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DDIT3 (CHOP) contributes to retinal ganglion cell somal loss but not axonal degeneration in DBA/2J mice

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Abstract

Glaucoma is an age-related neurodegenerative disease characterized by the progressive loss of retinal ganglion cells (RGCs). Chronic ocular hypertension, an important risk factor for glaucoma, leads to RGC axonal injury at the optic nerve head. This insult triggers molecularly distinct cascades governing RGC somal apoptosis and axonal degeneration. The molecular mechanisms activated by ocular hypertensive insult that drive both RGC somal apoptosis and axonal degeneration are incompletely understood. The cellular response to endoplasmic reticulum stress and induction of pro-apoptotic DNA damage inducible transcript 3 (DDIT3, also known as CHOP) have been implicated as drivers of neurodegeneration in many disease models, including glaucoma. RGCs express DDIT3 after glaucoma-relevant insults, and importantly, DDIT3 has been shown to contribute to both RGC somal apoptosis and axonal degeneration after acute induction of ocular hypertension. However, the role of DDIT3 in RGC somal and axonal degeneration has not been critically tested in a model of age-related chronic ocular hypertension. Here, we investigated the role of DDIT3 in glaucomatous RGC death using an age-related, naturally occurring ocular hypertensive mouse model of glaucoma, DBA/2J mice (D2). To accomplish this, a null allele of *Ddit3* was backcrossed onto the D2 background. Homozygous *Ddit3* deletion did not alter gross retinal or optic nerve head morphology, nor did it change the ocular hypertensive profile of D2 mice. In D2 mice, *Ddit3* deletion conferred mild protection to RGC somas, but did not significantly prevent RGC axonal degeneration. Together, these data suggest that DDIT3 plays a minor role in perpetuating RGC somal apoptosis caused by chronic ocular hypertension-induced axonal injury, but does not significantly contribute to distal axonal degeneration.

Introduction

Glaucoma is an age-related neurodegenerative disease characterized by the death of retinal ganglion cells (RGCs), the output neurons of the retina. An important risk factor for glaucomatous RGC death is elevated intraocular pressure (IOP), which leads to RGC axonal injury at the lamina cribrosa[1–5] (termed the glial lamina in mice[5]). This insult is thought to trigger molecular signaling within RGCs that regulates somal degeneration proximal to the site of injury and axonal degeneration distal to the site of injury[6–10]. Identifying the molecular signaling pathways that lead from ocular hypertensive injury to RGC death is critical for understanding the pathobiology of glaucoma. To date, a mechanism important in both proximal and distal RGC degeneration has not been identified. The pro-apoptotic molecule BAX was shown to be required for RGC somal death but not axonal degeneration after chronic ocular hypertension and acute optic nerve injury[6,11,12]. Thus, the mechanism triggered by ocular hypertension that regulates glaucomatous neurodegeneration must ultimately converge upon BAX induction.

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The adaptive response to endoplasmic reticulum (ER) stress (known as the unfolded protein response or the integrated stress response) has been implicated as a driver of neuronal death in many neurodegenerative diseases, including glaucoma[13–17]. After prolonged and unresolved ER stress, the unfolded protein response has been shown to promote apoptosis via induction of DNA damage inducible transcript 3 (DDIT3, also known as CHOP). DDIT3 has been shown to act as a pro-apoptotic transcription factor; DDIT3 promoted transcription of pro-apoptotic *Bbc3*[18,19], *Bim*[18,19], *Gadd34*[20], *Dr5*[19,21], and *Ero1α*[22] genes and inhibited transcription of the pro-survival gene *Bcl2*[19,20,23,24]. DDIT3 was also shown to be important for the translocation of activated BAX from the cytosol to the mitochondria[25,26]; allowing the intrinsic apoptotic cascade to ensue. Therefore, as a pro-apoptotic transcription factor upstream of BAX, DDIT3 may be an important regulator of RGC death after glaucomatous insult.

DDIT3 has been shown to regulate RGC death in glaucoma and various other neurodegenerative diseases[27,28]. DDIT3 was expressed by RGCs after glaucoma-relevant insults, including optic nerve crush[13–15,29] and the microbead model of acute ocular hypertension[13,14]. In addition, *Ddit3* was upregulated in both the retinas and optic nerve heads (ONHs) of mice with chronic ocular hypertension prior to the onset of glaucomatous neurodegeneration[30–32]. *Ddit3* deficiency or silencing was protective to RGC somas after mechanical axonal injury (optic nerve crush)[14,17,33] and the microbead model of acutely induced ocular hypertension[14,33]. Interestingly, despite not appearing to have a major role in RGC axonal degeneration after optic nerve crush[17], DDIT3 deficiency lessened axonal degeneration in an acute ocular hypertension model[33]. This protection, though minor, appeared roughly equal to the level of somal protection, suggesting that in some cells, *Ddit3* deficiency completely protected the RGC after an ocular hypertensive injury[33].

DDIT3 appears to be an important mediator of RGC viability after glaucoma-relevant injuries. However, the role of DDIT3 in glaucomatous neurodegeneration has not been tested in a model of stochastic, age-related ocular hypertension. Here, we critically tested the role of DDIT3 in RGC axonal degeneration and somal loss in an inherited, age-related mouse model of chronic ocular hypertension. We found DDIT3 played a minor role in RGC somal death but not axonal degeneration in the DBA/2J (D2) mouse model of chronic, age-related ocular hypertension[3,5,34–36].

