated gene transfer of BEST-1, both these approaches have limitations including low transfection/transduction efficiency, which would introduce a significant variable in every experiment and within the same experiment.

For these reasons the first goal of this project was to find a simple cellular model that is easier to manipulate than RPE cells. The first cell line considered to this end was HeLa. HeLa cells are widely used in research thanks to their high proliferation rate and because they are easily transfected. Previous work in the
Manson lab has shown that, in transiently transfected HeLa cells, WT bestrophin-1 is not localised at the plasma membrane (Forbes Manson, personal communication). This could be explained by the different nature of the cell lines. RPE cells have in fact a well-defined apical-basal polarity compared to HeLa cells which do not polarise. This difference in cell structure might allow bestrophin-1 to be processed and trafficked in different ways between the two cell lines. However another possible explanation could be that the expression level of the protein in transiently transfected cells is sufficiently high that it could interfere with its sorting. On the contrary stable transfection would allow a lower level of protein to be expressed and possibly to be correctly processed by the cell (Dalton and Barton 2014).

Among the different cell lines previously tested in the Manson lab such as HEK293, ARPE-19, hTERT RPE-1 and MDCKII, the latter showed a basolateral expression of WT bestrophin-1 following transient transfection (Davidson et al. 2011). MDCKII cells are epithelial cells with a clear apical-basolateral polarity. Although the orientation of the polarity in vivo is determined by the extracellular matrix, MDCKII cells grown in vitro on porous transwell filters orientate the basal surface against the filter (Mostov et al. 2003). In addition to this, these cells have well defined cell junctions, a rapid growth rate and thanks to their capability of growing in polarised monolayer, they are suitable for confocal analysis thus ideal for cellular trafficking studies (Dukes et al. 2011; Cao et al. 2012).

For these reasons both HeLa and MDCKII were used to generate stable cells expressing BEST-1 under the control of a tetracycline-inducible promoter. To this end the recombinase-base integration Flp-in T-REx system was used.

3.2 Generation of HeLa stable cell lines expressing WT or mutant bestrophin-1 proteins

The Flp-in T-REx system takes advantage of the of the Saccharomyces cerevisiae-derived DNA recombination system to allow a single copy of the gene of interest to be integrated into the genome of the host cell line (Ward et al. 2011). The gene of interest can be introduced in the genome of parental cell lines containing the Flp Recombination Target (FRT) site by co-transfection of the
pcDNA5/FRT/TO expression vector, and the pOG44 plasmid, which constitutively expresses the Flp recombinase (Fig. 3.1 A). Cells that have integrated the construct can then be selected for hygromycin resistance and zeocidin sensitivity.

The first step in generating HeLa stable cell lines was to establish optimal conditions for transfection. A range of commercially available transfection reagents (Lipofectamine LXT, GeneCellin, JetPEI and PEI) were tested for their ability to transfect a GFP construct in parental HeLa cells. Lipofectamine was the most effective among all reagents with ~80% of cells successfully transfected. As the following step in generating cell lines with the Flp-in T-REx system is the ability to select stably transfected cells using hygromycin, an assay of cell viability, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, was performed on parental cell lines in order to identify the minimum concentration of hygromycin needed to select stably transfected cells. HeLa parental cells were treated with increasing concentrations of hygromycin and viability was determined using MTT assay at 24 h, 48 h and 72 h following treatment. As a result, 200 µg/ml was selected as the minimum concentration needed to kill the majority of non-transfected cells within 72 h.

Once conditions for transfection and selection were established, parental HeLa T-REx cells were transfected with WT, p.L41P, p.L140V, p.R141H, p.A195V, p.R202W and p.M325T bestrophin-1 constructs (Fig. 3.1 B) and stably transfected cells were selected with hygromycin and blasticidin for 3-4 weeks. In the following two weeks, few hygromycin resistant colonies were observed for HeLa cells transfected with WT, p.L41P, p.L140V, p.R141H and p.M325T bestrophin-1 constructs. The stable HeLa cell lines generated were at this point expanded for storage and a portion of each cell line was tested for bestrophin-1 expression.

3.2.1 Expression of WT and ARB-associated bestrophin-1 proteins in stable HeLa cells

In order to test the expression of WT, p.L41P, p.L140V, p.R141H and p.M325T bestrophin-1 proteins, a western blot was performed as follow. HeLa cells were treated with tetracycline for 16-24 h to induce bestrophin-1 expression or cells were left un-induced prior to direct lysis in SDS sample buffer. Cell lysates were
separated on reducing SDS-PAGE gels, transferred onto a nitrocellulose membrane and blotted with an anti-bestrophin-1 antibody raised against the protein C-terminus. An anti-tubulin antibody was used as loading control. Infrared labelled secondary antibodies were used to visualise immunoreactive bands at the Odyssey® CLx Infrared Imaging System. In the absence of tetracycline no immunoreactive bands were detected with the anti-bestrophin-1 antibody in lysates of any of the five cell lines tested (Fig 3.2A, lanes 1, 3, 5, 7 and 9). However, an immunoreactive band of the expected molecular mass for bestrophin-1 was detectable in lysates of WT cells, and more weakly in p.L41P, p.L140V, p.R141H and p.M325 bestrophin-1 cells following induction (Fig 3.2A, lanes 2, 4, 6, 8 and 10).

The lower level of mutant bestrophin-1 expression indicated that possibly these mutant proteins were unstable and perhaps degraded more rapidly than the WT thus leading to a lower level of detectable protein following induction. To test this, the effect of proteasome and lysosomal protease inhibitors on the steady state level of WT and mutant bestrophin-1s was investigated. Stable cells were induced for 16-24 h, and treated with the proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leucinal (PSII) or a combination of lysosomal protease inhibitors, leupeptin A and pepstatin, for 6 h. Treatment with proteasome inhibitors resulted in a marked increase in protein levels of p.L41P, p.L140V, p.R141H and p.M325T bestrophin-1 compared to untreated cells (Fig. 3.2 C-F, compare lanes 2 and 4). A significant increase in the WT protein following proteasome inhibition could also be observed (Fig 3.2 B lane 4 and G). In contrast, inhibition of lysosomal proteases did not result in higher levels of mutant bestrophin-1 proteins (Fig. 3.2 C-F, lane 3). These results suggested that mutant bestrophin-1 proteins were not stable when expressed in HeLa cells and were degraded by a proteasome mediated pathway. A modest increase of WT bestrophin-1 level could be observed following lysosomal protease inhibitors treatment (Fig. 3.2 B, lane 3), however this was not significant according to the One-Way ANOVA and the Tukey multiple comparison tests (Fig 3.2 G).
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.1 Principal steps in the generation of Flp-In T-REx expression cell lines.

A. Integration of the plasmid containing the Flp Recombination Target (FRT) site and the plasmid expressing the Tet repressor. Co-transfection of the pcDNA5/FRT/TO/BEST1 expression vector and the pOG44plasmid in HeLa T-REx cells (B) (transfection procedure according to manufacturer instruction) or MDCKII T-REx cells (inverted transfection) (C).
3. Generation of a cell model to study bestrophin-1 trafficking

**Figure 3.2 Expression of WT and ARB-causing bestrophin-1 in stable HeLa cells.**

WT or mutant p.L41P, p.L140V, p.R141H, and p.M325T bestrophin-1 expression was induced in stable HeLa cells with tetracycline (Tet) for 24 h before direct lysis in SDS sample buffer for WB analysis (A). To test whether ARB mutants were recognised by quality control machinery in the ER and targeted for degradation by the proteasome, cells were treated with lysosomal protease inhibitors (LI) and proteasome inhibitors (PI) for 6 h before direct lysis in 1X sample buffer (B-F). An anti-tubulin antibody was used as a loading control. Quantification of WT bestrophin-1 protein levels following treatment with LI and PI is shown in (G). Results are presented as mean ± SEM from at least three independent experiments. Protein levels are normalised by tubulin and are expressed relative to +T. **p<0.01 indicate significant differences between +T and +PI and *p<0.05 indicate significant differences between +LI and +PI.
3.2.2 Localisation of WT and ARB-associated bestrophin-1 proteins in stable HeLa cells

In order for HeLa cells to be a useful model for studying bestrophin-1, it is important that these cells were able to support proper folding and trafficking of WT bestrophin-1. Thus, the subcellular localisation of WT bestrophin-1 was examined by immunofluorescence microscopy to determine whether, in these cells, bestrophin-1 was localised at the plasma membrane. Cells were treated with tetracycline for 24 h to induce the expression of bestrophin-1 and then fixed in paraformaldehyde, detergent permeabilised and stained with anti-bestrophin-1 antibody and the ER marker calreticulin. WT bestrophin-1 was observed at the plasma membrane (Fig. 3.3, panel B) as seen for the native protein RPE cells (Marmorstein et al. 2000). However, some intracellular bestrophin-1 was also detected in punctate structures at the periphery of the cells and in the juxtanuclear compartment (Fig. 3.3, panel B1) suggesting that the WT protein might be recognised by cellular QC systems. It has been shown that part of WT bestrophin-1 localises to the ER in RPE cells where it is suggested to play a role in SOCE (Strauss et al. 2014; Strauss et al. 2016), however no obvious overlap with calreticulin could be observed for WT bestrophin-1 in HeLa stable cell lines (Fig. 3.3, panel B3). When degradation through the lysosomes was inhibited, punctate structures were more apparent, suggesting that a portion of WT bestrophin-1 is degraded by lysosomes (Fig. 3.3, panel C). On the contrary the addition of proteasome inhibitors did not seem to affect WT bestrophin-1 localisation dramatically; however an enrichment of the protein in the juxtanuclear compartment could be observed (Fig. 3.3, panel D). This is consistent with the previous data from the western blots which show that WT bestrophin-1 is stabilised by addition of proteasome inhibitors (Fig. 3.2 B and G). No signal was detected in the absence of tetracycline (Fig. 3.3, panel A), confirming the specificity of the immunofluorescence staining.
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.3 Localisation of WT bestrophin-1 in stable HeLa cells.

Fluorescent microscope images showing WT bestrophin-1 localisation in stable HeLa cells before (NI) or after induction with tetracycline (+tet), treatment with lysosomal proteases inhibitors (+tet +LI) or treatment with the proteases inhibitor PSII (+tet +PI). Cells were fixed in 4% paraformaldehyde, detergent permeabilised and stained with anti-bestrophin-1 antibody (green) and the ER marker calreticulin (red). The nuclei are stained in blue. The overlap of the bestrophin-1 and the calreticulin signal is shown in panel 3 (merge). Scale bar = 5um.
The localisation of ARB-causing bestrophin-1 mutants was then investigated in the stable cells lines following induction with tetracycline (Fig. 3.4, panel 1), addition of LI (Fig. 3.4, panel 2) or addition of PI (Fig. 3.4, panel 3). Interestingly different mutants had different patterns of localisation compared to the WT or to each other. Of the four mutants analysed, only p.L41P appeared to localise partly at the plasma membrane (Fig. 3.4, panel 1A) although the majority of this protein was localised intracellularly. p.L140V and p.R141H were observed intracellularly and most of the protein was concentrated in juxtanuclear structures (Fig. 3.4, panels 1B and C) resembling the ERQC compartment, a subdomain of the ER specialised for the degradation of misfolded proteins (Kuijpers et al. 2013; Leitman et al. 2013; Kamhi-Nesher et al. 2001; Leitman et al. 2014; Huang et al. 2014). p.M325T was also observed in the cell by the nucleus, however it seemed to be accumulated in punctuate structures (Fig. 3.4, panel 1D). When cells were treated with lysosomal protease inhibitors the formation of punctate structures similar to lysosomes could be detected in p.L41P and p.M315T cells (Fig. 3.4, panels 2A and D) but not in p.L140V and p.R141H cell lines (Fig. 3.4, panels 2B and C). Every cell line showed bestrophin-1 localisation at the juxtanuclear compartment following inhibition with PSII (Fig. 3.4, panel 3A-D).

Although the different localisation pattern of p.L41P and p.M325T compared to p.L140V and p.R141H seemed interesting, the identity of the different structures observed under different condition was not investigated any further since the localisation of WT bestrophin-1 was not consistent with the one observed in RPE cells. Most of the WT protein appeared, in fact, to localise to juxtanuclear structures, potentially the ERQC compartment (Kuijpers et al. 2013; Leitman et al. 2013; Kamhi-Nesher et al. 2001; Leitman et al. 2014; Huang et al. 2014), following induction with tetracycline. This suggests that a considerable proportion of WT bestrophin-1 is recognised by ER quality control system and targeted for degradation, possibly by ERAD. Therefore HeLa cells were considered an unsuitable model system to the purpose of this study. The next step was then to generate stable cells lines by using MDCKII cells, which as discussed above, share a lot of similarities with RPE cells thus suggesting that bestrophin-1 might be processed in a similar way in both cell lines.
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.4 Localisation of ARB-causing bestrophin-1 proteins in stable HeLa cells.

Fluorescent microscope images showing p.L41P, p.L140V, p.R141H and p.M325T bestrophin-1 localisation in stable HeLa cells following induction with tetracycline (+tet; panel 1), treatment with lysosomal proteases inhibitors (+tet +LI, panel 2) or treatment with the proteases inhibitor PSII (+tet +PI; panel 3). Cells were fixed in 4% paraformaldehyde, detergent permeabilised and stained with anti-bestrophin-1 antibody. Scale bar = 10 um.
3.3 **Generation and characterisation of MDCKII stable cell lines expressing WT or mutant bestrophin-1 proteins**

As with HeLa cells, the first step in generating MDCKII stable cell lines was to establish optimal conditions for transfection. Transfection efficiency was significantly lower in MDCKII cells; however Lipofectamine LTX was once again the most effective transfection reagent with a transfection efficiency of 13.5%.

Parental MDCKII T-Rex cells were transfected with WT, p.L41P, p.L140V, p.R141H, p.A195V, p.R202W and p.M325T bestrophin-1 constructs with Lipofectamine LTX following manufacturer’s instructions. No hygromycin resistant cells could be observed after 4 weeks of selection. As transfection and selection were repeated three times without success, a reverse transfection protocol was then optimised in order to improve the transfection efficiency (Fig. 3.1 C, chapter 2.2.1.3). Briefly parental MDCKII T-Rex cells were added directly into a plate containing the transfection mixture (Fig. 3.1 C, day 1) and after 24 h the transfection mixture was replaced with complete medium to allow cells to recover for an additional 24 h (Fig. 3.1 C, day 2). The selection medium containing hygromycin and blasticidin was then added (Fig. 3.1 C, day 3). The transfection efficiency increased up to 30% when the reverse transfection protocol was used. Although MDCKII cells were more sensitive to Lipofectamine toxicity using this approach, after three weeks of selection 1-5 small colonies were observed for cells transfected with WT, p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 constructs. The stable MDCKII cell lines generated were then expanded for storage and a portion of each cell line was tested for bestrophin-1 expression by western blot.

