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Targeting the ER-Autophagy System in the Trabecular Meshwork to Treat Glaucoma

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Abstract

A major drainage network involved in aqueous humor dynamics is the conventional outflow pathway, which is gated by the trabecular meshwork (TM). The TM acts as a molecular sieve, providing resistance to aqueous outflow, which is responsible for regulating intraocular pressure (IOP). If the TM is damaged, aqueous outflow is impaired, IOP increases and glaucoma can manifest. Mutations in the *MYOC* gene cause hereditary primary open-angle glaucoma (POAG) by promoting the abnormal amyloidosis of the myocilin protein in the endoplasmic reticulum (ER), leading to ER stress-induced TM cell death. Myocilin accumulation is observed in approximately 70–80% of all glaucoma cases suggesting that environmental or other genetic factors may also promote myocilin toxicity. For example, simply preventing myocilin glycosylation is sufficient to promote its abnormal accretion. These myocilin amyloids are unique as there are no other known pathogenic proteins that accumulate within the ER of TM cells and cause toxicity. Moreover, this pathogenic accumulation only kills TM cells, despite expression of this protein in other cell types, suggesting that another modifier exclusive to the TM participates in the proteotoxicity of myocilin. ER autophagy (reticulophagy) is one of the pathways essential for myocilin clearance that can be impacted dramatically by aging and other environmental factors such as nutrition. This review will discuss the link between myocilin and autophagy, evaluating the role of this degradation pathway in glaucoma as well as its potential as a therapeutic target.

Introduction

Aqueous humor is a watery, ionic fluid, much like plasma in the blood, produced by the ciliary body that fills the anterior chamber of the eye (Abu-Hassan et al., 2014). Unlike the vitreous humor in the vitreous chamber of the eye, aqueous humor is constantly produced; therefore, in order to maintain a homeostatic environment, it must be constantly recycled out of the eye. Regulatory aqueous outflow occurs by two pathways in the anterior chamber: the conventional pathway, comprised mainly of the TM, and the unconventional outflow pathway, comprised of drainage channels located at the angle between the ciliary muscle

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and the iris. The conventional pathway regulates upwards of 85% of the aqueous outflow that occurs in the anterior chamber (Tamm, 2009).

TM is the major component of the conventional outflow pathway, comprised of TM cells, juxtacanalicular connective tissue (JCT), the inner walls of Schlemm's canal, Schlemm's canal, the collector channel, and finally the episcleral vein, which carries aqueous humor back to systemic circulation (Lutjen-Drecoll, 1999; Swaminathan et al., 2014). The TM is located at the iridocorneal junction of the anterior chamber, the area where the iris forms a junction with the cornea. In addition to giving the eye its shape, aqueous humor is responsible for providing nutrients to the avascular structures of the anterior chamber, including the lens and cornea (Abu-Hassan et al., 2014; Tamm, 2009). However, one of the more important functions aqueous humor provides to the microenvironment of the eye is producing IOP (Abu-Hassan et al., 2014), which is largely regulated by resistance of outflow provided by the TM in the conventional outflow pathway. Studies have shown that resistance to outflow is increased with age and in ocular disorders, such as glaucoma, where IOP elevation is a pathological hallmark (Tamm, 2009).

Glaucoma is a neurodegenerative protein misfolding disorder, characterized by retinal ganglion cell (RGC) death and optic nerve (ON) axon damage leading to progressive irreversible vision loss (Gupta and Yucel, 2007; Llobet et al., 2003; Porter et al., 2015; Stohtert et al., 2014; Wentz-Hunter et al., 2004). Glaucoma is the second leading cause of blindness worldwide, with over 60 million individuals suffering from the disease. In the United States alone, it is estimated that 2–3 million people have glaucoma, with over 120,000 individuals suffering blindness due to the disease (Fingert et al., 2002; Gupta and Yucel, 2007; Tamm, 2002). The most common forms of glaucoma include open-angle glaucoma, angle-closure glaucoma, normal-tension glaucoma, and congenital glaucoma. Although all forms of glaucoma are seen throughout the population, over 90% of cases involve a form of POAG (Bruttini et al., 2003; Shepard et al., 2007). In POAG aqueous drainage channels remain exposed, but the TM network is damaged preventing proper outflow (Bruttini et al., 2003). POAG arising from distinct mechanisms is often associated with accumulation of the glycoprotein myocilin, within the TM and Schlemm's canal (Burns et al., 2011; Jacobson et al., 2001; Konz et al., 2009; Lutjen-Drecoll et al., 1998; McDowell et al., 2012).