Materials and methods

Mice

DBA/2J (D2) mice and mice with a null allele of *Ddit3*[37] (B6.129S(Cg)-*Ddit3*$^{tm2.1Dron}$/J) were obtained from the Jackson Laboratory (Stock numbers 000671 and 005530, respectively). The *Ddit3* null allele was backcrossed to the D2 background 10 times (>99% D2). After this backcross was completed, the D2.*Ddit3* colony was maintained by D2.*Ddit3*$^{+/-}$ × D2.*Ddit3*$^{+/-}$ intercrossing. D2.*Ddit3*$^{+/+}$ environment-matched littermates were used as genetic controls for D2.*Ddit3*$^{-/-}$ mice, and each genotype group included roughly equal numbers of females and males (D2.*Ddit3*$^{+/+}$: 30 female, 34 male; D2.*Ddit3*$^{-/-}$: 29 female, 31 male). Mice were fed chow and water *ad libitum* and were housed on a 12-h light-to-dark cycle. All experiments were conducted in adherence with the Association for Research in Vision and Ophthalmology's statement on the use of animals in ophthalmic and vision research and were approved by the University of Rochester's University Committee on Animal Resources.

Retina processing for plastic sectioning

As previously described[9,17,38,39], eyes were enucleated and fixed for 24 h in a solution of 2.5% glutaraldehyde, 2% paraformaldehyde (PFA) in 1× phosphate buffered saline (PBS; BioRad, 161-0780) at 4 °C. Eyes were washed in 0.1 M PO_4, dehydrated in 50% ethanol for 1 h, and placed in 70% ethanol overnight at 4 °C. Eyes were incrementally dehydrated in 80, 95, and 100% ethanol for one hour each at room temperature. Eyes were placed in acetone for 1 h, washed with 100% ethanol for 1 h, and placed in 1:1 100% ethanol: Hardener 1 Technovit 7100 (Electron Microscopy Sciences 14653) overnight at 4 °C. Eyes were then placed in Hadner I Technovit 7100 for 24 h at 4 °C. Eyes were then incubated in 15:1 Hardener 1 Technovit 7100: Hardener 2 Technovit 7100 for 10 min on ice. Eyes were submerged in 15:1 Hardener 1 Technovit 7100: Hardener 2 Technovit 7100 and were allowed to harden in a plastic mold at room temperature. 2.5 µm coronal cross sections were cut and collected on microscope slides. Sections that included the ONH were stained with Multiple Stain Solution (Polysciences, Inc, 08824) for 1–2 min, washed with 100% ethanol, and cover-slipped with Permount (Fisher Scientific, SP15-500).

Optic nerve processing for plastic sectioning and grading

Optic nerves were harvested and processed as previously described[6,9,38]. In brief, optic nerves were fixed in situ in 2.5% glutaraldehyde, 10% formalin in 1× PBS for 24 h at 4 °C. Nerves were dissected from the brain and were incubated in 1% osmium for 2 h at room temperature. Otherwise, nerves were processed identically to eyes as described above. 1.5 µm cross sections were cut and collected on microscope slides. Nerve sections were stained with 1% paraphenylenediamine (PPD) in absolute methanol for 15 min, and washed with 100% ethanol for 10 min. PPD stains the myelin sheath of all axons but differentially darkly stains the axoplasm of dying axons. A

Table 1 Summary of antibodies

	Concentration	Company
Primary antibody (catalog #)		
Rabbit anti-cCASP3 (AF835)	1:1000	R&D Systems
Rabbit anti-RBPMS (GTX118619)	1:250	GeneTex
Guinea pig anti-RBPMS (1832-RBPMS)	1:250	PhosphoSolutions
Secondary antibody (catalog #)		
Donkey anti-rabbit 555 (A31572)	1:1000	ThermoFisher
Donkey anti-guinea pig 647 (706-605-148)	1:1000	Jackson ImmunoResearch

cCASP3 cleaved caspase 3, RBPMS RNA binding protein, mRNA processing factor

masked observer used a validated grading scale to assess the level of glaucomatous damage of each optic nerve. As previously described[5,6,9,40], nerves with <5% axons damaged or lost (consistent with axonal loss associated with normal aging) were judged to have no/early damage, nerves judged to have moderate damage had 5–50% axonal damage or loss (averaging ~30% loss) often with localized areas of gliosis, and nerves with >50% axonal damage or loss, often with large areas of glial scaring, were judged to have severe damage. A masked observer selected optic nerves with the most axonal damage (judged to have <5% axonal survival) for assessment of RGC somal survival.

Controlled optic nerve crush

Controlled optic nerve crush (CONC) was performed as previously described[6,38,39]. Briefly, mice were anesthetized with intraperitoneal 100 mg/kg ketamine and 10 mg/kg xylazine. Analgesic 2 mg/kg meloxicam was administered subcutaneously prior to surgery. The optic nerve was exposed and crushed immediately behind the eye with self-closing forceps for 5 s. Sham surgery was performed on the contralateral eye, where the optic nerve was exposed but not crushed. Antibiotic ointment was applied to the eyes following the procedure. Eyes were harvested 5 and 14 days post-CONC.

