Correlation of ER stress and retinal degeneration in *tubby* mice

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1. Introduction

Tubby mice carry a splicing mutation in the *Tub* gene which results in a malfunctioning intron coding protein (Bode and Wolfrum, 2003). Recently, a human ocular disease was shown to result from a mutation in the *Tub* gene and this disease exhibits the same phenotype as seen in *tubby* mice (Borman et al., 2014). One distinct characteristic of the *tubby* phenotype is the mislocalization of the photosensitive pigments (opsins) in the retina: they lose their polarized and restricted localization, and instead, the proteins are scattered throughout the photoreceptor cells (Cai et al., 2012; Kong et al., 2006; Sun et al., 2012). We have shown that oxidative stress is involved in, and accelerates, the retinal degeneration in *tubby* mice (Kong et al., 2006). Also prevention of photoreceptor cell death can be achieved via decreasing the amount of reactive oxygen species (ROS) by sulforaphane induced up regulation of antioxidant enzymes (Kong et al., 2007), overexpression of the thioredoxin (Trx) gene (Kong et al., 2010), or injection of catalytic nanoceria (Cai et al., 2012; Kong et al., 2011), which are inorganic direct antioxidants with properties mimicking superoxide dismutase and catalase (Karakoti et al., 2008).

ER (endoplasmic reticulum) stress has recently been implicated in many neurodegenerative diseases including ocular diseases and represents a distinct mechanism involved in cell death and other pathological activities (Cao and Kaufman, 2014; Sano and Reed, 2013; Tzekov et al., 2011). Over the past decade, targeting ER stress and the unfolded protein response

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(UPR) has emerged as a therapeutic strategy for a variety of disease treatments (Hetz et al., 2013; Kim et al., 2008). The ER is an important cellular organelle, which is responsible for protein synthesis, folding, secretion and Ca^{2+} homeostasis. The proper function of ER and its finely-tuned regulation of the maintenance of the proteome (refolding and/or degradation of the unfolded/misfolded proteins) play a critical role in determining cell survival or death (Cao and Kaufman, 2014; Szegezdi et al., 2006; Tzekov et al., 2011; Xu et al., 2005). Ample data demonstrate that many diseases are triggered by dysfunction of the protein quality control system in which accumulation of unfolded/misfolded proteins in the lumen of the ER, termed ER stress, causes the imbalance between the load of protein folding and ER capacity for protein folding (Chen et al., 2011). Oxidative stress has been reported to be the most dominant causative factor which cross-talks, interacts and is tightly linked with ER stress (Cao and Kaufman, 2014; Zhang, 2010). The UPR is initiated for self-protection in early ER stress conditions when the three ER stress sensors, PERK (protein kinase RNA-like ER kinase), ATF6 (activating transcription factor 6) and IRE1 (inositol-requiring enzyme 1), are activated following the release of GRP78/BiP (glucose regulated protein 78/ binding immunoglobin protein). However, in cases of persistent ER stress conditions, increases in accumulation of malfolded proteins and failure of ER homeostasis re-establishment result in the impairment of the UPR. This eventually causes the UPR to switch its function from anti-apoptotic to pro-apoptotic and the apoptosis signaling pathway is initiated (Lai et al., 2007; Szegezdi et al., 2006) through regulation of caspases and CHOP (CCAAT/enhancer-binding protein homologous protein) expression.

It is logical to think that the mislocalization of photoreceptor-specific proteins in *tubby* mice might be indicative of the involvement of ER stress. To investigate the role of ER stress in the retinal degeneration in *tubby* mice, we examined the expression levels of ER stress markers, their dynamics, and the alteration of the components of the UPR during retinal development from P4 through P28. Furthermore, the expression of several apoptosis associated factors was analyzed to elucidate the mechanism of ER stress action for photoreceptor degeneration in *tubby* mice.

2. Materials and Methods

2.1 Animals

tubby mice on C57BL/6J background and wild type (wt, C57BL/6J) were purchased from Jackson laboratory and used as breeders for the colonies. Animals were maintained and genotyped as previously reported (Cai et al., 2012; Kong et al., 2006). Animal care and handling strictly follow the ARVO statement for the Use of Animals in Ophthalmic and Vision Research (ARVO), and the protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) of University of Oklahoma Health Sciences Center and Dean A. McGee Eye Institute.

2.2 qRT-PCR and PCR array

Individual retinas (4 – 8) from *tubby* and C57BL/6J mice at postnatal day (P) P4 - P28 were collected; Total RNA isolation, cDNA reverse transcription, qRT-PCR performance and calculation of the relative expression of the target genes against the house-keeping gene

GAPDH are the same as previously reported (Cai et al., 2012). Primers for mouse Grp78/BiP and Chop are the same as previously reported (Lin et al., 2007). PCR array was carried out using the "mouse unfolded protein response" array plates (SABiosciences). Changes in gene expression were shown as fold changes of *tubby* compared to wt (*tub*/wt) and fold changes between two groups of *tubby* (P28/P7) using the array software with *P* values indicated (SABiosciences).

2.3 Immunocytochemistry

The protocol we used for immunocytochemistry is the same as previously reported (Cai et al., 2012). Briefly, eyes were enucleated and fixed, the cornea and lens were removed. After cryo-protection, the eyecups were embedded and 10 μm thick sections were cut. The slides were blocked with 5% BSA, then incubated in the following primary antibodies for 2 hrs at room temperature: mouse anti-rhodopsin (R1D4) (1:2000, generous gift from Dr. Robert Molday, Columbia University, Vancouver, Canada), rabbit anti-M-opsin (1:500, Millipore, AB5405) and rabbit anti-GRP78 BiP (1:300, Abcam, ab21685). The slides were rinsed with PBS buffer and incubated with the secondary antibodies of goat anti-mouse AlexaFluor 594 or goat anti-rabbit AlexaFluor 488 for 1 hr at room temperature. After mounting with anti-fade Vectashield mounting media with DAPI, the slides were coverslipped, observed and imaged under a Nikon Eclipse 800 epi-fluorescence microscope using a 20x or a 40x lens with proper filters.