3.3.1 **WT and mutant bestrophin-1 expression in stable MDCKII cells**

WT and ARB-causing bestrophin-1 stable cell lines were tested for tetracycline-inducible expression of bestrophin-1 by western blot. Cells were grown for 5 days on transwell filters in order to allow polarization (Fig.3.5 A). The attainment of a correct polarisation was confirmed by measuring the trans-epithelial electrical resistance (TEER) (Benson et al. 2013), which for MDCKII cells should be around 300 $\Omega \cdot \text{cm}^2$ (Dukes et al. 2011). Briefly $12.5 \times 10^5$ cells were seeded onto
12 mm transwell filters and TEER was measured at 24 h intervals for up to 6 days (Fig. 3.5 B and C). After three days a peak in the TEER which marked the formation of tight junctions between cells could be observed. Subsequently, with the establishment of oriented vesicular trafficking, a decrease in the TEER could be observed until a steady state level was achieved between days 4 and 6. (Fig. 3.5 C). Once established that MDCKII cells were polarised by day 5, Bestrophin-1 expression in either WT or ARB-causing bestrophin-1 cell lines was induced with tetracycline for 24 h or cells were left uninduced. Cells were then harvested and lysates were analysed by SDS-PAGE and immunoblotting with anti-bestrophin-1 antibody. An anti-tubulin antibody was used as a loading control (Fig.3.6)

When the expression of bestrophin-1 was induced MDCKII cell lines stably transfected with WT, p.L41P, p.R141H, p.R202W and p.M325T constructs, an immunoreactive band of the predicted size for bestrophin-1 could be detected in each cell lysate (Fig. 3.6, lanes 2, 4, 6, 8 and 10 respectively). However the immunoreactive band corresponding to bestrophin-1 was weaker in p.R141H, p.R202W and p.M325 cell lysates compared to WT (Fig 3.6, compare lane 2 with lanes 6, 8 and 10) and lysates of p.L41P cells showed almost no immunoreactive band (Fig 3.6, lane 4). No immunoreactive bands were detected in the absence of tetracycline in any of the cell lysates (Fig 3.6, lanes 1, 3, 5, 7 and 9) confirming that transgene expression was tightly regulated and that MDCKII cells do not express detectable levels of endogenous bestrophin-1.
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.5 Polarisation and TEER in stable MDCKII cells.

MDCKII cells expressing WT bestrophin-1 cells were seeded onto 12 mm transwell filters (A, day 0). TEER was measured at 24 h intervals for up to 6 days by using an epithelial voltohmmeter (EVOM, B). The electrodes were placed so that the longer (external) electrode could touch the bottom whilst the shorter (internal electrode) was prevented from reaching the bottom of the tissue culture cup (B). After three days a peak in the TEER could be observed (A, day 3 and C). This step marks the onset of tight junction formation. With the establishment of oriented vesicular trafficking, the TEER reaches a steady state level between days 4 and 6 (A, day 5 and C)
3. Generation of a cell model to study bestrophin-1 trafficking

![Western Blot Image]

**Figure 3.6 WT and ARB-causing bestrophin-1 expression in MDCKII stable cell lines.**

WT, p.L41P, p.R141H, p.R202W and p.M325T cells were grown on transwell filters for 5 days to allow polarisation. Bestrophin-1 expression was then induced with tetracycline for 24 h before direct lysis in SDS sample buffer. WT, p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 protein expression level was investigated by western blot. An anti-tubulin antibody was used as a loading control. An immunoreactive band at 58 kDa corresponding to bestrophin-1 was observed for WT, p.R141H, p.R202W and p.M325T induced cells (lanes 2, 6, 8 and 10) but not for p.L41P lysates (lane 4). No immunoreactive band for bestrophin-1 was detected in non-induced cells (lanes 1, 3, 5, 7 and 9).
3.3.2 WT and mutant bestrophin-1 localisation in stable MDCKII cells

The localisation of bestrophin-1 in the stable MDCKII cells was then investigated by confocal immunofluorescence microscopy. Cells were grown on transwell filters for 5 days to allow cells to polarise and then bestrophin-1 expression was induced with tetracycline for 16-24h. Cells were fixed in paraformaldehyde, detergent permeabilised and stained with anti-bestrophin-1 and anti-monocarboxylate transporter 1 (MCT1) primary antibodies, followed by fluorescently labelled secondary antibodies for observation by fluorescence microscopy. Although MCT1 localises to the apical plasma membrane in RPE cells (Philp et al. 1998; Philp et al. 2003), this transporter localises to the basolateral plasma membrane in MDCKII cells through a clathrin-dependent pathway (Castorino et al. 2011). Therefore MCT1 was used in this study to assess correct basolateral localisation of WT or ARB-causing bestrophin-1 proteins in stable MDCKII cells. Confocal analysis showed that a large proportion of WT bestrophin-1 co-localised with MCT-1, thus was correctly trafficked to the basolateral plasma membrane of polarised MCDKII cells (Fig 3.7, panel B). A small amount of WT bestrophin-1 was also observed intracellular. No signal was detected for bestrophin-1 in the absence of tetracycline, confirming the specificity of the immunofluorescence staining (Fig 3.7, panel A). Although following induction with tetracycline the presence of mutant bestrophin-1 proteins was poorly detected by western blot (Fig. 3.6), a faint bestrophin-1 staining could be detected in the mutant cell lines by confocal immunofluorescence microscopy (Fig.3.7, panels C-F), even for the p.L41P mutant which was not detectable by immunoblot (Fig. 3.6, lane 4). None of the mutant proteins appeared to co-localise with MCT-1 at the basolateral plasma membrane, but they all seemed to accumulate inside the cell (Fig 3.7, panels C-F). In particular p.L41P and p.M325T seemed to localise to small punctate structures in the cytoplasm (Fig. 3.7 panels C and F). This was especially noticeable in the XZ plane (Fig. 3.7 panels B and E). p.R141H and p.R202W were also observed intracellularly, however they seemed to accumulate into elliptical structures of variable length, ranging approximately between 1-4µm (Fig. 3.7 panels D and E, with particular focus on the XZ plane).
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.7 Bestrophin-1 localisation in MDCKII stable cell lines.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline for 24 h before fixation with 4 % paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in stably transfected polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker the basolateral plasma membrane. Nuclei are stained with DAPI (blue). Scale bar = 5 µm. l=lateral; b=basal. NI=not-induced
In order to test whether the reduction of mutant bestrophin-1 proteins compared to the WT observed by western blot and imaging was a consequence of reduced mRNA levels, expression levels of WT and mutant bestrophin-1 transcripts were investigated by RT-PCR (Fig. 3.8). Comparable amount of bestrophin-1 mRNA could be detected in all cell lines following tetracycline induction (Fig 3.8 lanes 2, 4, 6, 8 and 10). Interestingly a low level of transcript was detectable in uninduced cells (Fig 3.8 lanes 1, 3, 5, 7 and 9) but not in the untransformed parental cell lines (Fig 3.8 lane 11) suggesting that the tetracycline promoter was slightly leaky. However the small amount of transcript produced in absence of tetracycline was not enough to produce detectable level of bestrophin-1 protein as shown previously by western blot (Fig. 3.6).

### 3.3.3 ARB-causing bestrophin-1 are degraded by the proteasome

Another possible explanation for the reduced expression of mutant bestrophin-1 protein compared to the WT is that ARB-causing bestrophin-1 proteins were being recognised by QC systems and targeted for degradation. In order to test this hypothesis bestrophin-1 expression was induced in stable cell lines for 24 h and then proteasome and lysosomal proteases inhibitors were added to the medium 6h prior to harvesting. Noticeably WT bestrophin-1 protein level was increased by almost three fold following PSII treatment (Fig. 3.9 A, compare lane 2 and 4; Fig. 3.9 F). This suggests that WT bestrophin-1 protein folds with suboptimal efficiency and that those WT proteins that are not able to achieve a native structure are recognised by ERQC and are targeted for degradation by the proteasome. Similarly to the WT, following the addition of PSII, bestrophin-1 could be detected in all lysates extracted from ARB-causing MDCKII stable cell lines (Fig. 3.9 B-E, lane 4). This observation suggests that mutant bestrophin-1 proteins were also recognised by ERQC and degraded by a proteasome dependent pathway, possibly ERAD (Fig. 3.9 B-E, lane 4). The addition of lysosomal protease inhibitors had no significant effect on either WT or mutant bestrophin-1 proteins stabilisation (Fig. 3.9 A-E, lane 3; Fig 3.9 F).
3. Generation of a cell model to study bestrophin-1 trafficking

Figure 3.8 Bestrophin-1 mRNA expression in MDCKII cells following induction with tetracycline.

WT and mutant bestrophin-1 cell lines were tested for the expression of bestrophin-1 prior to (1, 3, 5, 7, 9) or following induction with tetracycline (Tet) (lanes 1, 3, 5, 7, 9 and 2, 4, 6, 8, 10 respectively). Cells were grown on transwell filter for 5 days to allow polarisation. Cells were then harvested in Trizol for total RNA extraction. RT-PCR was then performed to check bestrophin-1 expression. T-REx parental cell lines were used as negative control while GAPDH was used as control for total cDNA.
Figure 3.9 ARB-causing bestrophin-1 are recognised and degraded by ERAD.

WT, p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 expression in polarised stably transfected MDCKII cells was induced with tetracycline for 24 h. Cells were then treated with lysosomal proteases inhibitors or proteasome inhibitors for 6 h before direct lysis in SDS sample buffer. WT (A) p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 protein expression level was investigated by western blot. An anti-tubulin antibody was used as a loading control. The blots showed are representative of at least three independent experiments. The quantification of the immunoreactive bands (F) shows that WT and mutant bestrophin-1 proteins are stabilised by PI (** = p<0.01). The addition of LI only stabilised WT bestrophin-1. Tet = tetracycline; LI = lysosomal protease inhibitors; PI = proteasome inhibitors.
3.4 Discussion

The aim of this PhD project was to test the ability of small molecules to rescue bestrophin-1 function. To this end, a cellular system that could be readily manipulated and was capable of supporting proper bestrophin-1 trafficking to the plasma membrane was required. The suitability of two well characterised cell lines, HeLa cells and MDCKII cells, was therefore investigated as alternative model systems. HeLa cells have been used for studying trafficking and quality control of a wide range of heterologously expressed membrane proteins (McKibbin et al. 2012; Roboti et al. 2009). The advantages of using HeLa cells are that they are easy to culture and can be conveniently manipulated using a variety of techniques. Therefore the Flp-In T-REx expression system was used to generate stable HeLa cell lines expressing WT and disease-associated bestrophin-1 proteins under the control of a tetracycline inducible promoter.

In HeLa cells stably expressing WT bestrophin-1, a small amount of bestrophin-1 was observed at the plasma membrane, suggesting that some of the protein was correctly trafficked. However, the majority of WT bestrophin-1 was observed in intracellular punctate structures resembling lysosomes. One possible explanation for this could be that although bestrophin-1 is correctly trafficked to the plasma membrane in HeLa cells, it fails to be retained at this location and instead is internalised along the endocytic pathway. This could be due to HeLa cells not being polarised, unlike RPE cells (Cao et al. 2012; Stoops and Caplan 2014; Lehmann et al. 2014). Another possible explanation is that a peripheral QC system is recognising misfolded bestrophin-1 proteins at the plasma membrane and is targeting them to lysosomes for degradation (Satpute-Krishnan et al. 2014). It is also possible that misfolded bestrophin-1 is recognised at the Golgi and is targeted to lysosomes for degradation, as observed with some misfolded secretory and membrane proteins (Cortes et al. 2013).

In the absence of inhibitors, mutant bestrophin-1 proteins were expressed at lower levels compared to the WT protein. Furthermore different patterns of localisation were observed for the different mutants compared to the WT and to each other, suggesting that the mutant bestrophin-1 proteins are recognised by different QC mechanisms (Kausalya and Hunziker 2011). A proportion of p.L41P,
for example, seemed to escape QC at the ER and was capable of reaching the plasma membrane. Nevertheless, accumulation of p.L41P bestrophin-1 in punctuate structures, potentially lysosomes, could also be observed suggesting that p.L41P might be recognised by QC at the plasma membrane or at the Golgi and targeted to lysosomes for degradation. This interpretation is not supported by the western blot data of stable cells treated with proteasome inhibitors which showed that this mutant is mostly degraded by a proteosomal pathway. Inhibition of lysosomal proteases did not significantly increase levels of the mutant protein. A possible explanation for not seeing an increased amount of bestrophin-1 following lysosomal protease inhibition could be that a 6 h incubation with lysosomal protease inhibitors is not be enough to detect a significant difference in protein level by western blot compared to non-treated cells.

Since no punctuate structures could be observed for p.L140V and p.R141H bestrophin-1 proteins either before or after lysosomal protease inhibitor treatment, it is likely that these mutants are recognised earlier in the QC process, possibly at the ER, and are targeted for degradation by a proteosomal pathway. This would be consistent with the increase in p.L140V and p.R141H protein levels observed following proteasome inhibition.

Similar to the p.L41P mutant, p.M325T bestrophin-1 localised to a juxtanuclear compartment in absence of inhibitors suggesting that p.M325T bestrophin-1 is also recognised by an early QC system. However, the formation of punctate structures following lysosomal protease inhibition also suggests that a proportion of the mutant protein escapes the first QC checkpoint. Nevertheless, since it was not possible to observe p.M325T mutant protein at the plasma membrane, this mutant might be recognised and targeted to lysosomes for degradation at other cellular QC checkpoints, for example those regulated by the Golgi.

Although HeLa cells were able to support proper folding and trafficking of a small amount of WT bestrophin-1, the localisation of most of the WT protein to punctuate structures in the cells, as well as at juxtanuclear regions, was not consistent with the localisation of bestrophin-1 in RPE cells. For this reason HeLa cells were considered unsuitable for the purpose of this project.
Previous studies in the Manson lab showed that bestrophin-1 is trafficked to the basolateral plasma membrane in transiently transfected MDCKII cells, thus replicating the localisation in human RPE (Davidson et al. 2011). MDCKII cells are epithelial cells that exhibit polarised sorting, with a clear apico-basolateral polarity and well defined cell junctions and they have been often used for cell trafficking studies (Dukes et al. 2011). Therefore the possibility to use MDCKII cells as a model system to study the effect of small molecules on ARB-causing bestrophin-1 protein stabilisation, trafficking and function was investigated. The Flp-In T-REx expression system was again used to generate stable MDCKII expressing WT and disease-associated bestrophin-1 proteins under the control of a tetracycline inducible promoter. Although the process of generating stable cell lines by using the Flp-In T-REx system in MDCKII cells was not as rapid and efficient as for HeLa cells, five stable cell lines expressing WT, pL41P, p.R141H, p.R202W and p.M325T were successfully generated.