IOP measurement

As previously described[9,35,38], IOPs were measured by a masked observer using Tonolab (Colonial Medical Supply, Franconia, NH, USA) according to manufacturer's instructions 3–5 min after intraperitoneal administration of anesthetic 100 mg/kg ketamine and 10 mg/kg xylazine.

Immunofluorescence

As previously described[38,39], eyes were harvested and fixed in 4% PFA in 1× PBS for 90 min. Retinas were dissected free from the optic cup and blocked in 10% horse serum, 0.4% Triton™ X-100 (Fisher scientific, 9002-93-1) in 1× PBS overnight at 4 °C. Retinas were then incubated at 4 °C for 3 days in primary antibodies (Table 1) diluted in 10% horse serum, 0.4% Triton™ X-100 in 1× PBS. Retinas were then washed and incubated for 24 h at 4 °C in secondary antibodies (Table 1) diluted in 1× PBS. Retinas were washed and mounted on microscope slides ganglion cell layer-up in Flourogel in TRIS buffer (Electron Microscopy Sciences, 17985-11).

Cell quantification

As previously described[38,39], cCASP3+ RBPMS+ cells were quantified using eight 20x fields per retina, and RBPMS+ cell counts were assessed using eight 40x fields per retina. Images were taken approximately 220 µm from the peripheral edge of the retina and were equally spaced from each other. The manual cell counter plug-in in ImageJ was utilized for cell quantification. Retinal imaging and cell quantifications were performed by a masked observer. Cell quantifications were normalized to the total area measured and reported as cells/mm^2.

Statistical analysis

Data were analyzed using GraphPad Prism8 software. Comparisons between two groups (cCASP3+ RBPMS+ cells/mm^2 after CONC between genotypes, Fig. 1a and %RGC survival in retinas with severe optic nerves between genotypes, Fig. 4) were analyzed using an unpaired two-tailed student's *t* test. Comparisons across more than two groups (RGCs/mm^2 14 days after sham and CONC procedures between genotypes, Fig. 1b) or two groups across multiple timepoints (IOP measurements at multiple timepoints between genotypes, Fig. 2b) were analyzed using a two-way ANOVA followed by a Sidak post hoc test. For these statistical tests, multiplicity adjusted *P* values are reported. The comparison of the percent of optic nerves at each grade between genotypes (Fig. 3b) was analyzed using a Chi-square test. *P* values of <0.05 were considered statistically significant. Throughout the manuscript, results are reported as mean ± standard error of the mean (SEM).

Results

The D2 background did not alter protection conferred by *Ddit3* deletion after axonal injury

Ddit3 deficiency on the C57BL/6J background provided protection to some RGC somas after CONC[14,17,33]. However, genetic background may affect RGC death after axonal insult[41,42]. To determine if *Ddit3* deficiency lessened RGC death after axonal injury on the DBA/2J (D2) genetic background, CONC was performed on young (1.5–2.5 months of age, M) D2 mice. At this age, wildtype D2 mice do not yet have elevated IOP or any morphological glaucomatous damage[35]. D2.*Ddit3*$^{-/-}$ and D2.*Ddit3*$^{+/+}$ retinas were harvested 5 and 14 days after CONC. Consistent with CONC-induced RGC death in C57BL/6J mice, D2.*Ddit3*$^{-/-}$ retinas had 28.1% fewer cleaved caspase 3+ (cCASP3; cleavage of CASP3 is a