2.4 Western blot

Individual retinas (8–10) from P4 - P28 *tubby* and wt mice were collected, homogenized and proteins were quantified (Cai et al., 2012). Soluble proteins, 30 μg, were loaded in 10% SDS-page gels and electrophoresed. After electrophoretic transfer to the nitrocellulose membrane, the following primary antibodies at 1:1000 dilution were used: phospho-eIF2α (Ser51)(119A11) rabbit mAb and phospho-Bcl2 (Ser70)(5H2) rabbit mAb (Cell signaling technology, 3597 and 2827, respectively), mouse anti-ATF6 (Imgenex, IMG-273), rabbit anti-GRP78 BiP and rabbit anti-IRE1 (phospho S724) (Abcam, ab48187), rabbit NF-κB p65 (C-20) (Santa Cruz Biotechnology, sc-372) and mouse anti-XBP-1s (Biolegend, 647502). β-actin (13E5) rabbit mAb (HRP conjugate) (Cell signaling technology, 5125) was used as a loading control. The membrane development, imaging and densitometry of the bands were performed as previously reported (Cai et al., 2012; Kong et al., 2011).

2.5 Statistical analysis

Data are shown as mean ± SEM and indicated in each figure. The unpaired student *t*-test and/or one-way ANOVA with Bonferroni's post hoc test for multiple comparison were performed and significant differences were determined by $P<0.05$.

3. Results

3.1 Mislocalization of rod and cone opsins in an early age

The rod and cone opsins, and other phototransduction-associated proteins have a polarized location in the membrane of retinal outer segment (OS) where they function in the visual cycle. We previously reported that rhodopsin, arrestin and α-transducin are mislocalized in

tubby retinas when examined at P18 (Kong et al., 2006) and the same phenomenon was confirmed and extended to P28 or 1 month of age (Cai et al., 2012; Sun et al., 2012). However, there are no data indicating whether the protein mislocalization is a primary event or secondary response (they are transported to and distributed in the OS at the beginning of the OS formation but then the polarity is lost as the disease progresses). We performed immunocytochemistry to detect the expression and distribution of rod opsin (rhodopsin) and middle wave-length cone opsin (M-opsin) during retinal development (P4 - P28) in *tubby* and age-matched wt mice. Fig. 1 shows that rhodopsin in wt retinas was present at P4 as numerous small dots aligned in the nascent OS adjacent to the outer nuclear layer (ONL), while M-opsin was detectable at P12. The expression pattern of both proteins was retained and their levels increased with age. They reached the highest expression level and exhibited the typical expression pattern at P28. However in *tubby* retinas, rhodopsin appeared slightly later, compared to wt, at P7 and was widely distributed over the entire shorter OS, ONL and outer plexiform layer (OPL). M-opsin was also detectable at P12 in *tubby* retinas. In contrast to its proper localization (in the OS) in the wt, M-opsin in *tubby* retinas was localized in the short OS, ONL and OPL at P28. These data suggest that mislocalization of photoreceptor-specific proteins in *tubby* retinas is an early and possibly primary event following the loss of *tubby* function.

3.2 Activities of ER stress markers during *tubby* retinal development

We have previously reported that oxidative stress is involved in *tubby* retinal degeneration (Kong et al., 2006). Logically, we think that ER stress is most likely also involved in this process because oxidative stress and ER stress have been reported to be tightly correlated and interactive with each other (Cao and Kaufman, 2014; Zhang, 2010). Based on our previous report that rapid photoreceptor loss occurs at P14 through P34 (Kong et al., 2006), western blots were performed to examine the expression of ER stress markers during retinal development (P4 - P28) using wt and *tubby* retinas. As shown in Fig. 2, the expression of activated IRE1 (p-IRE1) (Fig. 2A) in *tubby* retinas is slightly higher than age-matched wt at P4 and is lower than the wt at P12 and thereafter, although its expression level continues to be up regulated. Cleaved ATF6 level (Fig. 2B) in *tubby* retinas is up regulated compared to age-matched wt from P4 and its elevated level peaks at P12. Although ATF6 expression is decreased at P20, it is still greater than the wt. elF2α is downstream of PERK, so its activation (by phosphorylation) and level of expression directly reflect the level of phosphorylated PERK. Our data (Fig. 2C) demonstrated that p-eIF2α in *tubby* retinas is greater than wt at P4 through P12 and becomes less than wt at P20 through P28.

XBP-1 (transcription factor X-box binding protein) is the downstream transcription factor of phosphorylated IRE1. Its active form (spliced XBP-1, XBP-1s) binds to the promoters of its targeted genes to regulate their expression and help in refolding and degrading the unfolded/misfolded proteins (Lee et al., 2003). In the current study, western blot assay (Fig. 2D) shows that XBP-1s is up regulated at P4 through P20 in *tubby* retinas compared to age-matched wt, although its expression gradually decreased to the wt level at P28.

GRP78/BiP is an ER resident chaperone protein and a key regulator of the UPR. Its cytoprotective/anti-apoptotic role has been reported in many tissues in a variety of diseases

(Gorbatyuk et al., 2010; Gorbatyuk et al., 2012; Morris et al., 1997; Pfaffenbach and Lee, 2011). To determine the dynamics of GRP78/BiP during retinal development, western blots and qRT-PCR were performed using P4 - P28 retinas from wt and *tubby*. Fig. 3 shows that the expression of GRP78/BiP protein and mRNA levels in *tubby* retinas are greater than the age-matched wt at P4 and peaks at P12. Its expression is lower than the wt and younger *tubby* mice at P20 through P28 (Fig. 3A–C). The expression and distribution of GRP78/BiP in the retinas were also assessed by immunocytochemistry (Fig. 4). The results show that weak expression of GRP78/BiP was seen in the RPE and it was stronger in the ganglion cell layer (GCL) in P4 wt retinas. It further distributed into the INL at P7. GRP78/BiP was distinct and strongly expressed in the RPE, IS, INL and GCL at P20 through P28. The expression pattern of GRP78/BiP in *tubby* retinas was similar to the wt. However, the fluorescence intensity in *tubby* retinas was more than the wt at P4 through P12, and the fluorescence intensity was greatly reduced in *tubby* retinas at P20 and thereafter (Fig. 4).