Western blot analysis showed that the expression of bestrophin-1 was extremely low in cell lysates of ARB-causing MDCKII cells compared to the WT. This suggests that bestrophin-1 mutants are less stable than the WT and that mutant proteins are recognised by QC system in the cells and targeted for degradation. Consistent with this hypothesis, when the proteasome inhibitor PSII was added to the cell media, mutant bestrophin-1 levels increased significantly compared to non-treated cells. Interestingly, an increase in WT bestrophin-1 level could also be observed following proteasome inhibition. This suggests that WT bestrophin-1 folds with sub-optimal efficiency even in MDCKII and is targeted for degradation through the proteasome.

Confocal immunofluorescence microscopy confirmed that MDCKII cells were polarised and that WT bestrophin-1 was localised to the basolateral plasma membrane where it co-localised with MCT-1 thus reflecting its localisation in human RPE cells. A proportion of WT bestrophin-1 was also observed intracellularly suggesting that a fraction of the protein did not reach the cell surface. This supports the hypothesis that WT bestrophin-1 folding is prone to error in MDCKII cells and intracellular QC systems retain the non-native protein therefore targeting it for degradation. However, the intracellular localisation of bestrophin-1 may also reflect the native distribution of bestrophin-1. Recent work
has shown that a proportion of bestrophin-1 appears to localise to the ER in RPE cells (Brandl et al. 2014) where it may play a role in the SOCE (Gomez et al. 2013; Smyth et al. 2010). Nonetheless, the data reported here confirm previous findings that MDCKII cells are able to support proper folding and basolateral trafficking of WT bestrophin-1 thus providing evidence that MDCKII cells are a better cell model than HeLa cells for the aim of this project.

Confocal immunofluorescence microscopy revealed that p.L41P, p.R141H, p.R202W and p.M325T mutant bestrophin-1 failed to reach the basolateral plasma membrane in polarised MDCKII cells but were instead observed to accumulate intracellularly. Furthermore, mutant protein levels were dramatically reduced compared to WT thus confirming the western blot data and providing further evidence that mutant bestrophin-1 proteins are recognised by QC systems in MDCKII cells and targeted for degradation by the proteasome.

Taken together, the western blot data and the confocal analysis suggest that MDCKII cells are a suitable model system for the study of bestrophin-1 stabilisation and trafficking. MDCKII cells share a lot of similarity with RPE cells as both cell lines have characteristics typical of an epithelial cell line (Lehmann et al. 2014). Although some proteins, such as MCT-1, do not have the same polarised distribution in MDCKII cells compared to RPE (Philp et al. 2003; Philp et al. 1998), bestrophin-1 localisation replicates that observed in RPE cells suggesting that polarised sorting might be controlled by similar mechanisms in both cell lines.

Of note, the expression level of WT and mutant bestrophin-1 proteins was significantly increased by treating cells with the proteasome inhibitor PSII. This was observed in both HeLa and MDCKII stable cell lines and indicates that WT bestrophin-1 folds with suboptimal efficiency. This is not surprising as protein folding is often subjected to error (Valastyan and Lindquist 2014). The cell has developed multiple QC system to ensure that only correctly folded proteins are able to reach their final destination. Temporarily misfolded proteins are normally retained in the ER where a range of molecular chaperones support proper folding. One of the best known QC systems responsible for retaining non-native protein in the ER is the calnexin/calreticulin chaperone system. The recognition of proteins by calnexin/calreticulin relies on the presence of N-glycans. However, no N-
glycosylation sites are present in bestrophin-1; therefore the mechanism by which bestrophin-1 is either retained in the ER or targeted for degradation is still unclear. This could make bestrophin-1 a suitable candidate for the study of non-glycosylated protein QC.

In conclusion, in order to investigate the ability of small molecules to rescue ARB-causing bestrophin-1 expression and trafficking, the suitability of two cell model systems was investigated: HeLa cells and MDCKII cells. To this end stable cell lines expressing WT and p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 proteins under the control of a tetracycline operator were generated in each cell line. Although both cell lines were able to partially support, to a greater or lesser extent, the proper trafficking of WT bestrophin-1 to the plasma membrane, HeLa cells also showed intracellular accumulation of bestrophin-1 to punctuate structures resembling lysosomes. This suggests that a portion of WT bestrophin-1 was recognised by QC at the plasma membrane or at the Golgi and was targeted to the lysosomes. The implication of this is that HeLa cells are incapable of supporting proper bestrophin-1 trafficking, possibly because they are not polarised. On the contrary, bestrophin-1 localisation in MDCKII cells reflects that observed in human RPE cells, suggesting that MDCKII are able to support the proper trafficking of bestrophin-1. For this reason MDCKII are a more suitable model for the purpose of this study.
4 Use of small molecules to restore mutant bestrophin-1 trafficking and function

4.1 Introduction

Small molecules such as chemical chaperones, pharmacological chaperones and proteostasis regulators have been used in the last decade as an alternative to gene therapy to correct the loss- or gain-of-function defects of mutant proteins associated with misfolded protein diseases.

Chemical chaperones, for example, have been reported to ameliorate the symptoms of several ocular diseases caused by protein misfolding such as Leber congenital amaurosis and retinitis pigmentosa. The chemical chaperone tauroursodeoxycholic acid (TUDCA) has been shown to preserve the photoreceptor structure and function by enhancing degradation of S-opsin aggregates thus reducing ER stress in mouse model of Leber congenital amaurosis (Zhang et al. 2012). The chemical chaperone trimethylamine N-oxide (TMAO) instead has been shown to reduce the accumulation of toxic aggregates in lens epithelial cells by preventing the aggregation of mutant α-crystallin thus preventing cataract development (Gong et al. 2009). Similarly, sodium 4-phenylbutyrate (4PBA) interacts with exposed hydrophobic region of mutant myocilin thus preventing its aggregation and accumulation in trabecular meshwork cells in a mouse model of open angle glaucoma (Zode et al. 2012).

Proteostasis regulators, unlike chemical chaperones, do not specifically interact with disease-causing proteins but they act by modulating the intracellular proteostasis network through four distinct mechanism: inhibition or enhancement of protein production; degradation of misfolded protein; modulation of chaperone activity; upregulation of molecular chaperones such as Hsp70 (Balch et al. 2008; Calamini et al. 2012). An example of the latter is the derivative of the antibiotic geldanamycin, tanespimycin or 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) which has been shown to have protective effect on retinal ganglion cells following nerve injury by increasing Hsp70 production (Kwong et al. 2015). 17-AAG can also alleviate the mutant rhodopsin
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phenotype in autosomal dominant retinitis pigmentosa inducing molecular chaperones production through HSF-1 activation (Aguila et al. 2014).

ARB-causing mutant bestrophin-1 proteins appear to be recognised by QC in the cell and degraded possibly by ERAD (chapter 3) therefore the use of small molecules to promote correct folding of mutant bestrophin-1 may prove beneficial for treating ARB. To this end the ability of BTZ, TMAO, TUDCA, 4PBA and 17-AAG to promote correct folding and trafficking of ARB-causing bestrophin-1 proteins was investigated in polarised MDCKII cells. Functional analyses were also performed to test whether small molecules treatment was able to restore the function of bestrophin-1 mutants.

4.2 Use of small molecules to promote bestrophin-1 stability

In order to test the effect of selected chemical chaperones and proteostasis regulators on ARB-causing bestrophin-1 proteins, stably transfected MDCKII cells were grown on transwell filters for 5 days to allow polarisation. Bestrophin-1 expression was then induced with tetracycline and simultaneously the cells were treated with LI, PSII, 25 nM BTZ, 50 mM TMAO, 1 mM TUDCA, 2.5 mM 4PBA or 50 nM 17-AAG. Controls were left untreated. Cells were then harvested in SDS sample buffer 24 h following treatment, apart from cells treated with LI and PSII that were harvested after 6 h due to a toxic effect that was observed following prolonged treatments with these two inhibitors. Cell lysates were analysed by western blot.

Treatment with the proteasome inhibitor BTZ resulted in an increase in WT bestrophin-1 protein levels compared to untreated WT cells (Fig. 4.1A, compare lane 2 and 5). The level of WT bestrophin-1 stabilisation was greater than that observed following lysosomal protease inhibition (Fig. 4.1A, compare lanes 3 and 5) or treatment with the proteasome inhibitor PSII (Fig. 4.1A, compare lanes 4 and 5). However treatment with lysosomal proteases inhibitors and proteasome inhibitors was for 6 h opposed to the 24 h for BTZ treatment. Therefore the different effect on WT bestrophin-1 stabilisation might be explained by the different length of treatments. Following treatment with BTZ additional immunoreactive bands, smaller than the immunoreactive band corresponding to bestrophin-1, could be observed (Fig. 4.1A, lane 5). These might represent
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degradation products and suggest that prolonged inhibition of the proteasome leads to the activation of a different degradation pathway, e.g. autophagy, in order to dispose of accumulated proteins potentially harmful to the cell. Furthermore multiple bands with a slightly higher molecular mass than bestrophin-1 could be detected in presence of some inhibitors (Fig. 4.1A, lanes 4 and 5), perhaps related to bestrophin-1 modification during trafficking along the secretory pathway. The observation that WT bestrophin-1 was stabilised upon addition of proteasome inhibitors provides further evidence that bestrophin-1 is recognised and degraded by a proteasome dependent pathway, such as ERAD. Of note, a major increase in the level of WT protein level was also observed following treatment with the proteostasis regulator 4PBA (Fig. 4.1A, lane 6). This suggests that 4PBA is either acting as a proteostasis regulator by promoting bestrophin-1 synthesis or as a chemical chaperone by supporting proper folding and reducing the degradation of the WT protein. However given 4PBA’s activity as a chemical chaperone and the observation that WT bestrophin-1 is stabilised by the proteasome inhibitors BTZ and PSII, it seems likely that it is acting as a chemical chaperone. Treatments with the chemical chaperones TMAO or TUDCA did not obviously affect WT bestrophin-1 protein levels (Fig.4.1A, lanes 7 and 8 respectively). Similarly treatment with the proteostasis regulator 17-AAG did not alter WT bestrophin-1 protein expression (Fig.4.1A, compare lanes 9 and 10).

When BTZ was used to inhibit proteosomal degradation in p.L41P, p.R141H, p.R202W and p.M325T MDCKII cell lines, an immunoreactive band corresponding to bestrophin-1 could be detected in all the mutant cell lines (Fig. 4.1 B-E, lane 3). This supports the hypothesis that p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 are degraded by a proteasome dependent pathway.

4PBA did not affect the levels of p.L41P, p.R141H and p.R202W bestrophin-1 proteins (Fig. 4.1 B-D, lane 4). However a faint immunoreactive band could be detected in p.M325T mutant cell lysates following 4PBA treatment (Fig. 4.1 E, lane 4). Neither the chemical chaperone TMAO (Fig. 4.1 B-E, lane 5) nor the proteostasis regulator 17-AAG (Fig. 4.1 B-E, lane 6) had any effect on the expression of any of the mutant bestrophin-1 proteins.
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**Figure 4.1 Effect of small molecules treatment on WT and ARB-causing bestrophin-1 proteins.**

WT, p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 expression in polarised stably transfected MDCKII cells was induced with tetracycline. Cells were treated with 25 nM BTZ, 2.5 mM 4PBA, 50 mM TMAO, 1 mM TUDCA or 50 nM 17-AAG for 24 h before direct lysis in SDS sample buffer. WT (A) p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 protein expression level was investigated by western blot. An anti-tubulin antibody was used as a loading control. BTZ treatment stabilised WT and mutant bestrophin-1 proteins. An increase in WT and p.M325T bestrophin-1 levels could be observed following 4PBA treatment whilst TMAO and TUDCA did not affect steady state level of bestrophin-1 proteins. Tet = tetracycline.
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As inhibition of mutant protein degradation might be sufficient to promote correct protein folding (Wang et al. 2011), the localisation of WT and mutant bestrophin-1 proteins was investigated by confocal microscopy to test whether mutant proteins stabilised by the addition of BTZ co-localised with MCT-1 at the basolateral plasma membrane. Cells were allowed to polarise on transwell filters for 5 days before the expression of bestrophin-1 was induced. Cells were then treated with 25 nM BTZ for 24h prior to fixation, permeabilisation and labelling with anti-bestrophin-1 and anti-MCT-1 antibodies. Accumulation of WT bestrophin-1 could be observed intracellularly in cells treated with BTZ (Fig 4.2 panel A), however no obvious enrichment at the plasma membrane was noticed compared to non-treated cells (Fig. 4.2 panel A). Confocal analyses revealed that only a small proportion of p.L41P and p.R141H co-localised with the plasma membrane marker MCT-1 (Fig. 4.2 panels B and C), whilst p.R202H and p.M325T were only observed intracellularly (Fig. 4.2 panels D and E). A possible explanation for the increased localisation of p.L41P and p.R141H bestrophin-1 is that preventing their degradation increases the time they have to fold successfully and therefore to be trafficked to the plasma membrane. However it is also possible that inhibiting mutant protein degradation results in an overloading of the folding machinery. This would cause the accumulation of partially folded/misfolded bestrophin-1 in the ER that would aggregate through hydrophobic interactions. Such aggregates would prevent further folding or trafficking of bestrophin-1. This is consistent with what was observed for the intracellular WT and mutant bestrophin-1 accumulation following BTZ treatment (Fig. 4.2 A-E).
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**Figure 4.2** BTZ treatment partially restored trafficking of p.L41P, p.R141H bestrophin-1 proteins but had no effect on p.R202W and p.M325T mutants.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline and cells were treated with 25 nM BTZ for 24 h before fixation with 4 % paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in stably transfected polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker for correct trafficking to the basolateral plasma membrane. Inhibition of protein degradation with BTZ alone was sufficient to allow partial localisation of p.L41P and p.R141H to the basolateral plasma membrane. p.R202W and p.M325T mutant bestrophin-1 proteins remained intracellular after BTZ treatment. Scale bar = 5 µm. l=lateral; b=basal.
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4.3 Combination of small molecules to restore bestrophin-1 folding and trafficking

A combination of proteostasis regulators and protein degradation inhibitors can support the proper folding of mutant proteins associated with disease (Mu et al. 2008), such is the case for lysosomal glucocerebrosidase (Wang and Segatori 2013). Mutations in glucocerebrosidase destabilise the enzyme’s native structure causing an active site loss-of-function resulting in Gaucher's disease (Hruska et al. 2008; Bae et al. 2015). Wang and Segatori showed that a combination of the L-type calcium channel blocker lacidipine and the ERAD inhibitor Eerl promotes correct glucocerebrosidase folding resulting in a rescue of its enzymatic activity (Wang and Segatori 2013). Therefore the hypothesis that BTZ in combination with 4PBA, TMAO, TUDCA or 17-AAG could enhance proper folding of mutant bestrophin-1 was tested.