1. Normal Intracellular Myocilin Processing

Understanding the normal features of myocilin protein could help to elucidate why this protein accumulates in the glaucomatous eye. Myocilin is a ubiquitous 504 amino acid (aa) secreted glycoprotein expressed in both ocular and non-ocular tissues. In the eye, myocilin is found in the TM, sclera, ciliary body, choroid, cornea, iris, retina, and ON (Karali et al., 2000). Outside the eye, myocilin is expressed in tissues of the peripheral nervous system (Kwon et al., 2013). Structurally, myocilin consists of an N-terminal region containing a signal peptide sequence (aa 1–32), a helix-turn-helix (aa 18–58) and a leucine zipper domain containing two coiled-coil domains (aa 74–110 and aa 118–186) responsible for coordinating protein-protein interactions (Resch and Fautsch, 2009; Tamm, 2002). Myocilin is a glycoprotein that normally undergoes asparagine-linked glycosylation at aa residues 57–

59 (Asn-Glu-Ser) (Amaravadi et al., 2011; Jacobson et al., 2001). The C-terminal globular region contains the olfactomedin (OLF) domain (aa 230–504) (Donegan et al., 2012; Tamm, 2002), an evolutionarily conserved region of amino acids first discovered in the olfactory neuroepithelium. In humans, the OLF domain is found within a group of glycosylated proteins important in the organization of the nervous system during development (Anholt, 2014; Burns et al., 2011). Though myocilin is expressed throughout the body, its physiological function is yet unknown. Recent studies have suggested the importance of myocilin in oligodendrocyte differentiation and axonal myelination (Kwon et al., 2014), and myocilin may play a role in retinal cell programmed death during development (Koch et al., 2014).

The secretory pathway is responsible for synthesis, folding, and delivery of a wide range of cellular proteins, including myocilin (Barlowe and Miller, 2013; Farhan and Rabouille, 2011). This pathway is particularly important in correctly coordinating the localization of newly formed proteins to specific organelles and to the extracellular space (Farhan and Rabouille, 2011). The pathway is comprised of the rough endoplasmic reticulum (ER), ER-exit sites, ER-to-Golgi intermediate compartment (ERGIC), the Golgi complex, and post-Golgi carriers (Barlowe and Miller, 2013). During ribosome synthesis of a polypeptide, proteins that are to be secreted are targeted by a signal recognition particle (SRP), a molecule that targets hydrophobic peptide signal sequences on the N-terminal domain of a protein. SRPs direct the translocation of newly forming polypeptides into the ER membrane via translocons, or channels in the ER membrane, for continued translation and post-translational modifications required for secretion. Once in the ER membrane, post-translational modifications such as signal peptide cleavage, glycosylation, disulfide bond formation, and glycosidase trimming occur to ensure proper protein folding.

The cleavage of the N-terminal signal peptide sequence is a common modification for many proteins that enter the ER, including myocilin. Cleavage of this sequence occurs via signal peptidase complex (SPC), a 4-subunit polypeptide. Most of the SPC subunits are required for cell viability, except the Sec11 subunit, which contains the protease active site (Barlowe and Miller, 2013). The SPC cleaves off the N-terminal signal peptide sequence, exposing glycosylation sites on the protein (Barlowe and Miller, 2013; Farhan and Rabouille, 2011). Asparagine –linked (N-linked glycosylation) is a post-translational modification where oligosaccharides are attached to asparagine residues in the N-terminal domain of the protein (Schwarz and Aebi, 2011). Oligosaccharyltransferase enzyme (OST), an 8-subunit polypeptide, carries out the addition of oligosaccharides. Cells require the majority of the OST subunits for viability, though the Stt3 domain is dispensable and only required for catalytic activity. N-linked glycosylation of a protein is thought to be important in thermodynamic stability, solubility, and protein folding (Schwarz and Aebi, 2011). Glycosylated proteins in the ER membrane then form disulfide bonds at free sulfhydryl groups on cysteine residue side chains due to the oxidizing environment within the ER lumen. A specialized family of disulfide isomerases is required for the formation, reduction and isomerization of these disulfide bonds, ensuring correct protein folding (Farquhar et al., 1991). Once the signal sequence is cleaved, proteins are glycosylated and disulfide bonds form. Following this process, the final step required for proper protein folding is the trimming of glucose residues from the protein oligosaccharide core. Glucosidases (Gls1/