3.3 UPR behavior during *tubby* retinal development

Given the results from western blot and immunocytochemistry assessment, we performed PCR array to assay the alterations of mRNA levels of the UPR components during retinal degeneration by selecting P7 and P28 retinas and using the "mouse unfolded protein response" array plates. A total of 89 genes were surveyed for the expression of ER stress-associated signaling. The genes to be tested on the array plates can be categorized as the following groups: 1) unfolded protein binding and/or protein folding (Canx, Cct4, Dnajb2, Dnajb9, Ero1l, Ero1lb, Hspa4l, Htra2, Scap, Sec63, Tcp1, Tor1a, Uggt1); 2) ER protein folding quality control (Edem1, Edem3, Ganab, Rpn1, Serp1, Uggt1); 3) protein disulfide isomerization (PDI) (Ddit3, Ero1l, Ero1lb, H47, Pdia3, Srebf1); 4) ER associated degradation (ERAD) (Amfr, Derl1, Derl2, Edem1, Fbxo6, H47, Herpud1, Mbtps1, Mbtps2, Nploc4, Nucb1, Os9, Sec62, Syvn1, Ubxn4); 5) heat shock proteins (Dnajb2, Dnajb9, Dnajc3, Hspa1l, Hsph1, Sec63); 6) ubiquitination (Amfr, Edem3, Herpud1, Rnf139, Rnf5, Sec62, Ube2g2, Ube2j2, Ubxn4, Ufd1l); 7) regulation of translation (Eif2α, Insig1, pp1r15b, Serp1); 8) transcription factors (Atf4, Atf6, Atxn3, Cebpb, Creb3, Creb3l3, Ddit3, Ern2, Mbtps1, Scap, Srebf1, Xbp1); and 9) apoptosis (Bax, Atxn3, Cebpb, Ddit3, Ern2, H47, Htra2, Manf, Mapk8, Mapk10, Pdia3, Ppp1r15b). In P7 *tubby* retinas, 44% of the surveyed genes (37 out of 84 genes) changed their expression (only those >1.5 fold are listed). Over 20% of them (Atf4, H47, Hspa1l, Mbtps1, Rnf5, Sec62, Sec63, Ube2j2) increased their expression by more than 2 fold compared to age-matched wt (Table 1). The remaining genes (28 of 37) had large changes (>1.5 but <2 fold) (Table 2). However, at P28, the majority of these UPR genes in *tubby* retinas were greatly down regulated compared to P7 *tubby* retinas. Among these genes, transcription factors and regulators of translation, such as Atf4, Atf6 and Eif2α, were up regulated at P7 in *tubby* retinas by 2.83, 1.88 and 1.32 fold compared to age-matched wt (tub/wt). However, they were down regulated in P28 *tubby* retinas by 2.54, 1.79 and 2.28 fold compared to P7 *tubby* retinas (tubP28/P7).

3.4 Expression of apoptotic regulators/effectors

It is well accepted that photoreceptor death results from the impairment of the balance between anti-apoptotic and pro-apoptotic effectors. To determine the mechanism(s) by which the photoreceptor death is regulated, the expression of apoptotic regulators/effectors,

such as B-cell lymphoma protein 2 (Bcl2), CHOP and nuclear factor kappa B (NF-κB), was examined by qRT-PCR or western blots using age-matched wt and *tubby* retinas at P4 - P28. As shown in Fig. 5, phosphorylated Bcl2 (Fig. 5A) in *tubby* retinas was significantly down regulated compared to the wt at P7 and remained there. Chop (Fig. 5C) expression in *tubby* retinas was greater than that of wt litter mates at P7 through P28. NF-κB is a key effector for promoting apoptosis (Khandelwal et al., 2011) and has been reported to be activated by ER stress and ROS (Kaneko et al., 2003; Pahl and Baeuerle, 1997). In the current study, our data show that NF-κB (Fig. 5B) expression is up regulated in *tubby* compared to age-matched wt at P4 and P7, but its level is similar to wt at P12 through P28.

4. Discussion

The mislocalization of the key phototransduction proteins (rhodopsin, M-opsin and arrestin) is a distinct characteristic of *tubby* phenotype (Cai et al., 2012; Kong et al., 2006; Sun et al., 2012). In this study, we showed that the mislocalization of rhodopsin and M-opsin in *tubby* retinas occurs as soon as they are synthesized at P4 and P12 respectively and the expression of the majority of the UPR components is increased at P7. This suggests that mutation of the *Tub* gene results in disturbance of the normal distribution of rod and cone opsins and that this event triggers the activation of the UPR signaling cascade. The expression of ER stress markers, IRE1, eIF2α and GRP78/BiP is elevated which demonstrated that ER stress is indeed involved in the process of *tubby* retinal development. Mislocalization or aggregation of photoreceptor-specific proteins caused by the mutations in ocular genes has been shown to be a very common phenomenon, for example, in $Rpe65^{-/-}$ (Rohrer et al., 2005), $Lrat^{-/-}$ (Zhang et al., 2011), $Cng3A^{-/-}$ and $Cng3B^{-/-}$ (Thapa et al., 2012), $Rp2^{null}$ (Li et al., 2013) and $Gnat2^{c.518A>G}$ (Jobling et al., 2013) mice. Increases in ER stress and activation of the UPR signaling were also reported in $Lart^{-/-}$ (Zhang et al., 2011) and $Cng3^{-/-}$ (Thapa et al., 2012) mice.