Bestrophin-1 expression was induced in MDCKII stable cell lines and BTZ was added to the culture media in combination with 2.5 mM 4PBA, 50 mM TMAO, 1 mM TUDCA or 50 nM 17-AAG. Following treatment for 24 h, cell lysates were collected for WB analysis or cells were fixed and stained for confocal microscopy. The combination of BTZ plus 4PBA resulted in the stabilisation of WT bestrophin-1 to a greater extent than that observed following treatment with BTZ alone (Fig. 4.3 A, compare lanes 3 and 4; Fig. 4.3 F). On the contrary, WT bestrophin-1 levels in lysates of stable WT cells treated with BTZ plus the chemical chaperone TMAO or the proteostasis regulator 17-AAG were comparable to those from cells treated with only BTZ (Fig. 4.3 A; Fig. 4.3 F).

The effect of the combinatorial treatments on p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 proteins was then investigated. The protein level of each mutant protein was a noticeably increased by the treatment with BTZ and the chemical chaperone 4PBA (Fig 4.3 B-E, lane 4; Fig. 4.3 F). Similarly to the WT, this increase was higher than the one observed following BTZ treatment only. In contrast, combination of BTZ plus TMAO (Fig. 4.3 B-E, lane 5; Fig. 4.3 F) or 17-AAG (Fig. 4.3 B-E, lane 6; Fig. 4.3 F) did not appreciably alter expression levels of mutant bestrophin-1. Therefore to summarise, the combination of BTZ plus chemical chaperones or proteostasis regulators produced similar effects on the
4. Use of small molecules to restore mutant bestrophin-1 trafficking and function

**Figure 4.3 Combination of BTZ plus small molecules stabilises WT and ARB-causing bestrophin-1 proteins levels.**

WT, p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 expression was induced in polarised stably transfected MDCKII cells with tetracycline. Cells were treated with 25 nM BTZ, or a combination of 25 nM BTZ plus 2.5 mM 4PBA, 50 mM TMAO or 50 nM 17-AAG for 24 h before direct lysis in SDS sample buffer. WT (A) p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 proteins expression level was investigated by western blot. An anti-tubulin antibody was used as a loading control. A synergical effect of BTZ plus 4PBA on bestrophin-1 stabilisation could be observed in all cell lines. Figure (F) shows the expression of bestrophin-1 normalised by the loading control and relative to bestrophin-1 level following induction with tetracycline. The mean of at least three different experiments is represented.
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steady state level of WT and mutant bestrophin-1 proteins (Fig. 4.3 A-E). However the best results were obtained following treatment with BTZ and 4PBA.

As correctly folded bestrophin-1 should be trafficked to the basolateral plasma membrane in polarised MDCKII cells, the localisation of WT and mutant bestrophin-1 following treatment with BTZ plus 4PBA was investigated by confocal microscopy (Fig. 4.4). Co-localisation of WT bestrophin-1 with MCT-1 was partially increased following treatment with BTZ and 4PBA compared to cells treated with BTZ only (Fig. 4.4, panel A) providing evidence of a BTZ and 4PBA synergistic effect on bestrophin-1 folding. Although most of p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 proteins remained intracellular following treatment with BTZ and 4PBA, a small portion of bestrophin-1 co-localised with MCT-1 suggesting that some mutant bestrophin-1 protein was correctly folded and transported to the basolateral surface (Fig. 4.4 panels B-E). This was particularly noticeable for the p.R202W and p.M325T proteins (Fig. 4.4, panels D and E respectively) which showed no co-localisation at the plasma membrane when treated with BTZ only (Fig. 4.4, panels D and E).
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**Figure 4.4** BTZ plus 4PBA treatment restores trafficking of ARB-causing bestrophin-1 proteins.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline and cells were treated either with 25 nM BTZ or a combination of 25 nM BTZ plus 2.5 mM 4PBA for 24 h before fixation with 4 % paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker for correct trafficking to the basolateral plasma membrane. 4PBA in combination with BTZ was able to restore proper trafficking of mutant bestrophin-1 proteins to the basolateral plasma membrane. Scale bar = 5 µm. l=lateral; b=basal.
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The effect of combining BTZ with the chemical chaperones TMAO and TUDCA or the proteostasis regulator 17-AAG on bestrophin-1 trafficking was also investigated. WT bestrophin-1 trafficking was not altered by treatment with BTZ plus TMAO, however a considerable increase in co-localisation with MCT-1 could be observed following treatment with BTZ plus TUDCA or 17-AAG (Fig. 4.6 and 4.7, compare panel A).

Although most of the mutant protein remained intracellular following treatment with each combination of BTZ with TMAO, TUDCA or 17-AAG, a differential effect of the combinations was discernible on the trafficking of mutant bestrophin-1 and its co-localisation with MCT-1. The combination of BTZ with TMAO promoted p.L41P and p.R202W co-localisation with MCT-1 suggesting that p.L41P and p.R202W bestrophin-1 trafficking to the basolateral membrane was enhanced following this treatment (Fig. 4.5, panels B and D). However no obvious co-localisation could be observed for p.R141H and p.M325T following treatment with BTZ and TMAO (Fig. 4.5 panels C and E).

Treatment with BTZ plus TUDCA resulted in improved trafficking of p.L41P and, to a lesser extent, of p.R141H and p.R202W, to the plasma membrane (Fig. 4.6, panels C-D). However no co-localisation of p.M325T with MCT-1 could be observed following addition of BTZ plus TUDCA to the culture medium (Fig. 4.6, panel E). When cells were treated with BTZ plus the proteostasis regulator 17-AAG, an increased amount of p.L41P and of p.R141H could be observed at the basolateral plasma membrane (Fig. 4.7 panels B and C). In contrast p.R202W and p.M325T remained intracellular (Fig. 4.7 panels D and E).
4. Use of small molecules to restore mutant bestrophin-1 trafficking and function

Figure 4.5 BTZ plus TMAO treatment restored trafficking of p.L41P, p.R202W bestrophin-1 proteins and a small proportion of p.R141H and p.M325T mutants.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline and cells were treated with 25 nM BTZ plus 50 mM TMAO for 24 h before fixation with 4% paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker for correct trafficking to the basolateral plasma membrane. Scale bar = 5 µm. l=lateral; b=basal.
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Figure 4.6 BTZ plus TUDCA treatment restored trafficking of p.L41P and partially restored p.R141H and p.R202W bestrophin-1 proteins.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline and cells were treated with 25 nM BTZ plus 1 mM TUDCA for 24 h before fixation with 4% paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker for correct trafficking to the basolateral plasma membrane. Scale bar = 5 µm. l=lateral; b=basal.
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**Figure 4.7** BTZ plus 17AAG treatment restored trafficking of p.L41P bestrophin-1 protein.

WT (A), p.L41P (B), p.R141H (C), p.R202W (D) and p.M325T (E) bestrophin-1 expression was induced with tetracycline and cells were treated with 25 nM BTZ plus 50 nM 17AAG for 24 h before fixation with 4 % paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins (green) localisation in polarised MDCKII cells. Representative XY and XZ scans for each mutant are shown. Co-localisation with MCT-1 (red) was used as a marker for correct trafficking to the basolateral plasma membrane. Scale bar = 5 µm. l=lateral; b=basal.
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A possible interpretation of the western blot and confocal data could be that BTZ provides more time for the mutant bestrophin-1 proteins to achieve their correct native conformation by inhibiting protein degradation through the proteasome. However these treatments would prevent protein degradation, leading to the accumulation of misfolded/partially folded bestrophin-1 intermediates that would favour protein-protein interactions through their hydrophobic domain. This would result in the formation of insoluble aggregates that are potentially toxic for the cell. 4PBA, by acting as a chemical chaperone, can temporarily interact with the hydrophobic region of bestrophin-1 thus preventing aggregation and allowing promoting its chance of folding correctly. These two mechanisms would have a synergistic effect on bestrophin-1 stabilisation by enhancing proper folding. This hypothesis is consistent with the western blot data (Fig. 4.3 A-E) and the confocal analysis that shows that WT bestrophin-1 and at least a portion of mutant bestrophin-1 proteins are correctly trafficked to the basolateral plasma membrane (Fig 4.4). The combination of BTZ with TMAO or 17-AAG did not show the same synergistic effect on mutant bestrophin-1 protein levels as BTZ plus 4PBA. This may be because these small molecules promote the degradation of misfolded proteins and aggregates rather than enhancing folding (Gong et al. 2009; Ho et al. 2013; Rusmini et al. 2011).

4.4 Investigating the function of ARB-causing bestrophin-1 proteins following treatment with small molecules

Confocal microscopy showed that correct trafficking of mutant bestrophin-1 proteins to the basolateral plasma membrane after treatment with BTZ and 4PBA was partially restored, indicating that correct folding was achieved. However this was not sufficient to prove that the function of the channels was restored. Exogenously expressed bestrophin-1 acts as an anion channel (Davidson et al. 2011) and its properties have been extensively characterised in HEK293 cells (Milenkovic et al. 2011; Kuo et al. 2014; Davidson et al. 2011). Therefore whole cell patch clamp (WCPC) was used to measure Cl− conductance in HEK293 cells transiently expressing WT and mutant bestrophin-1 (Fig 4.8 A). HEK293 cells were transfected with the WT bestrophin-1 construct 24 h prior to WCPC recording. A GFP construct was co-transfected to allow identification of
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Transfected cells. Cells expressing WT bestrophin-1 generated a large time-independent Cl\textsuperscript{−} current (Fig. 4.8 D). In contrast, non-transfected cells or cells transfected with only GFP showed did not exhibit a Cl\textsuperscript{−} conductance suggesting that no other channels were expressed or active in HEK293 cells (Fig. 4.8 B and C respectively). The reversal potential, which indicates the membrane potential where the net flow of an ion through an open channel is zero, was zero in non-transfected cells and in cells only expressing GFP (Fig. 4.8 E). Cells expressing WT bestrophin-1 had a reversal potential of -17.6 ± 0.87 mV (Fig. 4.8 E), consistent with previous observations (Milenkovic et al. 2011; Kuo et al. 2014; Davidson et al. 2011).

In order to demonstrate that the current observed was mediated by WT bestrophin-1 channels at the plasma membrane as opposed to channels that are in the ER, a di-lysine ER-retrieval motif (KKAA) was added at the C-terminus of WT bestrophin-1 to prevent trafficking to the cell surface (Vincent et al. 1998; Gao et al. 2014; Jackson et al. 1990). Transmembrane proteins expressing the di-lysine motif KKXX at the C-terminus are recognised by a cytosolic protein complex (COPI). COPI packages such proteins into vesicles for retrograde transport to the ER (Ma and Goldberg 2013; Stornaiuolo et al. 2003). Noticeably cells expressing WT bestrophin-1 tagged with KKAA (WT\textsuperscript{KKAA}) showed very little in Cl\textsuperscript{−} conductance (compare Fig. 4.8 D and Fig. 4.9 A and B) even though the WT\textsuperscript{KKAA} construct was expressed at a comparable level to WT as determined by WB (Fig. 4.9 C). The reversal potential of WT and WT\textsuperscript{KKAA} bestrophin-1 induced currents was also comparable to untagged WT protein (Fig. 4.9 B) suggesting that the gating property of the channel was not compromised. This together with the observation that the addition of a C-terminal YFP or GFP tag does not affect bestrophin-1 function (Johnson et al. 2014; Marmorstein et al. 2000), suggests that the lack of current is not due to the added KKAA tag altering the channel properties per se, but it is due to a reduction of the number of channels at the plasma membrane. This is an important finding as bestrophin-1 has been suggested to have a role in ER functions (Marmorstein et al. 2015). These data provide evidence that the Cl\textsuperscript{−} currents measured by patch clamp are due to bestrophin-1 present at the cell surface rather than in the ER.
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Figure 4.8 Chloride conductance of WT bestrophin-1 in transiently transfected HEK293 cells.

Schematic diagram of whole cell patch clamp (WCPC) (A). A mild suction is applied in the cell attached configuration to pass to the whole cell configuration thus allowing recording from the ion channels present at the plasma membrane. Representative whole-cell current responses of HEK293 cells NT = not transfected (B), transfected with a GFP construct (C) or transfected with WT bestrophin-1 plus GFP constructs in a 4:1 ratio (D). Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The voltage protocol is shown in (B). The holding potential was -50 mV. The mean current/voltage relationship (I/V) for each condition is shown in (E). Results are presented as the mean ± SEM. n=number of cells recorded in independent experiments.
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Figure 4.9 The addition of an ER-retention tag inhibits chloride conductance of WT bestrophin-1.

Representative whole-cell current responses of HEK293 cells transiently transfected with WTKKAA bestrophin-1 construct showed that the addition of an ER-retention motif reduced the number of active channels at the plasma membranes (A). Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with WT or WTKKAA bestrophin-1 constructs (B). Results are presented as mean ± SEM. n=number of cells recorded in independent experiments. Western blot analysis and quantification of whole cell lysates of HEK293 cells transiently transfected with WT or WTKKAA bestrophin-1 constructs showed that the addition of a retention signal did not alter the steady state levels of bestrophin-1 protein (C). Quantification data are representative of at least three independent experiments and are presented relative to untagged WT which has been normalised to 1.
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4.4.1 4PBA is able to restore chloride conductance of ARB-associated bestrophin-1 mutants

In order to investigate whether 4PBA treatment could restore mutant bestrophin-1 function, the Cl\(^-\) conductance of the four ARB mutant bestrophin-1 channels in the absence or presence of 4PBA was investigated. HEK293 cells were transiently transfected with p.L41P, p.R141H, p.R202W and p.M325T mutant bestrophin-1 constructs and cells were treated with 2.5 mM 4PBA for 24 h or left untreated. Cells were washed extensively with extracellular bath solution to remove any residual 4PBA to exclude the possibility that 4PBA might directly interact with the channel thus affecting channel properties. The Cl\(^-\) currents in cells transiently expressing the 4 ARB bestrophin-1 mutants were greatly reduced compared to those induced by the WT protein (compare Fig. 4.10 A and C and Fig. 4.11 A and C to Fig. 4.8 D). This suggests that the reduction in Cl\(^-\) conductance was caused by a reduced number of bestrophin-1 channels at the plasma membrane consistent with previous data from western blot and confocal microscopy that showed that mutant bestrophin-1 is not trafficked to the basolateral plasma membrane in MDCKII cells but it is recognised by ERQC and targeted to degradation (chapter 3). The possibility that mutations might affect the property of the channel was also considered, however since the reversal potentials of ARB-causing bestrophin-1 induced currents were not different to the WT, this suggests that the lack of Cl\(^-\) conductance most likely reflects a reduced number of mutant bestrophin-1 channels at the plasma membrane rather than a change in the voltage-sensitivity or channel kinetics of the mutant channels.