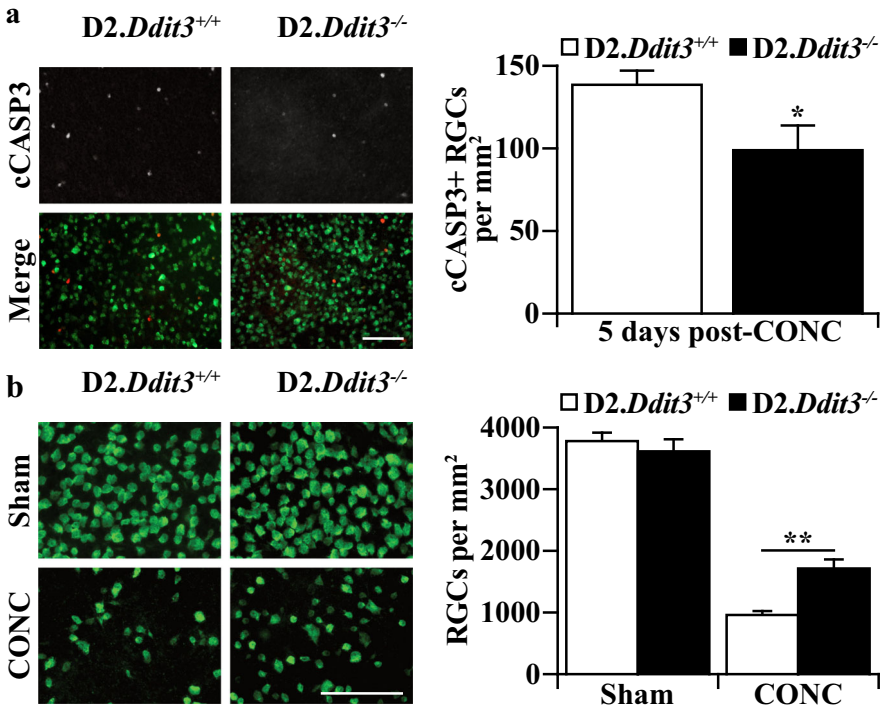

Fig. 1 The D2 background did not alter protection conferred by *Ddit3* deletion after axonal injury. a Retinal flat mounts 5 days post-CONC stained for the RGC marker RBPMS (green) and cleaved caspase 3 (cCASP3, red). There were significantly fewer cCASP3 + RGCs (cCASP3+ RBPMS+ cells) in D2.*Ddit3*$^{-/-}$ retinas compared with D2.*Ddit3*$^{+/+}$ retinas (cCASP3+ RBPMS+ cells/mm^2 ± SEM: D2.*Ddit3*$^{+/+}$, 139.7 ± 7.5, D2.*Ddit3*$^{-/-}$, 100.2 ± 13.8; $n = 6$ per genotype, $P = 0.031$, two-tailed t test). **b** Retinal flat mounts 14 days post-CONC stained for RBPMS. D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ retinas had similar RGC densities 14 days post-sham surgery (RBPMS+ cells/mm^2 ± SEM: D2.*Ddit3*$^{+/+}$, 3811.4 ± 109.5, D2.*Ddit3*$^{-/-}$, 3647.0 ± 166.2; $n =$ 6 per genotype; $P = 0.910$, two-way ANOVA, Sidak post hoc). Both genotypes had a significant reduction of RGCs 14 days post-CONC ($P < 0.001$ for both comparisons, $n = 6$ per condition per genotype, two-way ANOVA, Sidak post hoc), however, D2.*Ddit3*$^{-/-}$ retinas had significantly more surviving RGC compared to D2.*Ddit3*$^{+/+}$ controls (RBPMS+ cells/mm^2 ± SEM: D2.*Ddit3*$^{+/+}$, 991.0 ± 33.9, D2.*Ddit3*$^{-/-}$, 1745.8 ± 116.5; $n = 6$ per condition per genotype; $P = 0.001$, two-way ANOVA, Sidak post hoc). Error bars, SEM. Scale bars, 100 µm

critical step of apoptosis) RGCs compared to D2.*Ddit3*$^{+/+}$ retinas 5 days post-CONC (Fig. 1a). D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ retinas had similar RGC densities 14 days post-sham surgery (Fig. 1b) as judged by a specific marker for RGCs (RNA binding protein, mRNA processing factor; RBPMS)[43,44]. Retinas of both genotypes had significant RGC loss 14 days post-CONC as compared to sham, however, D2.*Ddit3*$^{-/-}$ retinas had 29.6% increased RGC survival compared to D2.*Ddit3*$^{+/+}$ controls (Fig. 1b). Thus, on the D2 genetic background, *Ddit3* deficiency provided similar protection to RGCs as previous reports using the C57BL/6J genetic background after axonal injury[14,17,33].

Ddit3 deletion did not alter D2-associated endophenotypes

The ONH is an important site in the pathobiology of glaucoma. In ocular hypertensive DBA/2J mice, the ONH is likely the site of an early critical axonal injury[4,5]. To ensure *Ddit3* deletion did not cause any developmental ONH or retinal abnormalities in D2 mice, D2.*Ddit3*$^{-/-}$ and D2.*Ddit3*$^{+/+}$ ONH and retinal morphologies were assessed at 1.5–3 M. *Ddit3* deletion caused no gross morphological ONH or retinal abnormalities in D2 mice as judged by semi-thin sections (Fig. 2a).

ER stress has been implicated in regulating IOP elevation for some genetic causes of glaucoma[45,46]. Since RGC degeneration in D2 mice depends on age-related IOP elevation[36,47–51], it was important to determine if *Ddit3* deficiency altered IOP elevation in D2 mice. IOP was assessed at 5, 7.5, 9, 10.5, and 12 M (Fig. 2b). As a population, IOP was not elevated at 5 and 7.5 M. Both genotypes had significant IOP elevations at 9, 10.5, and 12 M compared to baseline IOPs taken at 5 M. D2.*Ddit3*$^{-/-}$ mice had similar IOPs to D2.*Ddit3*$^{+/+}$ mice at each timepoint measured, thus, *Ddit3* deletion did not alter the stereotypic IOP profile of D2 mice.