Gls2) are enzymes that rapidly trim glucose residues, allowing for proper chaperone folding (Helenius and Aebi, 2004). Mns1 and Htm1, enzymes that cleave mannose residues, generate signals for protein degradation by recognizing proteins that are terminally misfolded (Jakob et al., 1998).

After post-translational modifications, proteins are exported to ER-exit sites, areas that have a high concentration of coat-protein complex II (COPII) (Farhan and Rabouille, 2011; Lee et al., 2004). COPII is a molecule that forms a coat around properly folded proteins, promoting vesicle formation for Golgi transport. COPII vesicle formation is activated by the ER GTPase Sar1, which interacts with Sec proteins responsible for protein recruitment to form Sec23–24 and Sec13–31 complexes. The Sec23–24 complex is vital for protein capture and the Sec13–31 complex is necessary for COPII coat formation (Lee et al., 2004; Matsuoka et al., 2001). Once COPII vesicles are formed around proteins, they can fuse with each other forming pre-Golgi intermediates (Xu and Hay, 2004), or fuse with already made ERGIC (Appenzeller-Herzog and Hauri, 2006) and directed to the Golgi complex (Fig. 1A), where they are further modified and processed, allowing delivery to their final destination (Emr et al., 2009; Jackson, 2009). Each step along this pathway represents a potential point where myocilin processing could go awry. This entire process is particularly relevant for understanding how a group of POAG cases that are caused by mutations in the secreted myocilin protein.

2. Myocilin Misfolding Propagates Glaucomatous Pathology

Mutations in the *MYOC* gene are observed in ~30% of juvenile onset glaucoma cases, and 2–4% of patients with POAG. To date, over 70 glaucoma-causing myocilin mutations have been identified, with >90% found in the C-terminal OLF domain (Resch and Fautsch, 2009). Less than 10% of disease-causing mutations are located in the N-terminal domain. Mutations in the C-terminus of the myocilin protein result in conformational changes in the tertiary structure of the protein, namely that charged residues important for regulating cleavage and secretion are no longer solvent-exposed (Aroca-Aguilar et al., 2005; Bruttini et al., 2003; Fingert et al., 1999; Fingert et al., 2002) (Table 1). These mutations thus induce a toxic gain of function for the cell via myocilin protein misfolding and abnormal amyloidosis in the ER of TM cells (Suntharalingam et al., 2012). Based on the genetic evidence, it is thought that the point mutations in the *MYOC* gene make the protein prone to misfolding, intracellular retention and subsequent aggregation somewhere along the secretory pathway in TM cells. This leads to ER stress, causing TM cell death, dysfunctional aqueous humor outflow, increased IOP and eventually optic nerve damage (Aroca-Aguilar et al., 2005; Jacobson et al., 2001; McDowell et al., 2012).

In addition to inherited mutations in myocilin, inhibition of post-translational modifications in WT myocilin can alter secretion leading to intracellular accumulation. Our laboratory examined this phenomenon by utilizing two pharmacological inhibitors, tunicamycin and brefeldin A. Tunicamycin is a well-documented inhibitor of N-linked glycosylation, leading to improper protein folding; Brefeldin A is an inhibitor of ER-Golgi transport. Treatment of cells overexpressing WT myocilin with Tunicamycin or Brefeldin A resulted in increased levels of intracellular myocilin (Suntharalingam et al., 2012). Moreover, prolonged