When 4PBA was added to the culture medium, the whole-cell current response of cells transfected with p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 constructs, increased significantly compared to the one observed in non-treated cells (Fig. 4.10, compare A and B, C and D; Fig. 4.11, compare A and B, C and D) suggesting that the mutant bestrophin-1 channels that reached the cell surface in the presence of 4PBA were functional. In particular p.L41P induced currents following 4PBA treatment were comparable to WT at each voltage point (Fig. 4.10 E). The responses generated by cells transfected with p.R141H, p.R202W and p.M325T constructs were significantly (p<0.001) above those generated by the WT channel at voltage values between +20 mV and +80 mV (Fig. 4.10 F; Fig
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4.11 E and F). One possible explanation for the apparent higher activity of p.R141H, p.R202W and p.M325T bestrophin-1 channel is that 4PBA promotes a conformation that is more active than the WT. However, as 4PBA treatment did not affect WT bestrophin-1 Cl\(^-\) conductance (Fig. 4.12 A and B), this is unlikely. Because only strongly expressing GFP cells were chosen for WCPC measurements another possible explanation for the increased Cl\(^-\) conductance is that the expression of bestrophin-1 was not comparable for all the constructs, meaning that some cells would express more functional channels at the plasma membrane following 4PBA treatment than others. Therefore, in order to investigate how the distribution of bestrophin-1 proteins was affected in HEK293 cells before or after 4PBA treatments biotinylation assays were performed.
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**Figure 4.10 4PBA treatment restore chloride conductance of p.L41P and p.R141H bestrophin-1 channels in transiently transfected HEK293 cells.**

Representative whole-cell current responses of HEK293 cells transiently transfected with p.L41P (A and B) or p.R141H (D and E) bestrophin-1 constructs to 2.5 mM 4PBA. Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with ARB-causing mutants plus and minus treatment, compared to WT (E and F). Results are presented as mean ± SEM. *p < 0.05, **p<0.01 and ***p<0.001 indicate significant differences between treated and untreated cells. n=number of cells recorded in independent experiments.
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**Figure 4.11** 4PBA treatment restore chloride conductance of p.R202W and p.M325T bestrophin-1 channels in transiently transfected HEK293 cells.

Representative whole-cell current responses of HEK293 cells transiently transfected with p.R202W (A and B) or p.M325T (D and E) bestrophin-1 constructs following treatment with 2.5 mM 4PBA. Recordings were taken from -120 mV to +80 mV in A20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with ARB-causing mutants plus and minus 4PBA compared to WT (E and F). Results are presented as mean ± SEM. *p < 0.05, **p<0.01 and ***p<0.001 indicate significant differences between treated and untreated cells. n=number of cells recorded in independent experiments.
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Figure 4.12 4PBA treatment had no effect on WT bestrophin-1 chloride conductance.

Representative whole-cell current responses of HEK293 cells transiently transfected with WT (A) bestrophin-1 constructs following treatment with 2.5 mM 4PBA. Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with WT bestrophin-1 constructs plus 4PBA compared to WT (C). Results are presented as mean ± SEM. n=number of cells recorded in independent experiments.
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4.4.2 4PBA treatment promotes trafficking of mutant bestrophin-1 channels to the plasma membrane in HEK293 cells

In order to determine more directly the levels of mutant bestrophin-1 reaching the cell surface in HEK293 cells and to further demonstrate that the trafficking of mutant bestrophin-1 proteins to the cell surface was adversely affected by mutation of bestrophin-1, the amount of bestrophin-1 at the plasma membrane HEK293 cells was determined by biotinylation (Fig. 4.13 A). HEK293 cells were transiently transfected with WT or p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 constructs and then treated with a membrane-impermeant biotinylation reagent in order to selectively modify proteins on the cell surface. The unreacted biotin was then quenched to avoid modification of intracellular proteins and cells were harvested in lysis buffer containing a cocktail of protease inhibitors. One tenth of the lysates was used as a control for the total protein input whilst streptavidin-agarose beads were added to the remaining fraction in order to isolate the biotinylated proteins (Fig. 4.13 A). SDS sample buffer was then used to elute the biotinylated proteins and the total fractions (Fig. 4.13 B, lanes 1-6) and the biotinylated fractions (Fig. 4.13 B, lanes 7-12). Samples were analysed by western blot. An anti-transferrin receptor (α-TfR) antibody was used as a control for the biotinylation assay.

Approximately 20% of the WT bestrophin-1 protein was observed in the streptavidin pulldowns (Fig. 4.13 B lane 8; Fig. 4.13 C) showing this fraction is at the cell surface. However the percentage of mutant bestrophin-1 proteins that was recovered from the plasma membrane was much lower and ranged between 0.4-2.6% (Fig. 4.13B lanes 9-12; Fig. 4.13 C). This is consistent with the hypothesis that the large majority of mutant bestrophin-1 was intracellular. When HEK293 cells expressing mutant bestrophin-1 proteins were treated with 4PBA, an increase in the amount of protein that could be biotinylated was observed (Fig. 4.14 A and B). The greatest difference was noticeable in cells expressing p.M325T bestrophin-1 where almost double the amount of protein was observed at the cell surface following 4PBA treatment (Fig. 4.14 A, compare lanes 11 and 12; Fig 4.14 B). However 4PBA treatment did not obviously affect WT bestrophin-1
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trafficking to the plasma membrane (Fig. 4.14 A, compare lanes 3 and 4; Fig. 4.14 B).

These data are consistent with the confocal analysis performed in MDCKII cells and provide further evidence that 4PBA promotes the correct folding and trafficking of mutant bestrophin-1 proteins. However a disparity is noticeable between the WCPC data and the biotinylation data. The p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 current responses following 4PBA treatment are comparable or higher than the WT (Fig. 4.10 E and F and Fig. 4.11 E and F) whereas the biotinylation data show that the amount of each mutant protein at the plasma membrane following 4PBA treatment is noticeably smaller compared to the WT (Fig. 4.14 A, compare lanes 4, 6, 8, 10 and 12). A possible explanation for the apparent lack of correlation between levels of bestrophin-1 at the cell surface and Cl- conductance is that biotinylation is used to analyse the whole cell population which was a mixture of transfected cells with a variable level of bestrophin-1 expression and non-transfected cells. In contrast, WCPC allows the selection of single cells that highly express GFP and, by association, bestrophin-1 therefore making direct comparison of the two techniques difficult.
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**Figure 4.13** Biotinylation of cell surface proteins showed that p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 mutants partially localise to the plasma membrane in HEK293 cells.

HEK293 cells transiently expressing WT or ARB-causing bestrophin-1 proteins were treated with a membrane-impermeant biotinylation reagent in order to selectively modify proteins on the cell surface. The unreacted biotin was then quenched and the cells lysed. Streptavidin-agarose beads were used to isolate the biotinylated proteins (A). Western blot was used to examine the bestrophin-1 level in whole cell lysates (B, 1-6) and precipitated biotinylated cell surface proteins (B, 7-12) One tenth of the lysate was used for the total input. An anti-transferrin receptor (α-TfR) antibody was used as a control for the biotinylation assay. An anti-tubulin antibody was used as loading control. Quantification of WT and mutant bestrophin-1 expression at the cell surface relative to total input is shown in (C). NT=non transfected cells.
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Figure 4.14 4PBA treatment promotes ARB-causing bestrophin-1 proteins trafficking to the cell surface in HEK293 cells.

Whole cell lysates and precipitated biotinylated cell surface proteins western blot analysis of HEK293 cells transfected with WT or p.L41P, p.R141H, p.R202W or p.M325T bestrophin-1 constructs. Cells were grown in absence (A 3, 5, 7, 9 and 11) or presence (4, 6, 8, 10 and 12) of 2.5 mM 4PBA for 24, before cell surface protein modification with biotin. An anti-tubulin antibody was used as a loading control. Quantification of WT and mutant bestrophin-1 protein expression at the cell surface relative to the respective untreated control is shown in (B). Data are normalised by tubulin and expressed relative to untreated cells. GFP=cells transfected with GFP construct only; NT=non transfected cells.
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4.4.3 4PBA fails to restore Cl⁻ conductance of WT^KKA and p.M325T^KKA bestrophin-1 channels

In order to further support to the hypothesis that 4PBA promotes correct folding of mutant bestrophin-1 and restores bestrophin-1 function at the plasma membrane, the effect of 4PBA on WT and p.M325T bestrophin-1 proteins expressing the ER retrieval motif KKAA was investigated.

HEK293 cells transiently co-transfected with either WT^KKA or p.M325T^KKA bestrophin-1 constructs and GFP were treated with 2.5 mM 4PBA for 24 h or left untreated. Cells expressing WT^KKA bestrophin-1 showed very little in Cl⁻ conductance following 4PBA treatment (Fig. 4.15 A) comparable to the one observed in non-treated cells (Fig. 4.15 B). Similarly the whole cell current response of HEK293 cells expressing p.M325T^KKA bestrophin-1 was significantly reduced despite 4PBA treatment (Fig. 4.15 C and D) even though the p.M325T^KKA construct was expressed at a comparable level to the p.M325T as determined by WB (Fig. 4.15, E and F).

The observation that 4PBA fails to restore Cl⁻ conductance of WT and p.M325T tagged with the ER retrieval motif KKAA provides further support to the hypothesis that the ability of 4PBA to rescue bestrophin-1 Cl⁻ channel activity is dependent upon the protein being transported along the secretory pathway to the plasma membrane. Furthermore these results demonstrate that Cl⁻ conductance is specifically induced by cell surface localised bestrophin-1 and not by intracellular protein.
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Figure 4.15 4PBA fails to restore chloride conductance of WT<Sup><Sup>KKAA</Sup></Sup> or p.M325T<Sup><Sup>KKAA</Sup></Sup> bestrophin-1.

Representative whole-cell current responses of HEK293 cells transiently transfected with WT<Sup><Sup>KKAA</Sup></Sup> or p.M325T<Sup><Sup>KKAA</Sup></Sup> bestrophin-1 construct showed that treatment with 4PBA cannot restore chloride currents (A and C). Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with WT<Sup><Sup>KKAA</Sup></Sup> or p.M325T<Sup><Sup>KKAA</Sup></Sup> bestrophin-1 constructs (B and D). Results are presented as mean ± SEM, n=number of cells recorded in independent experiments. Western blot analysis (E) and quantification (F) whole cell lysates of HEK293 cells transiently transfected with p.M325T or p.M325T<Sup><Sup>KKAA</Sup></Sup> bestrophin-1 constructs showed that the addition of a retention signal did not alter the steady state levels of the mutant bestrophin-1 protein. Quantification data are representative of at least three independent experiments and are presented relative to untagged p.M325T which has been normalised to 1.
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4.4.4 Effect of TMAO, TUDCA and 17-AAG on p.M325T bestrophin-1 Cl$^-$ conductance and trafficking in HEK293

As the chemical chaperones TMAO and TUDCA and the proteostasis regulator 17-AAG had differential effect on mutant bestrophin-1 trafficking, the ability of TMAO, TUDCA and 17-AAG to restore mutant bestrophin-1 function was investigated. HEK293 cells transiently transfected to express p.M325T bestrophin-1 were treated for 24 h with 50 mM TMAO, 1 mM TUDCA or 50 nM 17-AAG. The Cl$^-$ conductance was then measured by WCPC. Treatment with TMAO produced a small but significant increase in Cl$^-$ conductance at +60 mV and at +80 mV (Fig. 4.16); however the Cl$^-$ current at these voltage points was at least 4 fold lower than for WT bestrophin-1 (Fig. 4.16 C). None of the other small molecules tested significantly restored the Cl$^-$ conductance of p.M325T bestrophin-1 (Fig. 4.17) suggesting that only TMAO was able to partially support proper folding and therefore allow the trafficking of some p.M325T bestrophin-1 to the plasma membrane. This was consistent with the data obtained from the confocal immunofluorescence analysis (Fig. 4.5-7, panel E). Further support for these data was provided by biotinylation studies. Streptavidin pulldowns showed that the amount of p.M325T protein accessible for biotinylation increased slightly following treatment with TMAO (Fig. 4.18 A, lane 2 and 4.18 B) but was unchanged by treatment with TUDCA or 17-AAG (Fig. 4.18 A lanes 3 and 5 respectively and 4.18 B).
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Figure 4.16 TMAO treatment partially rescued p.M325T bestrophin-1 channel chloride conductance in HEK293 cells.

Representative whole-cell current responses of HEK293 cells transiently transfected with p.M325T bestrophin-1 construct before (A) or after (B) 50 mM TMAO treatment. Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. The mean current/voltage relationship (I/V) is shown for cells transfected with p.M325T construct plus and minus TMAO treatment, compared to WT (C). Results are presented as mean ± SEM. *p < 0.05 and **p<0.001 indicate significant differences between treated and untreated cells. n=number of cells recorded in independent experiments.
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Figure 4.17 TUDCA and 17-AAG treatment did not restore p.M325T bestrophin-1 channel chloride conductance in HEK293 cells.

The mean current/voltage relationship (I/V) is shown for cells transfected with p.M325T construct plus TUDCA or 17-AAG treatment, compared to WT. Recordings were taken from -120 mV to +80 mV in Δ20 mV steps of 450 ms each. The holding potential was -50 mV. Results are presented as mean ± SEM. n=number of cells recorded in independent experiments.
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Figure 4.18 TMAO treatment promotes trafficking of a small proportion of p.M325T bestrophin-1 proteins to the cell surface in HEK293 cells.