Three ER-resident transmembrane proteins (PERK, ATF6 and IRE1) are the sensors responding to ER stress. By activation (phosphorylation or cleavage), these sensor proteins initiate a self-defense UPR signaling network to regulate their downstream gene transcriptional and translational activities for maintaining the homeostasis and proper function of the ER (Doyle et al., 2011; Lai et al., 2007; Salminen et al., 2010). A recent publication reported that, by selective chemical-genetic activation of individual PERK, ATF6 or IRE1 pathways in HEK293 cell lines expressing Fv2E-PERK, TetON-ATF6f or IRE1[1642G], respectively, the aggregation of mutant rhodopsin was reduced (Chiang et al., 2012). Surprisingly, PERK also reduced the level of wt rhodopsin (Chiang et al., 2012). Many eye diseases including retinitis pigmentosa (Kunte et al., 2012; Shinde et al., 2012; Yang et al., 2007), age-related macular degeneration (AMD) (Salminen et al., 2010), glaucoma (Zode et al., 2014), oxygen-induced retinopathy (OIR) (Liu et al., 2013) and diabetic retinopathy (DR) (Yan et al., 2012), etc., have been shown to involve ER stress in their pathology. In the current study, the expression of ER stress markers and their dynamics during retinal development in *tubby* mice were assessed using wt as a reference. Our data demonstrated that IRE1, ATF6, eIF2α, XBP-1s and GRP78/BiP were up regulated in *tubby* retinas at early developmental stages (P4 through P12 or P20) compared to age-matched wt, and they were down regulated thereafter. Similar observations were also obtained from

transgenic P23H (at P6) (Lin et al., 2007), hT17M Rho (at P15) (Kunte et al., 2012) and rd1 mice (at P10) (Yang et al., 2007), and in the ER stress-associated indicator (ERAI)-transgenic mice expressing a variant of the gene for green fluorescent protein (F-XBP1DDBD-venus), in which fluorescent intensity increased after treatment with an ER stress inducer (Shimazawa et al., 2007). However, persistent ER stress caused decreased expression of these markers (Lin et al., 2007). The regulation of the UPR genes assayed with the "mouse unfolded protein response" PCR arrays (Table 1 and 2) was also in agreement with this result. In P7 *tubby* retinas (before the peak of photoreceptor death), a majority of the UPR genes, especially those having functions related to unfolding protein binding and protein folding, ER protein folding quality control, ER associated degradation (ERAD) and protein disulfide isomerization (PDI), showed increased expression levels. However, P28 retinas (an age after the peak of photoreceptor death) exhibited down regulation of these genes (Table 1 and 2). It should be noted that the heat shock protein family 70 (HSP70) is an important group of proteins for normal cellular activities. One group of the HSP70 family, CCT (Chaperonin containing Tcp1), was shown to be essential for elongation and extension of the rod outer segments, i.e. mice deficient in CCT were unable to build outer segments due to the impairment of ciliary trafficking (Posokhova et al., 2011; Sinha et al., 2014). It has been reported that *tubb*y mice exhibited blockage of transport of selective G protein-coupled receptors (GPCRs) to the neuronal primary cilia and aberrant rhodopsin trafficking, indicating that the Tubby protein is a required part of the transport machinery in retinal cells (Sun et al., 2012). These discoveries, in combination with our PCR array data, suggest that the primary cause of retinal degeneration in *tubby* retinas is the mislocalization of key phototransduction proteins which induces ER stress. The activated UPR signaling is an attempt to overcome the earlier ER stress condition in *tubby* retinas. However, the unmitigated prolonged ER stress triggers dysregulation of UPR genes, which in turn promotes the failure of trafficking and transportation of necessary components (structural proteins) for photoreceptor build up. Moreover, our data indicate that all three pathways of the UPR are elevated in the *tubby* retinas and ER stress involvement in *tubby* retinal degeneration is in a time window similar to that of apoptosis, in which TUNEL-positive apoptotic photoreceptor cells increased from P14 through P19 and decreased by P28 (Bode and Wolfrum, 2003), and also agrees with our report that significant loss of photoreceptors occurs in *tubby* at P14 through P34 (Kong et al., 2006).

The important role of the IRE1/XBP-1 pathway in mediating cell death and inflammatory signaling is well established (Tang et al., 2014). XBP-1 activation was reported to have a protective role in preventing injury-induced retinal ganglion cell death (Hu et al., 2012). Overexpression of spliced XBP-1 (XBP-1s) stimulated cell proliferation and inhibited ER stress-induced CHOP-involving apoptosis, whereas its knockdown reversed these effects (Guo et al., 2012). Overexpression of ATF6 up regulated IRE1 spliced XBP-1, which was negatively regulating apoptosis by down regulation of caspases, JNK and CHOP (Guo et al., 2014). In our current study, IRE1 expression was slightly higher in the *tubby* mutant retinas at P4 compared to wt; it continued to increase but was lower than age-matched wt at P12 and thereafter. ATF6 and XBP-1s are up regulated in comparison to age-matched wt at P4 through P20, although it tended to decrease with age and return to the wt level at P28. This suggests their adaptive protective role occurs at an early developmental age with the loss of

function at a later developmental stage following a prolonged ER stress condition in *tubby* retinas.

Although how ER stress induces apoptosis is not well defined, potential mechanisms involving multiple signaling pathways have been proposed and shown to involve the proteases (caspase 12), kinases (ASK1, JNK, and P38MAPK), transcription factors (CHOP) and Bcl2 family members (Lin et al., 2007; Nakanishi et al., 2013; Xu et al., 2005). It is commonly accepted that the communication between the ER and mitochondria for apoptosis is predominantly mediated by the Bcl2 family of proteins (Doyle et al., 2011). The anti-apoptotic Bcl2 protein protects against oxidative stress-induced damage and maintains intracellular Ca^{2+} homeostasis, which in turn controls the release of Ca^{2+} from the ER to the cytoplasm as a secondary messenger to prevent apoptosis (Rong and Distelhorst, 2008; Szegezdi et al., 2009). CHOP can be induced by all three UPR pathways. It can be regulated by ATF6 and XBP-1 through their binding to the promoter region of the Chop gene (Yoshida et al., 2001), and CHOP mediated cell death is directly linked with repressing the expression of Bcl2 protein (McCullough et al., 2001). Moreover, knockdown of Chop mRNA inhibits light-induced photoreceptor cell death (Nakanishi et al., 2013). NF-κB was activated by an ER overload (Pahl and Baeuerle, 1997) and it is the common downstream effector activated by both IRE1 and eIF2α (Hung et al., 2004; Jiang et al., 2003; Kaneko et al., 2003). In the current study, we showed that p-Bcl2 was down regulated and CHOP and NF-κB were up regulated in *tubby* retinas. These data suggest that retinal degeneration in *tubb*y mice results from the imbalance of the expression of multiple anti-apoptotic and pro-apoptotic regulator/effectors. In particular, persistent down regulation of Bcl2 protein and up regulation of CHOP and NF-κB expression contribute to the apoptotic death of photoreceptor cells, although transient increases in the expression of protective GRP78/BiP, ATF6 and XBP-1s occurred during earlier retinal degeneration.