Ddit3 deletion did not significantly prevent RGC axonal degeneration in a model of chronic ocular hypertension

DDIT3 has been implicated in driving both RGC somal and axonal degeneration after acute axonal injury[52] and

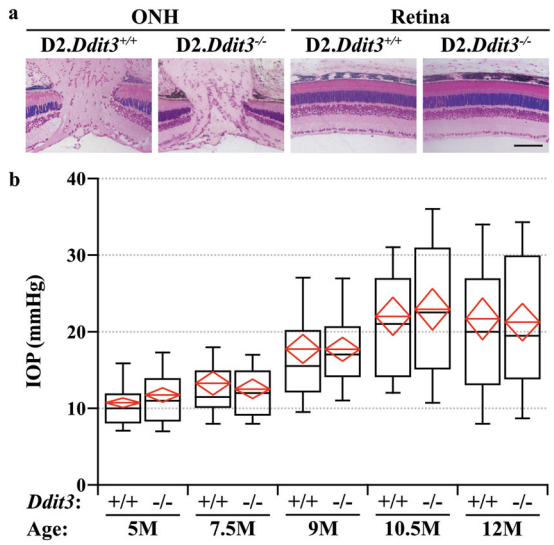

Fig. 2 *Ddit3* deletion did not alter D2-associated endophenotypes. a Semi-thin plastic sections of D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ ONHs and retinas. *Ddit3* deficiency did not cause gross morphological abnormalities of the ONH (left) or retina (right) in D2 mice ($n = 6$ per genotype). **b** D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ IOPs taken at 5 M ($n \geq 76$), 7.5 M ($n \geq 72$), 9 M ($n \geq 60$), 10.5 M ($n \geq 56$), and 12 M ($n \geq 66$). There was no difference in IOP between D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ eyes at any time point ($P > 0.05$ for all comparisons, two-way ANOVA, Sidak *post hoc*). Note, for both genotypes, 9 M, 10.5 M, and 12 M IOPs were significantly elevated compared to the 5 M timepoint ($P < 0.001$ for all comparisons, two-way ANOVA, Sidak post hoc). The black line represents the median, and the upper and lower bounds represent the 90th and 10th percentiles, respectively. The red line represents the mean, and the top and bottom points of the red diamond represent the 95% confidence interval. Scale bar, 100 µm

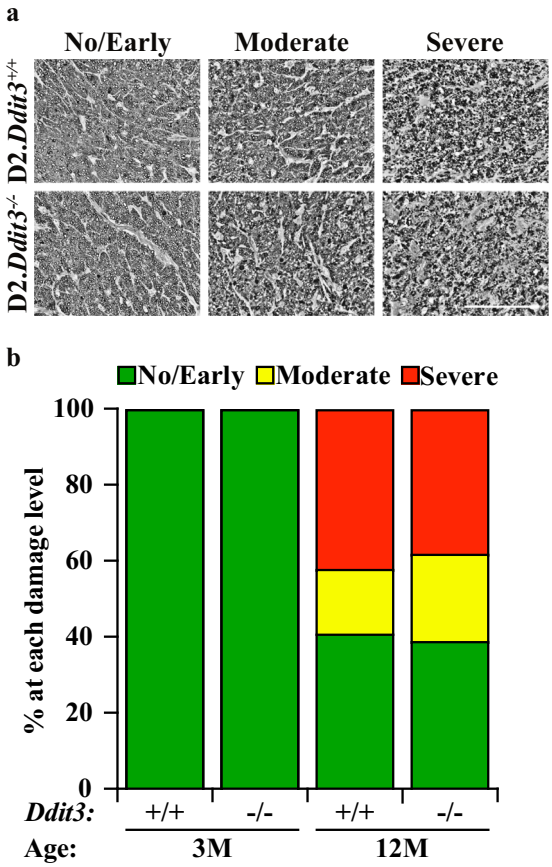

Fig. 3 *Ddit3* deletion did not prevent RGC axonal degeneration in a model of chronic ocular hypertension. a Representative optic nerve cross sections stained with PPD from 12 M D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ mice judged to have no/early (left), moderate (middle), and severe (right) axonal damage. **b** Percentages of D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ optic nerves judged to have no/early, moderate, and severe axonal damage at 3 M and 12 M. D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ optic nerves had no moderate or severe damage at 3 M ($n = 10$ per genotype). D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ optic nerves had similar levels of axonal damage at 12 M (D2.*Ddit3*$^{+/+}$, D2.*Ddit3*$^{-/-}$: no/early, 41%, 40%; moderate, 17%, 23%; severe, 42%, 37%; $n \geq 60$ per genotype, $P = 0.474$, Chi-square test). Scale bar, 100 µm

acute ocular hypertension[33]. To determine whether DDIT3 regulated glaucomatous neurodegeneration in a chronic model of age-related ocular hypertension, D2.*Ddit3*$^{-/-}$ and D2.*Ddit3*$^{+/+}$ littermate control optic nerves were assessed for glaucomatous damage at 12 M. At this time point, a significant proportion of D2 optic nerves had severe levels of axonal degeneration[9,35,38]. Optic nerves from young (1.5–3 M) D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ mice were also assessed to ensure no premature glaucomatous damage or axonal phenotype occurred in D2.*Ddit3*$^{-/-}$ mice. Optic nerve damage was graded as "no/early", "moderate", or "severe" using a validated grading scale[5,6,9,40] (Fig. 3a, see "Materials and methods" for grading details). Neither D2.*Ddit3*$^{+/+}$ nor D2.*Ddit3*$^{-/-}$ optic nerves exhibited any signs of axonal degeneration at 1.5–3 M (Fig. 3b). At 12 M, D2.*Ddit3*$^{-/-}$ mice had similar levels of optic nerve damage compared to D2.*Ddit3*$^{+/+}$ controls (Fig. 3b). Therefore, *Ddit3* deletion did not provide protection to RGC axons in D2 mice, suggesting that DDIT3 is likely not a critical regulator of axonal degeneration in a model of chronic age-related ocular hypertension.