HEK293 cells transfected with p.M325T bestrophin-1 constructs were grown in presence of 50 mM TMAO (A lane 2), 1 mM TUDCA (A lane 3), 2.5 mM 4PBA (A lane 4) or 50 nM 17AAG (A, lane 5) for 24 or left untreated (A lane 1) before cell surface protein modification with biotin. Western blot analysis of whole cell lysates and precipitated biotinylated cell surface proteins showed an increase of p.M325T bestrophin-1 at the cell surface following TMAO and 4PBA treatments. An anti-tubulin antibody was used as a loading control. Quantification of p.M325T bestrophin-1 proteins at the cell surface relative to the untreated control is shown in (B).
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4.5 Discussion

A crucial step in the acute response to protein misfolding is the decision between repair or degradation. In the ER, terminally misfolded proteins are processed by the ERAD pathway. This multistep pathway involves transport of the misfolded protein back across the ER membrane into the cytosol, a process known as retrotranslocation, and degradation by proteolysis through the ubiquitin proteasome system (Leitman et al. 2013; Nakatsukasa and Brodsky 2008; Olzmann et al. 2013; Smith et al. 2011)

ARB-causing bestrophin-1 proteins are recognised by the ERQC which prevent their trafficking to the plasma membrane and target them for degradation through a proteosomal pathway, possibly the ERAD (chapter 3). This suggests that the loss of function phenotype observed in ARB patients might be due to mutations reducing the amount of bestrophin-1 at the plasma membrane rather than disrupting the active site. A fraction of WT bestrophin-1 protein is also recognised by the ERQC suggesting that bestrophin-1 folds with suboptimal efficiency (chapter 3).

A large number of studies have shown that treatment with small molecules can correct protein folding defects caused by mutations and so alleviate the phenotype of protein conformational diseases (Burns et al. 2010; Calamini et al. 2012; Grey et al. 2011; Wang et al. 2011). These small molecules can be classified into three groups: chemical chaperones, pharmacological chaperones and proteostasis regulators. Chemical chaperones, such as TMAO, 4PBA and TUDCA, can promote protein folding by altering the solvent properties, for example by sequestering water molecules (TMAO) (Breydo et al. 2015), or by direct interaction with the hydrophobic domain of the nascent polypeptide (4PBA, TUDCA) (Cuadrado-Tejedor et al. 2013; Keene et al. 2002). Both mechanisms reduce protein aggregation by minimising off pathway reactions that could lead to stable misfolded/aggregated states (Vignaud et al. 2013; Liu et al. 2012), thus allowing the protein to fold into the native state (Cortez and Sim 2014). In contrast to chemical chaperones that have a non-selective effect on many different proteins, are able to bind to and stabilise any protein, pharmacological chaperones are designed specifically promote folding of particular target proteins (Parenti et
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Pharmacological chaperone therapy has given promising results for the treatment of lysosomal storage diseases, a group of genetic disorders due to mutations that affect lysosomal function. Clinical trials are ongoing to develop and select pharmacological chaperones for the treatment of lysosomal storage diseases such as Fabry, Gaucher and Pompe disease (Frustaci et al. 2001; Warnock et al. 2015; Khanna et al. 2014; Parenti et al. 2015) retinitis pigmentosa (Parfitt et al. 2014). Unlike chemical or pharmacological chaperones, proteostasis regulators mechanism of action is to alter the proteostasis network, a group of cellular pathways involved in proteostasis maintenance (Kim et al. 2013). To this end proteostasis regulators can to increase the expression of molecular chaperones such as Hsp70, to modify molecular chaperone function, and finally to enhance or inhibit degradation of misfolded proteins (Kim et al. 2013).

In order to test whether small molecule treatment could aid in restoring ARB-associated bestrophin-1 protein folding and function, the chemical chaperones TMAO, TUDCA and 4PBA and the proteostasis regulators BTZ and 17-AAG were tested. However only BTZ and in some cases 4PBA were able to stabilise WT and mutant bestrophin-1. As only very low levels of mutant protein could be detected following induction of bestrophin-1 expression, it is possible that the mutant protein is rapidly recognised and targeted for degradation. This means that the small molecules would not have the time to support the proper folding of mutant bestrophin-1. Increasing the time of treatment or the amount of the compound used might be helpful, however TMAO, TUDCA and 4PBA were already used at high concentration (millimolar level) and increasing the concentration further might cause cytotoxic effects thus making these compounds unable to be used for clinical trial. High TMAO level, for example, have been associated with high risk of cardio vascular disease(Koeth et al. 2013), whereas Although nanomolar concentrations of BTZ and 17-AAG were used to treat MDCKII cells, when higher concentrations of these compounds were tested on parental MDCKII cells, the cells only survived for 24 h or less following treatment (data not shown). Therefore using a combination of small molecules, in particular BTZ (to inhibit mutant bestrophin-1 degradation) with either TMAO, TUDCA, 4PBA or 17-AAG (to promote protein folding) was considered. Of note, although BTZ led to an increase in WT and mutant bestrophin-1 proteins, only a
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A negligible proportion of two of the four mutant proteins (p.L41P and p.R141H) could be detected at the basolateral plasma membrane. Although misfolded proteins should not be able to exit the ER, if degradation is blocked by proteasome inhibition, the QC machinery that retains misfolded proteins in the ER could be overwhelmed. Therefore, inhibiting degradation could allow more time for mutant protein to fold properly and a fraction of correctly folded proteins might escape the ER and reach the plasma membrane (Kincaid and Cooper 2007; Kota et al. 2007). This suggests that there may be some competition between degradation and productive folding/trafficking.

Misfolded proteins that manage to escape the ERQC can still be identified at other QC checkpoints, such as at the Golgi (Arvan et al. 2002) or the plasma membrane QC (PMQC) (Okiyoneda et al. 2010; Babst 2014; Apaja et al. 2010). Both these post-ER quality control pathways lead to the misfolded protein being targeted to lysosomes for degradation (Babst 2014), either directly or as part of the autophagy process (Wang et al. 2015; Marshall and Vierstra 2015). PMQC system can recognise non-native proteins by the interaction of Hsp70 with misfolded cytoplasmic domains which leads to the recruitment of the E3 ligase CHIP (Sharma et al. 2004; Okiyoneda et al. 2010). CHIP ubiquitinates the misfolded protein and the ubiquitin chains act as a signal for endocytosis and lysosomal targeting for degradation (Sharma et al. 2004; Okiyoneda et al. 2010).

Since bestrophin-1 has a large domain located in the cytoplasm, this PMQC mechanism could operate to remove misfolded bestrophin-1 from the plasma membrane. This would explain the presence of degradation products observed following proteasome inhibition.

When MDCKII cells expressing WT and mutant bestrophin-1 proteins were treated with combinations of BTZ and TMAO, TUDCA, 4PBA or 17-AAG, all mutant bestrophin-1 protein levels were increased under each combination. Treatment with BTZ and 4PBA was the most effective in stabilising the expression of mutant bestrophin-1 proteins. Furthermore, treatment with BTZ and 4PBA was the only combination that restored trafficking of all four of the mutant bestrophin-1 proteins to the basolateral plasma membrane, as determined by confocal analysis. The combination of proteasome and HDAC inhibitors has been recently used for the treatment of several type of cancer (Bhatt et al. 2013; Huang...
et al. 2015; Hideshima et al. 2011). However this is the first report about the use of the combined treatment of proteasome inhibitors and HDAC inhibitors to rescue misfolded proteins expression and trafficking. A similar mechanism of functional rescue was observed with the ΔF508 CFTR protein (Hutt et al. 2010) as inhibition of HDACs by SAHA, also known as vorinostat, restored channel trafficking to the cell surface, although the mutant channel activity levels were roughly 30% lower than the WT (Hutt et al. 2010). Furthermore siRNA-mediated silencing of HDAC7 resulted in correct trafficking of ΔF508 CFTR to the plasma membrane (Hutt et al. 2010). The mechanism through which 4PBA, , functions may be related to its activity as a chemical chaperone, its ability to inhibit histone deacetylases (HDACs) leading to changes in transcription of genes involved in protein folding/quality control, or a combination of both. In this respect, it is noteworthy that whilst treatment with an HDAC inhibitor (tubastatin) had no discernible effect, a second chemical chaperone TMAO was able to restore some Cl− conductance in cells expressing mutant p.M325T bestrophin-1 albeit to a much lesser extent than 4PBA. However, the expression of the molecular chaperones Hsp70 and Hsp90 was unaltered following 4PBA treatment. This suggests that the ability of 4PBA to restore mutant bestrophin-1 function is due to its activity as a chemical chaperone.

In order to demonstrate whether treatment with 4PBA could also restore protein function, its effect on Cl− conductance in HEK293 cells transiently transfected with WT and mutant bestrophin-1 was tested. Treatment with BTZ was not necessary in this system as both WT and mutant proteins could be easily detected without it. The four ARB-bestrophin-1 proteins used in this study produce significantly smaller Cl− currents compared to WT bestrophin-1 (Davidson et al. 2011). Treatment with 4PBA was able to restore Cl− conductance in cells expressing each of the mutant bestrophin-1 proteins, showing that restoration of mutant ARB bestrophin-1 activity is possible. As the addition of an ER retention signal to WT bestrophin-1 caused a dramatic reduction of the Cl− current, the restoration of Cl− conductance to the mutant channels can only be possible if the mutant protein is correctly folded and delivered to the plasma membrane.

In conclusion, the restoration of function to the four mutant bestrophin-1 proteins supports the hypothesis that the ARB-causing missense mutations result
4. Use of small molecules to restore mutant bestrophin-1 trafficking and function in bestrophin-1 loss of function by preventing the protein reaching the plasma membrane rather than altering its activity by disrupting the active site. Furthermore the results presented in this chapter provide evidence that 4PBA may be a promising therapy for the treatment of ARB and the other bestrophinopathies resulting from missense mutations in BEST1.
5  A possible role of Hsp70 in bestrophin-1 folding

5.1 Introduction

Protein folding can be supported by temperature reduction (Maljevic et al. 2011; Yang et al. 2005; Farinha et al. 2013; Gomes-Alves et al. 2009) or by brief temperature increase aimed to stimulate the heat shock response. The term heat shock response (HSR) describes a series of cellular response to exposure to abnormally high environmental temperature. The first evidence of HSR where presented in 1962 when a chromosomal puffin pattern indicating transcriptional activation was observed in drosophila cells following exposure to heat (Ritossa 1996). The proteins expressed as consequence of the HSR were thereafter referred to as heat shock proteins (HSP). It is possible to distinguish between two kind of cell response following heat shock, one at cellular level and one at molecular level. At cellular level, changes in the membrane fluidity, rearrangement of the cytoskeleton and morphological changes of cytoplasmic organelles can be observed. Furthermore the cell cycle stops in order to prevent damage to the DNA in replication. Not surprisingly, depending on the length and the strength of the heat shock, cell death can also occur. At molecular level is possible to observe the expression of molecular chaperones such as Hsp70 in order to help protein folding or facilitate the disposal of non-native proteins (Velichko et al. 2013).

The results presented in chapter 3 showed that mutant bestrophin-1 is recognised by QC systems, retained intracellularly and ultimately targeted for degradation, possibly through the ERAD. Rescue of the mutant protein expression and localisation was successfully achieved by the combination of the proteasome inhibitor BTZ and the chemical chaperone 4PBA. Furthermore function of the mutant channel could be restored by 4PBA treatments thus providing evidence that the loss-of-function observed in the 4 ARB-causing mutants was due to a structural defect of the channels caused by protein misfolding rather than a disruption of a functional domain (chapter 4). In this chapter temperature rescue
experiments were presented to further support the finding that mutant bestrophin-1 are misfolded and that it is possible to promote correct folding. Furthermore the ability of Hsp70 to bind to and support bestrophin-1 folding was investigated.

5.2 ARB-causing bestrophin-1 mutant proteins are stabilised by low temperature incubation and heat shock

WT, p.L41P, p.R141H, p.R202W and p.M325T MDCKII stable cell lines were grown on transwell filters for 5 days to allow polarisation. Bestrophin-1 expression was then induced with tetracycline. For low temperature rescue experiments cells were shifted to 26 °C immediately after induction and maintained at 26 °C for 24 h. For heat shock experiment cells were induced and incubated at 43 °C for 2 h, and then maintained at 37 °C for 22 h. At the end of the incubation cells were harvested in SDS lysis buffer and cell lysates were analysed by western blot. Anti-Hsp70 and anti-Hsp90 primary antibodies were used to monitor the heat shock response (Fig. 5.1 and Fig. 5.2). An increase in Hsp70 protein level could be observed following heat shock (Fig. 5.1, lane 4) suggesting that 2 h incubation at 43 °C were sufficient to activate the heat shock response in MDCKII cells. However the steady state level of Hsp90 was the same under the different conditions (Fig. 5.1).

Noticeably three bands that migrated around the predicted size of bestrophin-1 were observed in WT cells following tetracycline induction (Fig. 5.1, lane 2). This was also observed in previous blots, especially following proteasome inhibition (compare Fig 5.1, lane 2-5 and Fig. 4.1 A, lane 4). Although WT bestrophin-1 did not seem to be stabilised by low temperature incubation or heat shock (Fig. 5.1, compare lane 2 and lanes 3 and 4), the immunoreactive band corresponding to the highest molecular weight became fainter following incubation at low or high temperature (Fig. 5.1, lanes 3 and 4). On the contrary, when degradation through the proteasome was inhibited by the addition of PSII, an increase in the second and third bands could be observed, whilst the band at the lowest molecular weight became fainter (Fig. 5.1, compare lanes 3 and 4 with lane 5).
One possible explanation for this is that different bands could perhaps represent post-translational modification of bestrophin-1. Two phosphorylation sites at the protein C-terminus were predicted, one at S358 and another one at T536, furthermore immunoprecipitation experiments showed that bestrophin-1 one interact with the serine/threonine phosphatase PP2A (Xiao et al. 2008) suggesting that bestrophin-1 might be phosphorylated. However attempts to demonstrate that phosphorylation was occurring were not successful. The observation that an increase in the higher molecular weight bands could be detected following PSII treatment suggests that perhaps accumulation of bestrophin-1 in the cell might induce protein modification. Recently has been shown that proteins that are retained in the ER for prolonged time might be subjected to O-mannosylation, which is the transfer of mannose to serine or threonine residues (Xu and Ng 2015). Bestrophin-1 does not contain serine residues in its extracellular domain; however two threonine residues are present in the first extracellular loop of the protein which could be potential sites for mannosylation. In order to investigate whether mannosylation was occurring concavalin-A (conA) lectins immobilised on sepharose beads were used to isolated mannosylated protein in WT MDCKII cell lysates. Elution buffer was used to elute proteins from the conA sepharose beads and samples were analysed by western blot (Fig. 5.2). 10% of the cell lysates was used as control of total protein loading and 10% of the unbound fraction was used as control of unbound proteins. In order to control for non-specific binding to the resin, sepharose beads were incubated with WT cell lysates and processed as for the conA sepharose beads (Fig. 5.2, lanes 7 and 8). An immunoreactive band corresponding to bestrophin-1 could be detected in conA pulldown from induced WT cell lysates (Fig. 5.2, lane 9). However a faint immunoreactive band corresponding to tubulin was also detected in the same fraction (Fig. 5.2, lane 9). Furthermore the majority of bestrophin-1 was observed in the unbound fraction (Fig. 5.2, lane 6) thus indicating that, perhaps, binding to the beads was non-specific and that bestrophin-1 might not be subjected to mannosylation. Therefore the nature of the additional bestrophin-1 forms observed by western blot is still unknown.