It should be emphasized that GRP78/BiP expression was up regulated in *tubby* mice compared to age-matched wt at P4 until P12, and was down regulated thereafter. Accumulated data suggest that GRP78/BiP is a neuroprotector and survival factor which prevents neurodegeneration (Gorbatyuk et al., 2010; Gorbatyuk et al., 2012; Morris et al., 1997; Pfaffenbach and Lee, 2011). As a new strategy for a therapeutic reagent, GRP78/BiP has been shown to successfully rescue retinal structure and function in P23H rats (Gorbatyuk et al., 2010), and other forms of neurodegenerative diseases (Inokuchi et al., 2009). We think that supplementation of the GRP78/BiP protein before its expression is decreased, or a combination of increased GRP78/BiP protein with other antioxidants, will inhibit retinal degeneration in *tubby* mice.

5. Conclusion

Our data demonstrate that ER stress is involved in *tubby* retinal degeneration and the dynamics of ER stress markers match the time window of photoreceptor death in *tubby* mice. This finding supports potential therapeutic strategies for treatment of *tubby* retinal degeneration by up regulation or down regulation of specific ER stress markers before significant changes occur during *tubby* retinal development.

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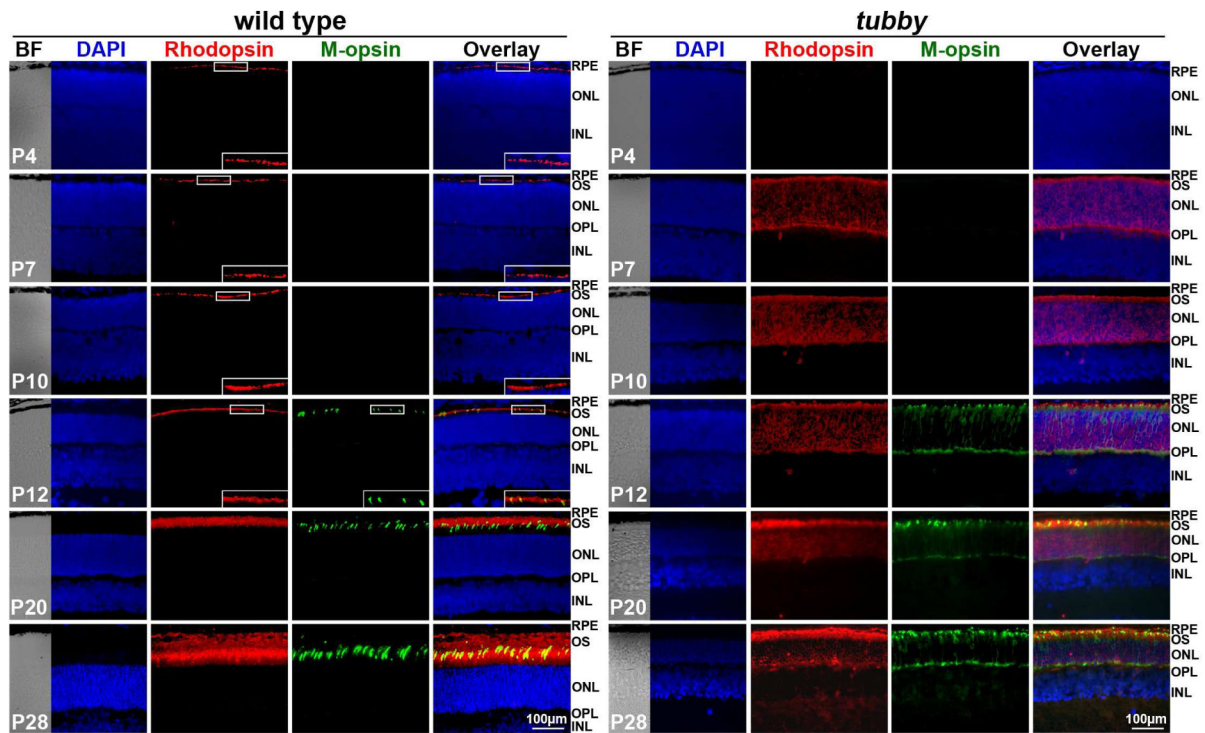
- Protein mislocalization in *tubby* retinal development correlates with ER stress

- Dynamics of ER stress parallel temporal degeneration of *tubby* retina

- Potential mechanisms for retinal degeneration are proposed

Figure 1.