treatment with glucocorticoids can result in increased myocilin deposition within the ER of TM cells, which has been documented to cause glaucoma pathology in humans (Kersey and Broadway, 2006; Mao et al., 2013; Whitlock et al., 2010). We have shown that WT myocilin, similar to other secreted proteins, is typically degraded through the proteasomal-dependent ER-associated degradation (ERAD) mechanism (Fig. 1b) (Barlowe and Miller, 2013; Farhan and Rabouille, 2011; Suntharalingam et al., 2012). ERAD is a process that uses the VCP/p97 complex to typically triage misfolded proteins from the ER lumen to the cytosol for ubiquitination and subsequent proteasomal degradation (Ye et al., 2001). But in cases where misfolded myocilin is produced, either by mutation or altered post-translational modification, this clearance mechanism fails, leading to myocilin aggregation, ER stress and TM cell toxicity. Therefore, if ERAD could be restored for these misfolded myocilin species, or another clearance mechanism could be engaged, such as autophagy, myocilin toxicity in the TM could be reduced.

3. Autophagic Degradation of Misfolded Proteins Promotes Cellular Homeostasis

Even in normally functioning cells, if there is a proteotoxic stress leading to accumulation of misfolded proteins in the ER, the unfolded protein response (UPR) is activated. If the activation is acute, then the UPR is beneficial to the cell, but if the activation is chronic, it can facilitate cell death (Born et al., 2013; Gorbatyuk and Gorbatyuk, 2013; Zhu and Lee, 2014). In cases of glaucoma caused by mutations in the *MYOC* gene, the UPR seems to be chronically activated, suggesting that this could be a primary mechanism of TM cell death. While trying to stimulate ERAD of mutant myocilin is one approach for overcoming chronic UPR activation, one caveat of this mechanism is that ubiquitin-proteasome degradation is limited to monomeric proteins. For an amyloidogenic protein like myocilin, this simply may never work. When the cell needs to degrade damaged organelles or aggregated proteins, a less specific but more robust ER autophagic mechanism of clearance (reticulophagy) is available. Engaging this system may be much more efficient for facilitating removal of myocilin aggregates from the ER (Klionsky and Codogno, 2013).

Autophagy is a general term used for the process involving degradation by lysosome-containing vesicles. There are at least three types of autophagy, differing by their means of delivering material to the lysosome for degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy (Mizushima, 2007; Porter et al., 2014). During chaperone-mediated autophagy, substrates are recognized by a particular amino acid peptide sequence and are translocated into the lumen of a lysosome for degradation (Cuervo and Wong, 2014). Microautophagy is a less specific process where the lysosomal membrane invaginates or sequesters material in the cytosol for degradation within the lysosome (Mijaljica et al., 2011). In addition, organelles such as the mitochondria and the ER can degrade their protein content via distinct routes that ultimately utilize macroautophagy (Cebollero et al., 2012).

Macroautophagy is a catabolic process necessary for cell homeostasis (Frost et al., 2014; Mizushima, 2007; Porter et al., 2015). It is upregulated when cells require intracellular nutrients and energy such as during starvation, growth factor withdrawal, or in the context of high-energy demand (Amaravadi et al., 2011). Additionally, macroautophagy can be

upregulated in times of cellular proteotoxic stress, when misfolded proteins accumulate. Macroautophagy is a multi-step process consisting of four stages: initiation, nucleation, maturation, and degradation. Initiation occurs by the down-regulation of the mammalian target of rapamycin (mTOR) pathway (Porter et al., 2014). In times of starvation autophagy-related protein 13 (Atg13) and unc51-like autophagy activating kinase (ULK1/2) are upregulated leading to the inhibition of the mTOR pathway. Conversely, when growth factors, such as insulin-like growth factor, bind to growth factor receptors, initiation of receptor tyrosine kinase signaling and subsequent activation of protein kinase B (AKT) occurs. AKT activation inhibits the major inhibitory proteins of the mTOR pathway, tuberous sclerosis 1 and 2 (TSC1/2), resulting in the activation of the mTOR pathway and subsequent inhibition of macroautophagy (Rubinshtein et al., 2011). Additionally, mTOR inhibition leads to ULK1 phosphorylation and activation of autophagic effectors Beclin1 and phosphatidylinositol 3-kinase (Vps34) (Amaravadi et al., 2011).