The effect of low temperature incubation and heat shock on ARB-causing bestrophin-1 proteins expression was then investigated. Although mutant
bestrophin-1 levels were extremely low following tetracycline induction (Fig. 5.3 A-D, lane 2), growth at reduced temperature resulted in the increase of all the 4 ARB-causing bestrophin-1 analysed (Fig. 5.3 A-D, compare lanes 2 and 3) thus suggesting that the mutant proteins were more stable. A similar result was obtained following heat shock (Fig. 5.3 A-D, lane 4). However the intensities of the immunoreactive bands corresponding to mutant bestrophin-1 proteins after heat shock were lower than the ones detected following low temperature incubation (Fig. 5.3 A-D, compare lanes 3 and 4). As Hsp70 protein levels were increased following heat shock, this indicates that Hsp70 could play a role in bestrophin-1 stabilisation following heat shock.
5. A possible role of Hsp70 in bestrophin-1 folding

Figure 5.1 Effect of low temperature incubation and heat shock on WT bestrophin-1 protein level

MDCKII cells stably transfected with WT bestrophin-1 were grown on transwell filter for 5 days to allow polarisation. Bestrophin-1 expression was then induced and cells were either grown at 37 °C (lane 2), at 26 °C (lane 3) for 24 h or at 43 °C for 2 h, then at 37 °C for 22 h. Cell extract were used for western blot analysis. Uninduced cells (lane 1) and cells treated for 6 h with the proteasome inhibitors PSII (lane 5) were grown at 37 °C. Samples were blotted with anti-bestrophin-1 antibody to investigate bestrophin-1 levels; anti-Hsp90 and anti-Hsp70 antibodies to monitor the cold/heat shock response; and anti-tubulin antibody which used as loading control.
5. A possible role of Hsp70 in bestrophin-1 folding

**Figure 5.2 Bestrophin-1 does not seem to be O-mannosylated in MDCKII**

WT bestrophin-1 expression in polarised stably transfected MDCKII cells was induced with tetracycline for 24 h before direct lysis IP buffer. 10% of the sample was used as control for the expression of bestrophin-1 (total). ConA sepharose beads were added to the remaining 90% of the lysates in order to bind o-mannosylated protein. WT bestrophin-1 protein level was investigated by western blot. An anti-tubulin antibody was used as a loading control. ConA= concavalin A; B= sample extracted by the addition of 1xSDS sample buffer to the sepharose beads; U= unbound fraction; E= sample collected by elution.
5. A possible role of Hsp70 in bestrophin-1 folding

Figure 5.3 Mutant bestrophin-1 protein levels increase following low temperature incubation or heat shock.

MDCKII cells stably transfected either with p.L41P (A), p.R141H (B), p.R202W (C) or p.M325T (D) bestrophin-1 constructs, were grown on transwell filter for 5 days to allow polarisation. Bestrophin-1 expression was then induced and cells were either grown at 37 °C (lane 2), at 26 °C (lane 3) for 24 h or at 43 °C for 2 h, then at 37 °C for 22 h. cell extract were used for western blot analysis. Uninduced cells (lane 1) were grown at 37 °C. Samples were blotted with anti-bestrophin-1 antibody to investigate bestrophin-1 levels. Anti-Hsp90 and anti-Hsp70 antibodies were used to monitor the cold/heat shock response and the anti-tubulin antibody was used as loading control.
To investigate whether the low temperature incubation was able to stabilise mutant bestrophin-1 by promoting correct folding, the localisation of the mutant proteins to the basolateral plasma membrane was investigated by confocal immunofluorescence microscopy in polarised MCDKII cells (Fig 5.4). No obvious difference could be detected in WT bestrophin-1 localisation following incubation at 26 °C compared to incubation at 37 °C (Fig. 5.4 panel A and B). However both p.L41P and p.R141H bestrophin-1 proteins showed clear evidence of co-localisation with MCT-1 following incubation at 26 °C (Fig. 5.4, panels B and C). This suggests that the mutant protein were able to achieve native confirmation following low temperature incubation (Fig. 5.4 panels B and C), as opposed to growth at 37 °C where proteins were observed in intracellular compartment (Fig. 5.4 panels B and C). Also an increase in the intensity of bestrophin-1 staining could be observed in p.L41P and p.R141H cells following incubation at 26 °C compared to incubation at 37 °C consistent with the western blot results (Fig. 5.3 B and C, compare lanes 2 and 3) and further suggesting that the mutant proteins were more stable at 26 °C.
Figure 5.4 Low temperature incubation partially restores trafficking of p.L41P, p.R141H bestrophin-1 proteins

WT, p.L41P and p.R141H bestrophin-1 expression was induced with tetracycline in polarised stable MDCKII cells. Cells were incubated at 37 °C (A, C, and E) or at 26 °C (B, D and F) for 24 h before fixation with 4 % paraformaldehyde and staining. Confocal immunofluorescence microscopy was used to visualise WT or mutant bestrophin-1 proteins localisation in stably transfected polarised MDCKII cells. Representative XY and XZ scans for each protein are shown. Co-localisation with MCT-1 was used as a marker for correct trafficking to the basolateral plasma membrane. The merged and the YZ panels show bestrophin-1 in green and MCT-1 in red. Scale bar = 5 µm. l=lateral; b=basal.
5.3 A possible role of Hsp70 in bestrophin-1 stabilisation

The results presented in the previous section showed that, following heat stress, mutant bestrophin-1 proteins could be detected by western blot. The increase in bestrophin-1 protein level correlated with an increase of the molecular chaperone Hsp70 thus suggesting that Hsp70 could potentially have a role in supporting mutant bestrophin-1 folding.

Hsp70 and its co-chaperones play a critical role in alleviating the phenotype caused by misfolded protein by attempting to promote their correct folding or, when this is not possible, by binding to misfolded intermediate thus preventing formation of non-native protein aggregates or targeting misfolding proteins for degradation (Duncan et al. 2015; Young 2014; Vabulas et al. 2010). Overexpression of Hsp70 has been showed to ameliorate the neurodegenerative symptoms caused by accumulation of mutant proteins aggregates (Duncan et al. 2015; Lu et al. 2014; Eroglu et al. 2014; Brinkmeier and Ohlendieck 2014; Ebrahimi-Fakhari et al. 2013; Cummings et al. 2001) or to improve trafficking and stabilisation of mutant ion channels such as CFTR (Young 2014). Therefore the possibility that Hsp70 is involved in the stabilisation of mutant bestrophin-1 was investigated.
5.3.1 **Exogenous overexpression of Hsp70 stabilise WT and at least two mutant bestrophin-1 proteins**

WT, p.L41P, p.R14H, p.R202W and p.M325T stale MDCKII cells were grown on transwell filter for 5 days to allow polarisation then cells were transfected with a V5 tagged Hsp70 (HSPA2A) construct or left non transfected. 24 h following transfection, expression of bestrophin-1 and Hsp70 was induced for 24 h with 1X SDS sample buffer. Over expression of Hsp70, which was calculated relative to non-transfected cells and normalised by tubulin level, was doubled in cell expressing WT and p.R141H bestrophin-1 and transfected with Hsp70/V5 compared to non-transfected cells (Fig. 5.5 and Fig. 5.6, compare lanes 1 and 4 in immunoblots for total Hsp70). However Hsp70 was increased only by a third in cell expressing p.L41P, p.R202W and p.M325T (Fig. 5.6 and Fig. 5.7, compare lanes 1 and 4 in immunoblots for total Hsp70). As only a faint immunoreactive bands corresponding to Hsp70/V5 could be detected in transfected cells lysates (Fig. 5.6 and Fig. 5.7, lane 4 in immunoblots for Hsp70/V5), it is possible that MDCKII transfection efficiency was quite low (see chapter 3) and thus only some cells will have Hsp70 overexpressed. Nonetheless, as western blot looks at whole cell population, any increase in bestrophin-1 is likely to be an underestimate of the increase that occurs in cells that actually overexpressed Hsp70.

Although overexpression of Hsp70 was low it was possible to observe an increase of WT, p.R141H and p.M325T bestrophin-1 following Hsp70/V5 transfection compared to non-transfected cells (Fig 5.5 A, 5.6 B and 5.7 B respectively; compare lanes 2 and 4). This was confirmed by the quantification data which are expressed as WT, p.R141H or p.M325T bestrophin-1 protein expression normalised by tubulin and relative to each non-transfected control (Fig 5.5 B, 5.6 B and 5.7 B). On the contrary p.L41P and p.R202W did not seem to be stabilised by overexpression of Hsp70/V5 (Fig. 5.6 A and 5.7 A respectively; compare lanes 2 and 4). This suggesting that perhaps Hsp70 is not the only molecular chaperone involved in the recognition of mutant bestrophin-1. It is possible in fact that co-chaperones or perhaps other classes of molecular chaperones are involved in the recognition of p.L41P and p.R202W bestrophin-1 proteins.
5. A possible role of Hsp70 in bestrophin-1 folding

MDCKII cells stably transfected with WT bestrophin-1 construct, were grown on transwell filter for 5 days to allow polarisation. An Hsp70/V5 construct, in pcDNA5/FRT/TO vector, was then transfected into cells by using Lipofectamine. Bestrophin-1 and Hsp70/V5 expression was then induced with tetracycline for 24 h (lanes 2 and 4 respectively) or cells were left uninduced (lanes 1 and 3). Cell extract were harvest with 1xSDS and lysates were used for western blot analysis (A). Anti-bestrophin-1 antibody was used to investigate bestrophin-1 levels. Anti-V5 and anti-Hsp70 antibodies were used to detect exogenous and total amount of Hsp70 respectively. Anti-tubulin antibody was used as loading control. Bestrophin-1 protein levels were normalised by tubulin and expressed relative to induced non-transfected cell lysates (lane 2) which was normalised to 1 (B). The bar chart represents the average value of 2 independent experiments.

Figure 5.5 WT bestrophin-1 protein levels increase following exogenous overexpression of Hsp70
5. A possible role of Hsp70 in bestrophin-1 folding

Figure 5.6 Overexpression of Hsp70 result in the increase of p.R141H bestrophin-1 protein levels but not of the ones of p.L41P bestrophin-1

MDCKII cells stably transected with p.L41P and p.R141H bestrophin-1 constructs were grown on transwell filter for 5 days to allow polarisation. An Hsp70/V5 construct, in pcDNA5/FRT/TO vector, was then transected into cells using Lipofectamine. Bestrophin-1 and Hsp70/V5 expression was induced with tetracycline for 24 h (lanes 2 and 4 respectively) or cells were left uninduced (lanes 1 and 3). Cell extract were harvest with 1xSDS and lysates were used for western blot analysis (A). Anti-bestrophin-1 antibody was used to investigate bestrophin-1 levels. Anti-V5 and anti-Hsp70 antibodies were used to detect exogenous and total amount of Hsp70 respectively. Anti-tubulin antibody was used as loading control. Bestrophin-1 protein levels were normalised by tubulin and expressed relative to induced non-transfected cell lysates (lane 2), which was normalised to 1 (B). The bar charts represent the average value of 2 independent experiments.
A possible role of Hsp70 in bestrophin-1 folding

Figure 5.7 Overexpression of Hsp70 result in the increase of p.M325T bestrophin-1 protein levels but not of the ones of p.R202W bestrophin-1

MDCKII cells stably transfected with p.R202W and p.M325T bestrophin-1 constructs were grown on transwell filter for 5 days to allow polarisation. An Hsp70/V5 construct, in pcDNA5/FRT/TO vector, was then transfected into cells using Lipofectamine. Bestrophin-1 and Hsp70/V5 expression was induced with tetracycline for 24 h (lanes 2 and 4 respectively) or cells were left uninduced (lanes 1 and 3). Cell extract were harvest with 1xSDS and lysates were used for western blot analysis (A). Anti-bestrophin-1 antibody was used to investigate bestrophin-1 levels. Anti-V5 and anti-Hsp70 antibodies were used to detect exogenous and total amount of Hsp70 respectively. Anti-tubulin antibody was used as loading control. Bestrophin-1 protein levels were normalised by tubulin and expressed relative to induced non-transfected cell lysates (lane 2), which was normalised to 1 (B). The bar charts represent the average value of 2 independent experiments.
5. A possible role of Hsp70 in bestrophin-1 folding

5.3.2 Hsp70 binds to WT and p.R141H bestrophin-1 proteins

To investigate whether Hsp70 interacts with bestrophin-1, immunoprecipitation of bestrophin-1 was performed with WT and p.R141H stable cell lysates. Briefly WT and p.R141H bestrophin-1 expression was induced in polarised MDCKII and cells were treated with BTZ for 24 h. In order to control that Hsp70 was not co-immunoprecipitated solely as a consequence of non-specific interactions with sepharose beads, parental T-REx MDCKII cells treated with BTZ for 24 h were also used. Lysis buffer containing apyrase to deplete ATP was then added to the cells in order to extract total protein lysates. Hsp70 affinity for the substrate is higher when Hsp70 is bound to ADP in what is known as Hsp70 closed conformation (Kim et al. 2013; Hartl et al. 2011). As the apyrase catalyses the hydrolysis of ATP into AMP and inorganic phosphate, the addition of apyrase to the lysis buffer helps to keep Hsp70 in its closed conformation thus preventing Hsp70 dissociation form the substrate. The anti-bestrophin-1 primary antibody was used to immunoprecipitate WT or p.R141H bestrophin-1 together with any potential interactors. The immunoprecipitated fraction was then analysed by western blot with SDS sample buffer. 10% of the cell lysates was used as control of total protein loading (Fig 5.8). An immunoreactive band of the size of Hsp70 could be observed in the WT (Fig 5.8 lanes 2 and 5) and p.R141H (Fig 5.8 lanes 3 and 6) immunoprecipitated fractions. Interestingly, the amount of Hsp70 co-immunoprecipitated with p.R141H bestrophin-1 appeared to be higher than the amount observed in the WT immunoprecipitate material. A very faint band was also observed in immunoprecipitates from parental T-REx MDCKII cell lysates suggesting that a negligible amount of Hsp70 may have background binding.