DDIT3 contributed to chronic ocular hypertension-induced RGC somal degeneration

Because RGC distal axonal degeneration and proximal somal apoptosis are regulated by molecularly distinct pathways after axonal insult[6,8–10,17,53], it was important to determine whether DDIT3 governs proximal RGC somal apoptosis in a model of age-related ocular hypertension. To accomplish this, retinas with corresponding optic nerves judged to have the most severe levels of degeneration (<5% axons remaining) from both genotypes were assessed for RGC somal survival. While D2.*Ddit3*$^{+/+}$ retinas with corresponding severe optic nerves had only 11.0 ± 1.8% somal survival, D2.*Ddit3*$^{-/-}$ retinas had

28.8 ± 1.4% somal survival (Fig. 4), consistent with levels of protection conferred by *Ddit3* deletion after mechanical axonal injury (Fig. 1b and ref. [17]). Therefore, DDIT3 played a minor role in chronic ocular hypertension-induced RGC somal apoptosis.

Discussion

Chronic ocular hypertension is an important risk factor for the development of glaucomatous neurodegeneration. Ocular hypertension is thought to injure RGCs as they exit the eye at the lamina cribrosa[1–5]. Axonal injury is thought to trigger both RGC somal and axonal degeneration pathways[6–10]. ER stress, specifically DDIT3, has been implicated as a driver of RGC death after glaucoma-relevant injuries[13–15,17,33]. Importantly, DDIT3 was shown to regulate both RGC axonal degeneration and somal apoptosis in models of mechanical axonal injury and acutely induced ocular hypertension[33]. In the present work, the role of DDIT3 in age-related, chronic ocular hypertension-induced RGC death was investigated. While *Ddit3* deletion in D2 mice provided mild protection to RGC somas, it did not significantly prevent RGC axonal degeneration. These data suggest DDIT3 has a minor role in regulating RGC somal death after axonal injury induced by chronic ocular hypertension. Therefore, the molecular process triggered by ocular hypertension that governs both RGC somal apoptosis and axonal degeneration remains unknown.

DDIT3 played a minor role in RGC somal degeneration in ocular hypertensive D2 mice; *Ddit3* deletion protected ~20% of RGC somas in retinas with severe RGC axonal degeneration. The pro-apoptotic molecule BAX was shown to be required for RGC somal degeneration after CONC[6,54] and in ocular hypertensive D2 mice[6], however, BAX did not regulate RGC axonal degeneration in these models[6]. DDIT3 is important in the translocation of BAX from the cytosol to the mitochondria during prolonged ER stress[25,26]. However, because *Ddit3* deletion only protected ~20% of RGC somas in D2 mice, another mechanism must work in tandem with DDIT3 to induce BAX. The mitogen-activated protein kinase effector and transcription factor JUN was shown to be an important regulator of ocular hypertension-induced RGC somal apoptosis. In fact, *Jun* deficiency protected ~2.5 times more RGC somas compared to *Ddit3* deficiency in 12 M D2 mice with severe optic nerve degeneration[9]. Interestingly, JUN and DDIT3 were shown to additively contribute to RGC somal apoptosis after CONC; dual deletion of *Jun* and *Ddit3* conferred 75% somal protection 120 days post-CONC (*Jun* and *Ddit3* deletion alone allowed 48% and 25% protection at this timepoint, respectively)[17]. Therefore, identifying the ocular hypertension-induced upstream regulator of both JUN and DDIT3 may be an important step in determining an upstream mechanism driving glaucomatous RGC death. Further, the role of both *Jun* and *Ddit3* in glaucomatous neurodegeneration should be tested in a model of age-related chronic ocular hypertension.