Following initiation, nucleation is the process in which the autophagosome, the unique organelle associated with macroautophagy, forms. Phosphorylated Beclin1/Vps34, along with p150, form a stable complex that initiates formation of the, pre-autophagosome, or phagophore (Rubinshtein et al., 2011). The phagophore matures into the autophagosome and fully surrounds the target cytosolic components. This process involves the elongation of the phagophore membrane via the incorporation of microtubule-associated protein 1A/1B-light chain 3 (LC3) into the membrane. LC3 mediates autophagosome growth and cytoplasmic cargo recruitment, and provides a scaffold to transport fully formed autophagosomes to the lysosomes. Once the autophagosome has fully formed around cytoplasmic constituents, LC3 mediates dynein-dependent microtubule trafficking to lysosomes. Finally, the autophagosome fuses with the lysosome via the SNARE proteins VAMP8, Vti1B, and Lamp2, and results in the formation of the autolysosome. Following autolysosome formation, the autophagic cargo is degraded and then recycled back to the cytosol for cell re-uptake (Amaravadi et al., 2011; Rubinshtein et al., 2011).

Until recently macroautophagy was thought of as a non-selective degradation pathway, but current research has proposed the idea of organelle-specific autophagy, including ribophagy (ribosomes), mitophagy (mitochondria) and reticulophagy (ER). While specific details on individual organelle autophagy remain largely uncharacterized, it is postulated that cellular constituents are targeted for these distinct autophagic pathways by Atg upregulation leading to autophagosome formation or possibly by accumulation of intra-ER ubiquitinated proteins (Cebollero et al., 2012). The latter is supported by work from our laboratory showing that only mutant/misfolded myocilin becomes ubiquitinated within the ER following treatment with epoxomicin, a proteasome inhibitor (Suntharalingam et al., 2012). In this way, the accumulation of misfolded, ubiquitinated myocilin could activate reticulophagic degradation.

4. TM Autophagic Dysregulation Promotes Ocular Disorders

Studies have shown that increasing macroautophagy can delay the aging process, and extend tissue longevity. This was first observed in the *C.elegans* model, where inhibition of the insulin-like growth factor receptor induced autophagy (Melendez et al., 2003). Conversely,

mutation of Atg genes resulted in decreased tissue longevity (Melendez et al., 2003). Moreover, age-related reductions in Beclin1 (Pickford et al., 2008), Sirtuin1 (A modulator of mTOR inhibition)(de Kreutzenberg et al., 2010), and Atg5/7 (Lipinski et al., 2010) protein levels have been observed in aged tissue. Additionally, decreased levels of ULK1 and LC3 have been observed in aged tissue. As glaucoma is a slowly progressing ocular disorder and pathology increases are associated with aging, it is possible this could largely be an effect of reduced autophagic protein clearance in TM cells.

Studies in primary human TM (hTM) cells have also shown that during glaucoma, the autophagic mechanism in TM cells is dysregulated. Recent research has indicated a dysregulation of the autophagic pathway and reduction of the autophagic response to oxidative stress in TM cells isolated from patients with glaucoma (Porter et al., 2015). Interestingly, this research determined an overall reduction of LC3 in TM cells of glaucoma patients compared to controls (Porter et al., 2015). Additionally, there was an increase in senescence-associated- β -galactosidase (SA- β -Gal) activity, which is shown to be upregulated when autophagy is inhibited in primary human fibroblasts (Kang et al., 2011). Moreover, they observed an increase in lipofuscin, or non-degradable lysosomal content, in TM cells of glaucoma patients compared to control cells (Porter et al., 2015). Other proteins associated with autophagy found to be down regulated during glaucoma were sequestosome-1 (p62), a scaffold that targets ubiquitinated proteins for autophagic degradation, scCTSB, a lysosomal protein, and LC3B-II (Porter et al., 2015). Taken together, these data show that autophagic clearance mechanisms are dysfunctional in TM tissue of glaucoma patients. Thus, restoring these pathways could be beneficial in disease.