Mislocalization of rhodopsin and M-opsin in *tubby* retinas. Immunocytochemistry of rhodopsin (red) and M-opsin (green) in wt (left) and *tubby* (right) retinas from P4 -P28 revealed that in wt retinas, rhodopsin is detectable at P4 and M-opsin at P12, whereas in *tubby* retinas, rhodopsin appears at P7 and M-opsin at P12 with mislocalization. Images shown are representative of 5–8 eyes per group. Nuclei were counterstained with DAPI. Inserts are the enlarged part of the boxed regions of the corresponding images. BF: bright field, RPE: retinal pigment epithelium, OS: outer segment, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer. N=5–8, Scale bar, 100 μm.
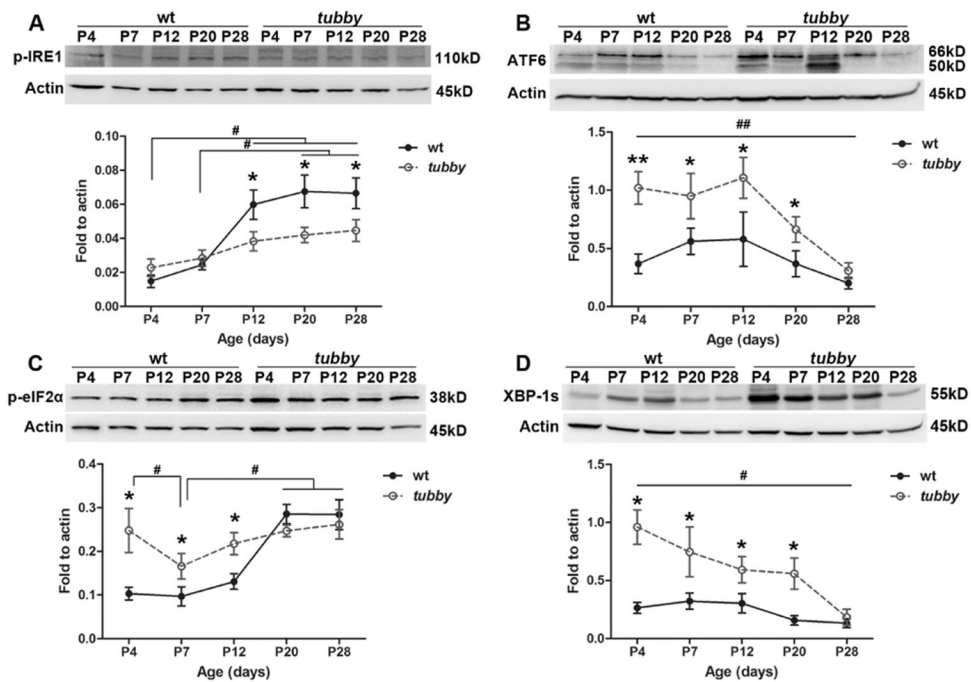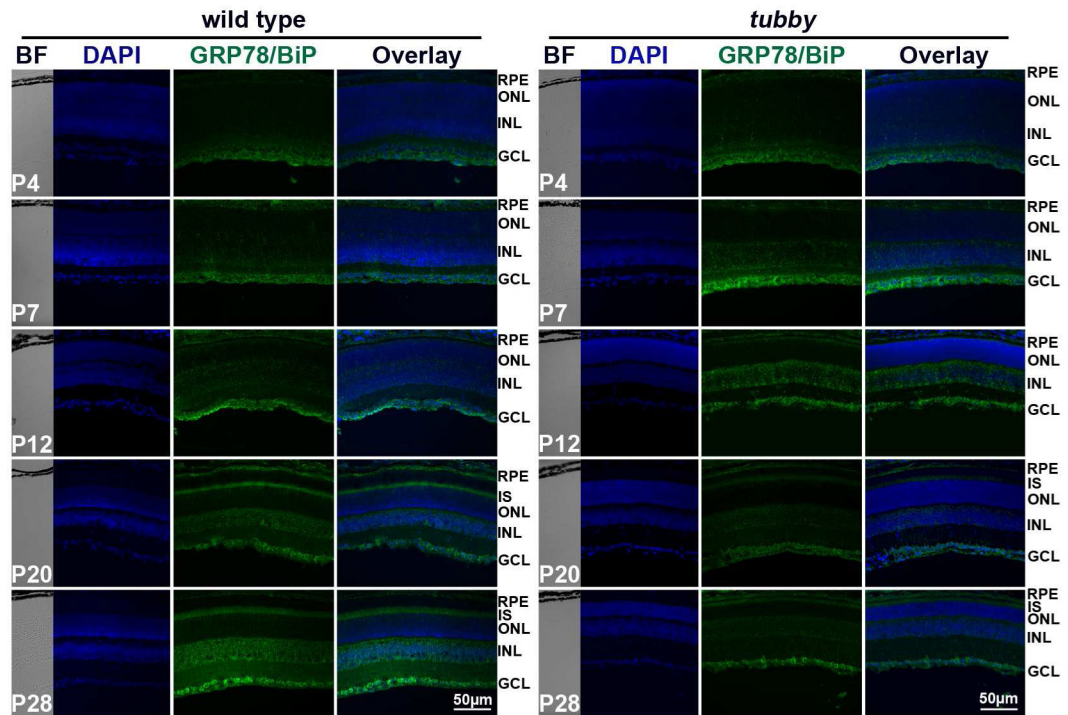
Figure 2.
Dynamic changes of ER stress markers during retinal development. Western blots show the expression of p-IRE1 (A), ATF6 (B), p-eIF2α (C), and XBP-1s (D) at selected developmental ages for wt and *tubby* retinas. Representative bands from each group are shown. Densitometric analysis of the bands is standardized to actin and shown as mean ± SEM beneath each western blot figure. N=8–10, *P<0.05, **P<0.005 (compared between *tubby* and age-matched wt using the unpaired student *t*-test), #P<0.05, ##P<0.005 (compared among *tubby* at different ages using one way ANOVA for B and D, or compared between each two groups using the unpaired student *t*-test for A and C).
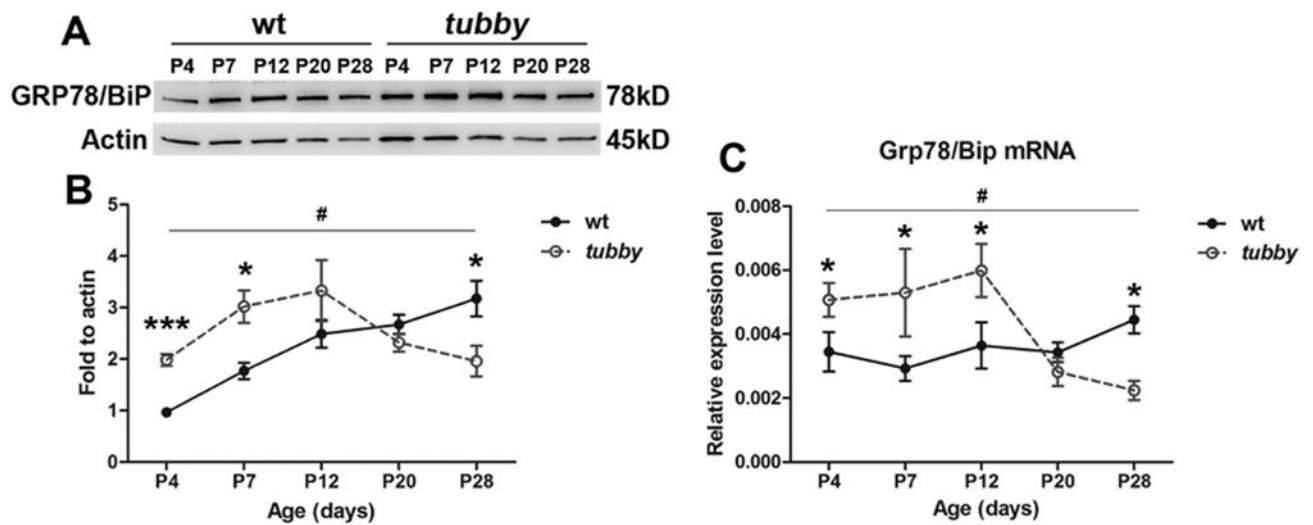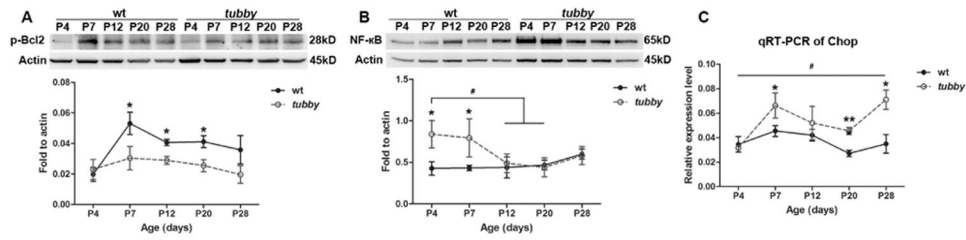
Figure 3.
Dynamics of GRP78/BiP expression during retinal development. GRP78/BiP protein is increased at P4 through P12 in *tubby* retinas compared to age-matched wt, but has significantly decreased expression at P20 through P28. (A) Data shown are representative of 8–10 western blots and (B) the average (mean ± SEM) of band intensity from 8–10 retinas. (C) qRT-PCR analysis demonstrated a similar alteration of Grp78/Bip mRNA level (mean ± SEM). *P<0.05, ***P<0.0001 (compared between *tubby* and age-matched wt using the unpaired student *t*-test), #P<0.05 (compared among *tubby* at different ages using one way ANOVA).