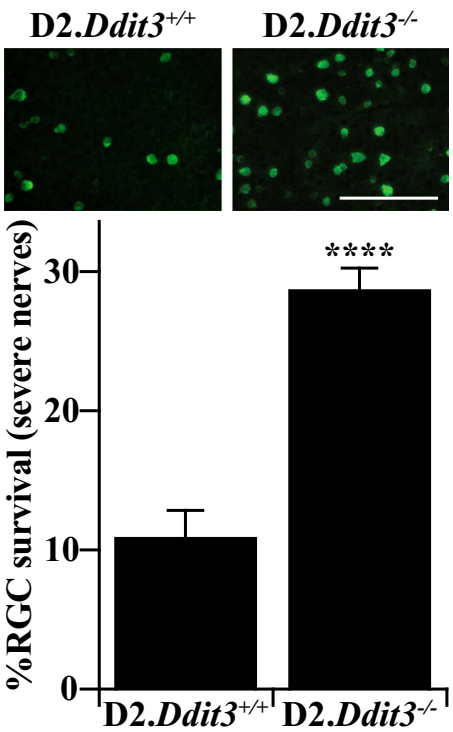

Fig. 4 DDIT3 contributed to chronic ocular hypertension-induced RGC somal degeneration. RBPMS + RGC somal density was assessed for 12 M D2.*Ddit3*$^{+/+}$ *and* D2.*Ddit3*$^{-/-}$ retinas with corresponding optic nerves judged to have the most severe levels of axonal degeneration (judged to have <5% axons remaining). D2.*Ddit3*$^{-/-}$ retinas had significantly more surviving RGC somas compared to D2.*Ddit3*$^{+/+}$ controls with equally severe optic nerves (% survival ± SEM; D2.*Ddit3*$^{+/+}$: 11.0 ± 1.8%, D2.*Ddit3*$^{-/-}$: 28.8 ± 1.4%, $n \geq 10$ per genotype, $P < 0.001$, two-tailed *t* test). RGC counts were normalized to young (1.5–2.5 M) control RGC counts of the respective genotype, see Fig. 1b. Error bars, SEM. Scale bar, 100 µm

Previous reports have shown that DDIT3 deficiency lessened both RGC axonal degeneration and somal loss in the microbead model of acute IOP elevation and after CONC[33]. However, we report no difference in RGC axonal degeneration between D2.*Ddit3*$^{+/+}$ and D2.*Ddit3*$^{-/-}$ mice at 12 M. This result is consistent with our previous report that *Ddit3* deletion did not protect from loss of RGC axonal conductance after CONC in C57BL/6J mice[17], unlike manipulation of molecules known to protect axons from degeneration (*Wld*^{S} and *Sarm1*[10]). The differences between these results could perhaps be explained by the nature and/or duration of the insults. In the microbead model of acute ocular hypertension, optic nerves had only moderate neurodegeneration (~29% axonal loss) after 8 weeks[33]. It is

possible that DDIT3 deficiency can delay axonal degeneration after an ocular hypertensive injury, but not prevent degeneration after long term ocular hypertensive insult or severe mechanical injury. It is also conceivable that the differences in findings are explained by the age-related nature of the DBA/2J disease, as acute induction of ocular hypertension was performed on young animals[33]. Finally it is possible that there is a small number of axons surviving in the D2. *Ddit3*$^{-/-}$ optic nerve that were not detected using a grading system. Regardless, our findings suggest that DDIT3 does not play a major role in axonal degeneration in an age-related, chronic ocular hypertension model of glaucoma.

In conclusion, the role of DDIT3 in glaucomatous neurodegeneration was tested in the DBA/2J (D2) inherited model of chronic, age-related ocular hypertension. DDIT3 deficiency did not alter retinal or optic nerve morphology, nor did it alter the IOP profile of the D2 model. In this model, DDIT3 did not contribute to RGC axonal degeneration, but it was responsible for ~20% of RGC somal apoptosis. Future work should focus on the roles of both JUN and DDIT3 together in perpetuating glaucomatous RGC death and should elucidate upstream regulators of both JUN and DDIT3 after glaucoma-relevant injury.

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Conflict of interest
The authors declare that they have no conflict of interest.