5. Grp94 Prevents Misfolded Myocilin Degradation via Autophagy

ER homeostasis, including ERAD and reticulophagy pathways, is mediated by chaperone activity. Chaperones aid in the proper folding of proteins under physiological conditions as well as in times of ER stress (Cuervo and Wong, 2014; Rikhvanov et al., 2007; Verchot, 2014). Stress caused by accumulation of terminally misfolded proteins activates the UPR, resulting in cessation of protein translation (Gorbatyuk and Gorbatyuk, 2013; Verchot, 2014) and increased production of molecular chaperones (Born et al., 2013). We recently performed a small-interfering RNA (siRNA) screen on several ER-associated chaperones to examine their effects on overexpressed I477N mutant myocilin or WT myocilin in Hek293T cells (Suntharalingam et al., 2012). Interestingly, only knockdown of glucose-regulated protein 94 (Grp94), the ER-specific isoform of Hsp90, caused intracellular I477N mutant myocilin reduction. Under physiological conditions, Grp94 is inactive (Marzec et al., 2012). During ER stress, Grp94 is associated with ER quality control and aids in the proper folding of misfolded proteins. Grp94 also associates with the ERAD-associated osteosarcoma amplified 9 (OS-9) protein and acts to shuttle terminally misfolded monomeric protein to the ER membrane for subsequent proteasomal degradation (Dersh et al., 2014; Marzec et al., 2012).

This intracellular reduction was also observed in cells overexpressing four other common glaucoma-associated myocilin mutations (Stohtert et al., 2014). Interestingly, Grp94 knockdown had no effect on intracellular or secreted levels of WT myocilin. Moreover,

following myocilin immunoprecipitation (IP), we found that Grp94 associated with mutant protein but not WT myocilin (Suntharalingam et al., 2012), highlighting the specificity offered by this chaperone. Using a cycloheximide (a potent inhibitor of protein translation) chase experiment, we were able to determine that the half-life of intracellular mutant myocilin was decreased after Grp94 inhibition. Additionally, we observed an increase in high-molecular weight myocilin species with Grp94 overexpression, and conversely, reduction in high-molecular weight myocilin species after Grp94 knockdown (Suntharalingam et al., 2012). This led us to believe that Grp94 was preserving mutant myocilin, and possibly driving protein aggregation.

Importantly, Grp94 inhibition also reduced intracellular mutant myocilin levels, as well as cellular toxicity associated with mutant myocilin overexpression, in primary hTMs (Stothert et al., 2014). Interestingly, Grp94 inhibition had no effect on normally folded WT myocilin levels. However, when N-linked glycosylation of WT myocilin was blocked by tunicamycin, we observed an increased association with WT myocilin and Grp94. This was accompanied by sensitization to Grp94 inhibition (Suntharalingam et al., 2012). Moreover, Grp94 could stimulate aggregation of WT myocilin OLFs *in vitro*, a process that could be mitigated by the addition of Grp94 inhibitor (Stothert et al., 2014). These data led us to the conclusion that Grp94 recognizes misfolded myocilin species within the ER, but iteratively attempts to triage the protein through ERAD unsuccessfully. This leads to myocilin protein aggregation in the ER and subsequent TM cell toxicity (Fig. 2).

To explore the feasibility of Grp94 inhibition as a potential therapeutic mechanism, one first must examine the importance of Grp94 in an organism. Grp94, as a molecular chaperone of the ER, has relatively few client proteins (Duerfeldt et al., 2012). Interestingly, despite this Grp94 is necessary for embryonic development. This has been shown in studies looking at both heterozygous (+/-) and homozygous (-/-) knockdown of Grp94 in murine models and of gp93 in drosophila (Maynard et al., 2010). While the -/- mice have lethality and a number of other functional deficits, +/- mice survive and seem healthy, suggesting that partial but not complete depletion of Grp94 may be a viable therapeutic strategy (Mao et al., 2010). Moreover, recent studies have shown that targeted knockout in discreet tissues does not impact viability (Zhu et al., 2014). However, Grp94 does play important roles in immune cell and liver function (Chen et al., 2014; Chen et al., 2014), and others, suggesting that caution will be necessary moving forward with therapeutic approaches targeting this protein.