Figure 4.
Distribution and alteration of GRP78/BiP protein during retinal development.
Immunocytochemistry showed that in wt and *tubby* retinas, GRP78/BiP is mainly expressed
in the RPE, INL and GCL at P4 through P12 with expression being stronger in *tubby* retinas
than the wt, but at P20 through P28, it was also expressed in the IS. However, *tubby* retinas
have much less GRP78/BiP fluorescence compared to wt and to younger *tubby* retinas at
P20 and P28. Representative images from each group are shown. BF: bright field, RPE:
retinal pigment epithelium, IS: inner segment, ONL: outer nuclear layer, INL: inner nuclear
layer, GCL: ganglion cell layer. N=8–10, Scale bar, 50 μm.

Figure 5.
Expression of anti-apoptotic and pro-apoptotic effectors/regulators during retinal development. Western blot assay demonstrated that the activation of p-Bcl2 (A) decreases with age. The expression of NF-κB (B) is increased in the early developmental stages in *tubby* retinas (P4 and P7), but decreases thereafter. Densitometric analysis of the bands is shown beneath the corresponding western figure. (C) qRT-PCR analysis showed that the level of Chop mRNA in *tubby* is higher than the wt from P4 through P28. Data shown are mean ± SEM, N=8–10. *P<0.05, **P<0.005 (compared between *tubby* and age-matched wt using the unpaired student *t*-test), #P<0.05 (compared among *tubby* at different ages using one way ANOVA for A and C, or compared between each two groups using the unpaired student *t*-test for B).

Table 1

Mouse unfolded protein response genes with changes in expression in *tubby* mice (≥ 2.0 fold).

Symbol	Gene name	Function	tub/wt(P7)		tub(P28/P7)	
			fold	P value	fold	P value
Atf4	Activating transcription factor 4	*g*	2.83	0.1301	−2.54	0.0140*
Bax	Bcl2-associated X protein	*k*	1.15	0.4472	−2.95	0.0012**
Canx	Calnexin	*a,b*	1.63	0.3210	−2.06	0.2201
Cct4	Chaperonin containing Tcp1, subunit 4 (delta)	*a,b*	1.90	0.1393	−2.49	0.0139*
Derl1	Der1-like domain family, member 1	*e*	1.49	0.3704	−2.01	0.0740
Eif2a	Eukaryotic translation initiation factor 2a	*f*	1.32	0.3732	−2.28	0.0179*
Ern2	Endoplasmic reticulum (ER) to nucleus signalling 2	*g,k*	1.60	0.1774	−3.24	0.0479*
Ganab	Alpha glucosidase 2 alpha neutral subunit	*c*	1.34	0.0180*	−2.13	0.0253*
H47	Histocompatibility 47	*e, h, k*	2.30	0.0671	−1.33	0.1094
Hspa1l	Heat shock protein 1-like	*j*	1.96	0.1665	−2.12	0.2263
Mbtps1	Membrane-bound transcription factor peptidase, site 1	*e, g, i*	2.47	0.0019**	−2.19	0.0002**
Rnf5	Ring finger protein 5	*d*	2.12	0.1821	−1.55	0.1063
Sec62	SEC62 homolog (S. cerevisiae)	*e, d*	2.62	0.0734	−1.73	0.0399*
Sec63	SEC63-like (S. cerevisiae)	*a, b, j*	3.06	0.0203*	−1.65	0.0328*
Serp1	Stress-associated endoplasmic reticulum protein 1	*c, f*	1.43	0.0530	−2.27	0.0016**
Ube2j2	Ubiquitin-conjugating enzyme E2, J2 homolog (yeast)	*d*	2.90	0.0085**	−2.19	0.0290*
Creb3l3	CAMP responsive element binding protein 3-like 3	*g*	1.76	0.2692	1.97	0.0335*
Dnajb2	DnaJ (Hsp40) homolog, subfamily B, member 2	*a, b, j*	1.36	0.4107	2.37	0.0008**
Insig1	Insulin induced gene 1	*f*	1.65	0.3395	3.92	0.0125*
Nploc4	Nuclear protein localization 4 homolog (S. cerevisiae)	*e*	1.37	0.3617	1.95	0.0864
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	*g,k*	−1.13	0.5732	2.69	0.0381*
Dnajb9	DnaJ (Hsp40) homolog, subfamily B, member 9	*a, b, j*	−1.70	0.0978	2.52	0.0108*