In conclusion the Hsp70 overexpression and the co-immunoprecipitation analysis taken together suggest that Hsp70 might play a role in the stabilisation of WT, p.R141H and possibly p.M325T by direct interaction with partially folded/misfolded bestrophin-1 intermediates. However since overexpression of Hsp70 did not seem to affect p.L41P and p.R202W bestrophin-1 expression levels, it is possible that other molecules are involved in the QC of bestrophin-1 folding.
Figure 5.8 Hsp70 interact with bestrophin-1 in stable MDCKII cells.

Immunoprecipitation of bestrophin-1 was performed with anti-bestrophin-1 antibody in WT (lanes 2 and 5) or p.R141H (lanes 3 and 6) MDCKII cell lysates or from parental MDCKII cells (lanes 1 and 4) following induction and treatment with BTZ for 24 h. One tenth of the cell lysates was used as a control of total loading. The total and the precipitated fractions were blotted with anti-bestrophin-1 antibody, anti-Hsp70 antibody and anti-tubulin antibody which was used as loading control. The arrow indicates the Hsp70 fraction isolated as a result of non-specific interaction. *IgG HC= IgG heavy chains
5.4 Discussion

In vitro and in vivo studies have shown that low temperature incubations can provide a kinetic advantage during the ΔF508 CFTR folding (Farinha et al. 2013; Qu and Thomas 1996), thus preventing its degradation by the proteasome. Similarly, when polarised MDCKII cells expressing p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 proteins were grown at 26°C, bestrophin-1 could be detected in all cell lysates in contrast to cells were incubated at 37 °C which showed no evidence of bestrophin-1 protein. Furthermore when the localisation of p.L41P and p.R141H mutant proteins was investigated in polarised MDCKII cells grown at the lower temperature of 26°C, a proportion of the mutant proteins were localised to the basolateral plasma membrane. The western blot results and the confocal analyses indicate that low temperature incubation can support correct folding of mutant bestrophin-1 possibly by providing a kinetic folding advantage towards bestrophin-1 native structure other than misfolded intermediates. However another possible explanation is that cells activate a cold shock response following prolonged incubation at 26 °C. Cold exposure can have similar effects on the cell physiology to the ones observed following heat shock. These effects include upregulation of genes encoding for molecular chaperones to assist protein folding or DNA/RNA binding proteins (Matz et al. 1995; Kljashtorny et al. 2015; Al-Fageeh et al. 2006). Nevertheless the mechanisms that regulate the cold shock response (CSR) remain unclear. Although there is a possibility that bestrophin-1 stabilisation following low temperature incubation is due to an increase of CSPs, this hypothesis was not investigated. However a better understanding of the events that regulate the CSR might pinpoint to specific proteins/pathways whose regulation might prove beneficial or feasible in terms of potential therapeutic avenues for treating misfolding diseases such as bestrophinopathies.

When the heat shock response was induced in polarised MDCKII cells expressing WT or mutant bestrophin-1 by a 2 h incubation at 43 °C followed by a recovery at 37 °C for 22 h, an increase in WT and mutant bestrophin-1 proteins could be observed. As mutant bestrophin-1 protein levels could not be detected in induced cells maintained at 37 °C, the appearance of a band corresponding to bestrophin-1 suggests that a least a proportion of the mutant protein was correctly
folded and therefore was not rapidly degraded by the cellular QC systems. As expected (Velichko et al. 2013), Hsp70 levels were increased following heat shock, indicating that Hsp70 may be directly involved in the stabilisation of mutant bestrophin-1 proteins. In order to investigate this hypothesis, Hsp70 overexpression experiments were performed. When Hsp70 was overexpressed, stabilisation of WT, p.R141H and p.M325T bestrophin-1 proteins was observed. No obvious change could be observed for the p.L41P and p.R202W mutants. As polarised cells are particularly resistant to transfection, a possible explanation for the apparent lack of effect of Hsp70 on the stabilisation of p.L41P and p.R202W bestrophin-1 could be that its overexpression was insufficient. Furthermore recent work from Yurinskaya and colleagues showed that exogenous Hsp70 is recognised by the cell and targeted for degradation, most likely via the proteasome (Yurinskaya et al. 2015). The reduced transfection efficiency in polarised cells and Hsp70 degradation may explain why overexpression of exogenous Hsp70 by transfection is not as effective as increased expression of endogenous Hsp70 following heat shock, and provides a reason why all of the mutants were stabilised heat shock.

Bestrophin-1 structure (Yang et al. 2014;Dickson et al. 2014) reveals that p.L41P bestrophin-1 is located by the first loop of the protein, which faces the lumen of the ER. This suggests that other chaperones might be involved in the recognition of this mutant and could explain why p.L41P is not stabilised by overexpression of Hsp70. On the contrary, p.R141H, p.R202W and p.M325T proteins are located in the cytoplasmic portion of bestrophin-1 where Hsp70 is more abundant (Dickson et al. 2014;Bharill et al. 2014), suggesting that Hsp70 may be involved in their recognition. Co-immunoprecipitation experiments showed that Hsp70 binds to WT and p.R141H bestrophin-1 proteins thus supporting the hypothesis that Hsp70 may promote the correct folding of p.R141H, p.R202W and p.M325T bestrophin-1. Nonetheless, p.R202W protein levels are not increased following Hsp70 overexpression, suggesting that the mechanism by which this mutant is recognised is different from the others. The R202 residue is close to the R205 one, which is involved in the control of the gating of the channel. It is possible that this region of the protein is subjected to
strict QC and therefore that p.R202W mutant recognition and degradation is more efficient compared to the others.

In conclusion, the results presented in this chapter provide evidence that misfolding of ARB-causing bestrophin-1 mutant proteins can be modulated by manipulating the temperature and expression of Hsp70 chaperones. Therefore, it is possible to speculate that the induction of HSPs, in particular Hsp70, by proteostasis regulators in RPE cells might provide a therapeutic target for future studies that aim to correct mutant bestrophin-1 associated with ARB. This may offer an alternative treatment strategy to 4PBA. To this end it would be interesting to perform WCPC on cells HEK293 cells expressing mutant bestrophin-1 channels grown at low temperature or following Hsp70 overexpression. Also it would be interesting to investigate, by co-immunoprecipitation, whether there are other chaperones able to bind to bestrophin-1 and support proper folding.
6 General Discussion

Autosomal Recessive Bestrophinopathy (ARB) represents the null phenotype of \textit{BEST1}, a gene encoding for bestrophin-1, a homopentameric Ca\(^{2+}\)-activated Cl\(^-\) channel that mainly localise to the basolateral plasma membrane of RPE cells. Mutations in bestrophin-1 are associated with a wide range of retinal dystrophies named bestrophinopathies. A common characteristic of bestrophinopathies is the presence of an altered EOG, which might be caused by differences in the chloride conductance of the mutant channel. To date there is no treatment these retinal dystrophies. The available therapeutic options aim to alleviate the symptoms of the disease and to delay the disease progression in order to improve the quality of life of the patient. Recent studies and previous works in the Manson lab have shown that the majority of disease-causing bestrophin-1 proteins mislocalise and are target to degradation thus suggesting that the mutant protein does not fold efficiently. Promoting the folding and the stability of mutant bestrophin-1 might restore trafficking and functionality of the mutant protein and might present a promising strategy to treat a group of different condition that share some similarities such as bestrophinopathies. The work presented in this thesis aimed to generate and validate a model system for RPE cells in order to easily test the ability of chemical chaperones and proteostasis regulators to restore expression, trafficking and function of four ARB-causing bestrophin-1 proteins: p.L41P, p.R141H, p.R202W and pM325T bestrophin-1.

In order to fulfil their role in cell function, proteins must fold in the correct three-dimensional structure which usually is represented by the most thermodynamically stable conformation with low free energy gain (Lindquist and Kelly 2011). The information needed for the achievement of the native structure is encoded in the protein amino acid sequence (Anfinsen et al. 1954), nonetheless a changes in the protein sequence caused by one or more mutations can dramatically affect protein folding by supporting a misfolded state because thermodynamically more stable (Anfinsen et al. 1954). Inefficient protein folding is often associated with disease. There are several mechanisms by which protein misfolding can cause disease, however, it is possible to group them in 2
categories: disease caused by a loss of functional activity of the protein due either to improper folding, degradation or mislocalisation and gain-of function diseases which are due to either mutations that cause a toxic novel function, dominant-negative mutations or amyloid accumulation (Valastyan and Lindquist 2014). The bestrophin-1 crystal structure (Dickson et al. 2014; Bharill et al. 2014) suggests that none of the mutations in the four ARB-causing bestrophin-1 proteins analysed in this study lie within the functional domains of the channel. However, the analysis of the amino acid substitutions p.L41P, p.R141H, p.R202W and p.M325T (Forbes Manson, personal communication) suggests that the mutations are likely to disrupt protein folding by increasing the Gibbs free energy. This confirms the hypothesis that p.L41P, p.R141H, p.R202W and p.M325T bestrophin-1 mutants cause disease by loss of function activity due to improper folding which leads to mislocalisation and rapid degradation of the mutant protein. A case of ocular disease caused by similar mechanism is the one of the fatty acid elongase ELOVL4. ELOVL4 is expressed at high level in the ER of both rod and cone photoreceptor outer segments where it is responsible for the elongation of long fatty acid chains. Mutations in the ELOV4 gene are associated with the autosomal dominant disease Stargardt-like macular degeneration. Mutant ELOVL4 proteins were shown to mislocalise to the cytoplasm and to form aggregates. Furthermore co-transfection of the WT protein resulted in the WT protein being sequestered into the aggregates thus demonstrating that the mutant protein acted in a dominant-negative manner (Karan et al. 2005; Karan et al. 2004)

In order to ensure effective detection and degradation of potentially toxic misfolded/non-native proteins, the cell has developed a complex series of QC checkpoints at the ER (ERCQ), Golgi, plasma membrane (PMQC) and the nucleus (Nielsen et al. 2014). Molecular chaperones play a key role in QC pathways by virtue of their ability to recognise non-native features exposed by misfolded/unfolded proteins. Recognition by molecular chaperones will initially attempt to promote folding. However, if the protein still fails to achieve the correct structure, then it will be targeted for degradation. Misfolded membrane proteins can be targeted for degradation by three different proteolytic pathways: the ER associated degradation (ERAD) mediated by the proteasome, vesicular targeting to lysosomes or autophagy (Ciechanover and Kwon 2015).
Although the recognition and degradation of mutant proteins by the QC systems is essential to maintain cellular and organismal homeostasis, in some cases it may be overactive and cause disease by promoting degradation of mutant proteins that still retain some functionality. In chapter 3 evidences were presented that mutant bestrophin-1 is recognised by QC control systems, retained intracellularly and targeted for degradation, possibly through ERAD. In order to do this MDCKII cells stably transfected with either WT or ARB-causing bestrophin-1 constructs were used as a cell model of the RPE. The observation that mutant bestrophin-1 is mislocalised in stable MDCKII cells is consistent with previous research showing that ARB-causing bestrophin-1 proteins are mislocalised in either MDCKII or porcine/human RPE cells (Johnson et al. 2014; Johnson et al. 2013; Milenkovic et al. 2011; Davidson et al. 2011) thus supporting the hypothesis that the different phenotypes observed in bestrophinopathies may be a consequence of the mutant protein being recognised by different QC mechanisms, retained in the cell and/or prematurely degraded, even thought they might still be functional. A similar case is the one of ΔF508 CFTR. Similarly to mutant bestrophin-1, ΔF508 CFTR is mislocalised, recognised by Hsp70 and transferred to the E3 ligase CHIP which promotes its degradation (Meacham et al. 2001). However, if degradation of the channel is inhibited, a portion of ΔF508 CFTR can get trafficked to the plasma membrane where it is partially functional (Wang et al. 2006). Promoting protein folding by the use of chemical and pharmacological chaperones has therefore become a valid therapeutic strategy to be used as alternative to or together with gene replacement therapy and could be a promising approach for the treatment of bestrophinopathies.

In chapter 4 evidences are presented that treatments of MDCKII cells stably expressing ARB-causing bestrophin-1 proteins with the chemical chaperone 4PBA are able to restore trafficking and function of mutant bestrophin-1 proteins. 4PBA, a hydrophobic short chain fatty acid, is a drug approved by the FDA for the treatment of the urea cycle disorders (Batshaw et al. 2001). The mechanism by which 4PBA is able to restore mutant bestrophin-1 expression, trafficking and function can be different. Thanks to his ability to inhibit histone deacetylases, 4PBA can act as a transcriptional regulator thus promoting
transcription of genes involved in protein folding. Nonetheless 4PBA can also inhibit expression of genes that encodes for QC protein involved in the recognition of mutant bestrophin-1 (Rubenstein and Zeitlin 2000). Furthermore 4PBA can assist protein folding (de Almeida et al. 2007; Cho et al. 2014) and prevent protein aggregation in the ER (Valastyan and Lindquist 2014) by shielding hydrophobic regions of the nascent protein. Although 4PBA appears to be a promising candidate for the treatments of bestrophinopathies, there are few considerations that have to be made. The fact that 4PBA has different mechanism of action suggests that his effect is non-specific. This means that further studies are needed for a better understanding of how this molecule might affect function of other biological systems. Furthermore 4PBA and chemical chaperones in general, are usually effective at high concentration and such concentrations are often toxic. Therefore alternative strategies need to be considered. Manipulating the intracellular proteostasis pathways can alleviate and, in some cases, restore the loss of function phenotype caused by protein misfolding (Brandvold and Morimoto 2015; Parfitt and Cheetham 2016). In chapter 5, evidenced are shown that WT and mutant bestrophin-1 interact with the molecular chaperones Hsp70. This suggests that an alternative therapeutic strategy to treat ARB and perhaps bestrophinopathies in general, may be to use proteostasis regulators to induce the heat shock response in order to increase Hsp70 production.

In conclusion, the data presented in this thesis showed evidences that mutant bestrophin-1 proteins are recognised by QC and are mislocalised and targeted for degradation thus justifying the loss of function phenotype observed in ARB. Restoration of protein expression, trafficking and function was possible by the use of the chemical chaperone 4-PBA. This is the first time that restoration of function has been shown for ARB-causing bestrophin-1 proteins. The work presented in this thesis pave the way for the use of small molecules to rescue misfolded protein of the RPE that cause retinal degeneration.
7 References


References


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References