While previous data suggests that the inhibition of Grp94 association with misfolded or mutant myocilin leads to intracellular degradation of the myocilin protein and therefore could be a potential therapeutic target to treat glaucoma, the mechanism by which degradation of this protein occurs was still not known. To determine if the autophagic pathway was involved in misfolded myocilin protein clearance, cells overexpressing mutant myocilin (I477N) were co-transfected with Grp94 siRNA and either Lamp2 or Beclin1 siRNA, two well-characterized components of the autophagy pathway. Indeed the addition of Lamp2 or Beclin1 siRNA abrogated intracellular myocilin clearance following Grp94 knockdown (Suntharalingam et al., 2012). Thus, inhibiting Grp94 shunts misfolded myocilin towards the autophagic degradation pathway, a pathway that must be functional for mutant

myocilin turnover (Fig. 3). In fact, since TM cell dysfunction prevents normal aqueous humor outflow, nutrient delivery to these cells is impaired, which would normally trigger macroautophagy and possibly facilitate misfolded myocilin clearance, restoring balance to the outflow pathway. But, because of the interaction of Grp94 with myocilin, its autophagic degradation is prevented, allowing glaucomatous pathology to perpetuate. Therefore, discovering therapeutic measures to enhance the ER autophagic mechanism, allowing for misfolded myocilin to be degraded, could lead to restoration of TM cell function and reduction in glaucomatous pathology. In fact, perhaps combining Grp94 inhibition with enhancers of reticulophagy could work together synergistically to promote myocilin clearance and rescue glaucoma caused by myocilin toxicity.

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- Mutations in the *MYOC* gene lead to intra-ER protein accumulation and aggregation resulting in TM cell toxicity
- Misfolded myocilin associates with Grp94, preventing autophagic degradation of aggregated protein
- Grp94 inhibition allows misfolded myocilin aggregates to be processed and degraded through an autophagic mechanism
- Treatment with Grp94 inhibitions in coordination with autophagic enhancers could be useful in treating glaucoma

Figure 1. Normal intracellular processing of myocilin

After signal recognition particle (SRP) targeting and translocation of a newly forming myocilin polypeptide to the endoplasmic reticulum lumen, postranslational modifications occur to ensure proper folding of the protein. If these modifications are successful (A), the newly formed, properly folded, protein will be sent to the golgi apparatus via COPII coat formation for packaging and delivery to specific cellular organelles or the extracellular space. However, if these modifications do not properly occur (B), and the protein becomes terminally misfolded, the intra-ER degradation mechanism, known as ERAD, will degrade the protein through a proteasomal dependent manner for peptide recycling. This method requires the ubiquitination of misfolded proteins, and is only substantial if the misfolded protein is monomeric.

Figure 2. Grp94 association with mutant myocilin promotes intracellular accumulation
 Mutations in the MYOC gene lead to the production of myocilin protein that readily misfolds and is prone to aggregation. Grp94 is an ER-associated co-chaperone that recognizes pre-aggregated misfolded myocilin, preventing ERAD proteasomal degradation, leading to aggregation within the ER. Proteotoxicity caused by aggregated myocilin in the ER leads to TM cell death and aqueous outflow dysfunction, promoting the pathologies associated with glaucoma.

Figure 3. Inhibition of Grp94 interaction with mutant myocilin promotes autophagic degradation

Through siRNA knockdown or pharmacological inhibition, Grp94 association with misfolded myocilin can be prevented, inhibiting protein aggregation. After inhibition, misfolded myocilin can be processed into transport vesicles for transport out of the ER lumen to the cell cytosol, where these vesicles fuse with lysosomes promoting autophagic degradation of the misfolded protein. As aggregated misfolded myocilin is cleared from the ER of TM cells, aqueous outflow becomes restored.

Table 1

List of known mutations in the *MYOC* gene that lead to glaucoma.