Symbol	Gene name	Function	tub/wt(P7)		tub(P28/P7)	
			fold	P value	fold	P value
Edem3	ER degradation enhancer, mannosidase alpha-like 3	*c, d*	−2.14	0.2194	2.77	0.0211[*]
Syvn1	Synovial apoptosis inhibitor 1, synoviolin	*e*	−1.21	0.3540	2.01	0.0440[*]

N=3–4,

[*] P<0.05,

[**] P<0.01

[a] Unfolding protein binding;

[b] Protein folding;

[c] ER protein folding quality control;

[d] Ubiquitination;

[e] ER associated degradation (ERAD);

[f] Regulation of translation;

[g] Transcription factor;

[h] Protein disulfide isomerization (PDI);

[i] Regulation of cholesterol metabolism;

[j] Heat shock protein;

[k] Apoptosis

Table 2

Mouse unfolded protein response genes with changes in expression in *tubby* mice (1.5 fold< 2.0 fold).

Symbol	Gene name	Function	tub/wt(P7) fold	P value	tub(P28/P7) fold	P value
Amfr	Autocrine motility factor receptor	e,d	1.64	0.0888	-1.87	0.0708
Manf	Mesencephalic astrocyte-derived neurotrophic factor	k	-1.31	0.4786	-1.81	0.2844
Atf6	Activating transcription factor 6	g	1.88	0.2989	-1.79	0.3563
Atxn3	Ataxin 3	g,k	1.59	0.2715	-1.35	0.3849
Creb3	CAMP responsive element binding protein 3	g	1.85	0.1243	-1.83	0.1152
Derl2	Der1-like domain family, member 2	e	1.51	0.0379*	-1.49	0.0171*
Dnajc3	DnaJ (Hsp40) homolog, subfamily C, member 3	j	1.84	0.0401*	-1.52	0.0471*
Edem1	ER degradation enhancer, mannosidase alpha-like 1	c,e	1.64	0.0360*	-1.34	0.0249*
Fbxo6	F-box protein 6	e	1.75	0.0040**	1.15	0.5788
Htra2	HtrA serine peptidase 2	a,e,k	1.40	0.3803	-1.41	0.0012*
Mapk8	Mitogen-activated protein kinase 8	k	1.21	0.6105	-1.70	0.0423*
Mbtps2	Membrane-bound transcription factor peptidase, site 2	i,e	1.64	0.1199	-1.65	0.1137
Nucb1	Nucleobindin 1	e	1.70	0.3499	-1.80	0.3027
Rnf139	Ring finger protein 139	d	1.05	0.9995	-1.67	0.0090**
Rpn1	Ribophorin I	c	1.50	0.1036	-1.27	0.0540*
Scap	SREBF chaperone	a,g	1.86	0.1465	-1.42	0.0108*
Srebf1	Sterol regulatory element binding transcription factor 1	g,h,i	1.91	0.0569	-1.72	0.0876
Tcp1	T-complex protein 1	a,b	1.14	0.1114	-1.67	0.0039*
Tor1a	Torsin family 1, member A (torsin A)	a,b	1.21	0.4038	-1.57	0.0316*
Ube2g2	Ubiquitin-conjugating enzyme E2G 2	d	-1.82	0.1978	-1.18	0.1275
Ubxn4	UBX domain protein 4	e,d	1.83	0.0915	-1.34	0.1228
Ufd1l	Ubiquitin fusion degradation 1 like	d	1.28	0.3043	-1.93	0.0278*
Uggt1	UDP-glucose glycoprotein glucosyltransferase 1	a,b,c	1.57	0.0876	-1.54	0.0419*

Symbol	Gene name	Function	tub/wt(P7)		tub(P28/P7)	
			fold	P value	fold	P value
Ddit3	DNA-damage inducible transcript 3	g, h, k	1.48	0.0528	1.61	0.0492*
Herpud1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	d, e	1.91	0.0893	1.74	0.0066**
Hspa4l	Heat shock protein 4 like	b	1.35	0.1475	1.80	0.0234*
Hsph1	Heat shock 105kDa/110kDa protein 1	j	1.71	0.3653	1.39	0.3202
Mapk10	Mitogen-activated protein kinase 10	k	1.43	0.1494	1.81	0.0153*
Os9	Amplified in osteosarcoma	e	1.74	0.2344	1.83	0.0622
Pdia3	Protein disulfide isomerase associated 3	h, k	1.71	0.2060	1.28	0.3002
Ero1l	ERO1-like (S. cerevisiae)	b, h	−1.42	0.2597	1.57	0.0278*
Ero1lb	ERO1-like beta (S. cerevisiae)	a, h	−1.17	0.5778	1.66	0.0340*
Ppp1r15b	Protein phosphatase 1, regulatory (inhibitor) subunit 15b	f, k	−1.30	0.4214	1.51	0.0582
Xbp1	X-box binding protein 1	g	−1.46	0.5196	1.47	0.0983

N=3-4,

*
P<0.05,

**
P<0.01

[a] Unfolding protein binding;

[b] Protein folding;

[c] ER protein folding quality control;

[d] Ubiquitination;

[e] ER associated degradation (ERAD);

[f] Regulation of translation;

[g] Transcription factor;

[h] Protein disulfide isomerization (PDI);

[i] Regulation of cholesterol metabolism;

[j] Heat shock protein;

$k_{Apoptosis}$