Mutation (AA)	Gene Location	Ref
C25R	Exon1	(Bruttini et al., 2003)
R46X	Exon1	(Fingert et al., 1999)
R82C	Exon1	(Alward et al., 1998) (Fingert et al., 1999)
R91X	Exon1	(Fan et al., 2004)
R126W	Exon1	(Faucher et al., 2002)
<u>C245Y</u>	Exon 3	(Yam et al., 2007)
<u>G246R</u>	Exon 3	(Fingert et al., 2002)
<u>V251A</u>	Exon 3	(Michels-Rautenstrauss et al., 2002)
<u>G252R</u>	Exon 3	(Fingert et al., 2002)
<u>R272G</u>	Exon 3	(Fingert et al., 2002)
<u>P274R</u>	Exon 3	(Markandaya et al., 2004)
<u>W286R</u>	Exon 3	(Fingert et al., 1999)
<u>T293K</u>	Exon 3	(Fingert et al., 1999)
<u>E300K</u>	Exon 3	(Fan et al., 2004)
<u>S313F</u>	Exon 3	(Fan et al., 2005)
<u>E323K</u>	Exon 3	(Fingert et al., 2002)
<u>Q337R</u>	Exon 3	(Fingert et al., 2002)
<u>R342K</u>	Exon 3	(Challa et al., 2002)
<u>I345M</u>	Exon 3	(Michels-Rautenstrauss et al., 2002)
<u>Y347X</u>	Exon 3	(Fingert et al., 1999)
<u>I360N</u>	Exon 3	(Izumi et al., 2003)
<u>P361S</u>	Exon 3	(Fingert et al., 1999)
<u>A363T</u>	Exon 3	(Izumi et al., 2003)
<u>G364V</u>	Exon 3	(Fingert et al., 1999)
<u>G367R</u>	Exon 3	(Kanagavalli et al., 2003)
<u>Q368X</u>	Exon 3	(Fingert et al., 1999)
<u>P370L</u>	Exon 3	(Fingert et al., 2002)
<u>T377M</u>	Exon 3	(Fingert et al., 1999) (Kanagavalli et al., 2003)
<u>D380G</u>	Exon 3	(Gong et al., 2004)
<u>D380H</u>	Exon 3	(Gong et al., 2004)
<u>D380N</u>	Exon 3	(Gong et al., 2004)
<u>D380A</u>	Exon 3	(Fingert et al., 2002)
<u>S393N</u>	Exon 3	(Michels-Rautenstrauss et al., 2002)
<u>S393R</u>	Exon 3	(Fingert et al., 1999)
<u>K423E</u>	Exon 3	(Fingert et al., 2002)
<u>V426F</u>	Exon 3	(Fingert et al., 2002)
<u>A427T</u>	Exon 3	(Gobeil et al., 2006)

Mutation (AA)	Gene Location	Ref
<u>C433R</u>	Exon 3	(Nagy et al., 2003)
<u>Y437H</u>	Exon 3	(Fingert et al., 1999)
<u>A445V</u>	Exon 3	(Fingert et al., 1999)
<u>T448P</u>	Exon 3	(Fingert et al., 2002)
<u>N450D</u>	Exon 3	(Michels-Rautenstrauss et al., 2002)
<u>Y453del</u>	Exon 3	(Fingert et al., 1999)
<u>I465M</u>	Exon 3	(Fingert et al., 1999)
<u>R470C</u>	Exon 3	(Fingert et al., 1999)
<u>Y471C</u>	Exon 3	(Fan et al., 2004)
<u>I477N</u>	Exon 3	(Fingert et al., 1999)
<u>I477S</u>	Exon 3	(Fingert et al., 2002)
<u>N480K</u>	Exon 3	(Fingert et al., 2002)
<u>P481S</u>	Exon 3	(Fingert et al., 1999)
<u>P481T</u>	Exon 3	(Fingert et al., 1999)
<u>P481R</u>	Exon 3	(Fingert et al., 1999)
<u>E483X</u>	Exon 3	(Fingert et al., 1999)
<u>I499F</u>	Exon 3	(Fingert et al., 2002)
<u>S502P</u>	Exon 3	(Fingert et al., 2002)

BOLD denotes mutations in the N-Terminal domain.

Underline denotes mutations in the C-Terminal domain.