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References

1. Anderson, D. R. & Hendrickson, A. Effect of intraocular pressure on rapid axoplasmic transport in monkey optic nerve. *Invest Ophthalmol.* **13**, 771–783 (1974).
2. Quigley, H. A., Hohman, R. M., Addicks, E. M., Massof, R. W. & Green, W. R. Morphologic changes in the lamina cribrosa correlated with neural loss in open-angle glaucoma. *Am. J. Ophthalmol.* **95**, 673–691 (1983).
3. Jakobs, T. C., Libby, R. T., Ben, Y., John, S. W. & Masland, R. H. Retinal ganglion cell degeneration is topological but not cell type specific in DBA/2J mice. *J. Cell Biol.* **171**, 313–325 (2005).
4. Schlamp, C. L., Li, Y., Dietz, J. A., Janssen, K. T. & Nickells, R. W. Progressive ganglion cell loss and optic nerve degeneration in DBA/2J mice is variable and asymmetric. *BMC Neurosci.* **7**, 66 (2006).
5. Howell, G. R. et al. Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. *J. Cell Biol.* **179**, 1523–1537 (2007).
6. Libby, R. T. et al. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. *PLoS Genet.* **1**, 17–26 (2005).
7. Beirowski, B., Babetto, E., Coleman, M. P. & Martin, K. R. The WldS gene delays axonal but not somatic degeneration in a rat glaucoma model. *Eur. J. Neurosci.* **28**, 1166–1179 (2008).
8. Fernandes, K. A., Harder, J. M., John, S. W., Shrager, P. & Libby, R. T. DLK-dependent signaling is important for somal but not axonal degeneration of retinal ganglion cells following axonal injury. *Neurobiol. Dis.* **69**, 108–116 (2014).
9. Syc-Mazurek, S. B., Fernandes, K. A. & Libby, R. T. JUN is important for ocular hypertension-induced retinal ganglion cell degeneration. *Cell Death Dis.* **8**, e2945 (2017).
10. Fernandes, K. A. et al. Role of SARM1 and DR6 in retinal ganglion cell axonal and somal degeneration following axonal injury. *Exp. Eye Res.* **171**, 54–61 (2018).
11. Li, Y., Schlamp, C. L., Poulsen, K. P. & Nickells, R. W. Bax-dependent and independent pathways of retinal ganglion cell death induced by different damaging stimuli. *Exp. Eye Res.* **71**, 209–213 (2000).
12. Maes, M. E., Schlamp, C. L. & Nickells, R. W. BAX to basics: How the BCL2 gene family controls the death of retinal ganglion cells. *Prog. Retinal Eye Res.* **57**, 1–25 (2017).
13. Doh, S. H., Kim, J. H., Lee, K. M., Park, H. Y. & Park, C. K. Retinal ganglion cell death induced by endoplasmic reticulum stress in a chronic glaucoma model. *Brain Res.* **1308**, 158–166 (2010).
14. Hu, Y. et al. Differential effects of unfolded protein response pathways on axon injury-induced death of retinal ganglion cells. *Neuron* **73**, 445–452 (2012).
15. Fernandes, K. A., Harder, J. M., Kim, J. & Libby, R. T. JUN regulates early transcriptional responses to axonal injury in retinal ganglion cells. *Exp. Eye Res.* **112**, 106–117 (2013).
16. Ojino, K. et al. Involvement of endoplasmic reticulum stress in optic nerve degeneration after chronic high intraocular pressure in DBA/2J mice. *J. Neurosci. Res.* **93**, 1675–1683 (2015).
17. Syc-Mazurek, S. B., Fernandes, K. A., Wilson, M. P., Shrager, P. & Libby, R. T. Together JUN and DDIT3 (CHOP) control retinal ganglion cell death after axonal injury. *Mol. Neurodegeneration* **12**, 71 (2017).
18. Ghosh, A. P., Klocke, B. J., Ballestas, M. E. & Roth, K. A. CHOP potentially co-operates with FOXO3a in neuronal cells to regulate PUMA and BIM expression in response to ER stress. *PLoS ONE* **7**, e39586 (2012).
19. Jung, K. J., Min, K. J., Bae, J. H. & Kwon, T. K. Carnosic acid sensitized TRAIL-mediated apoptosis through down-regulation of c-FLIP and Bcl-2 expression at the post translational levels and CHOP-dependent up-regulation of DR5, Bim, and PUMA expression in human carcinoma caki cells. *Oncotarget* **6**, 1556–1568 (2015).
20. Teske, B. F. et al. CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol. Biol. Cell* **24**, 2477–2490 (2013).
21. Yamaguchi, H. & Wang, H. G. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *J. Biol. Chem.* **279**, 45495–45502 (2004).
22. Li, G. et al. Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J. Cell Biol.* **186**, 783–792 (2009).
23. McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y. & Holbrook, N. J. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol. Cell. Biol.* **21**, 1249–1259 (2001).
24. Zhang, M. et al. Chop deficiency prevents UUO-induced renal fibrosis by attenuating fibrotic signals originated from Hmgb1/TLR4/NFkappaB/IL-1beta signaling. *Cell Death Dis.* **6**, e1847 (2015).

25. Gotoh, T., Terada, K., Oyadomari, S. & Mori, M. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ.* **11**, 390–402 (2004).
26. Zou, X. J., Yang, L. & Yao, S. L. Endoplasmic reticulum stress and C/EBP homologous protein-induced Bax translocation are involved in angiotensin II-induced apoptosis in cultured neonatal rat cardiomyocytes. *Exp. Biol. Med.* **237**, 1341–1349 (2012).
27. Huang, H. et al. Neuroprotection by eIF2alpha-CHOP inhibition and XBP-1 activation in EAE/optic neuritiss. *Cell Death Dis.* **8**, e2936 (2017).
28. Nashine, S., Liu, Y., Kim, B. J., Clark, A. F. & Pang, I. H. Role of C/EBP homologous protein in retinal ganglion cell death after ischemia/reperfusion injury. *Investigative Ophthalmol. Vis. Sci.* **56**, 221–231 (2014).
29. Ha, Y. et al. AAV2-mediated GRP78 Transfer Alleviates Retinal Neuronal Injury by Downregulating ER Stress and Tau Oligomer Formation. *Investigative Ophthalmol. Vis. Sci.* **59**, 4670–4682 (2018).
30. Howell, G. R., Walton, D. O., King, B. L., Libby, R. T. & John, S. W. Datgan, a reusable software system for facile interrogation and visualization of complex transcription profiling data. *BMC Genomics* **12**, 429 (2011).
31. Howell, G. R. et al. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. *J. Clin. Investig.* **121**, 1429–1444 (2011).
32. John, S. S. W., Libby, R. T. & Howell, G. R. *Glaucoma Discovery Platform*, <http://glaucomadb.jax.org/glaucoma> (2009).
33. Yang, L. et al. Rescue of glaucomatous neurodegeneration by differentially modulating neuronal endoplasmic reticulum stress molecules. *J. Neurosci.* **36**, 5891–5903 (2016).
34. Anderson, M. G. et al. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. *Nat. Genet.* **30**, 81–85 (2002).
35. Libby, R. T. et al. Inherited glaucoma in DBA/2J mice: pertinent disease features for studying the neurodegeneration. *Vis. Neurosci.* **22**, 637–648 (2005).
36. Howell, G. R. et al. Absence of glaucoma in DBA/2J mice homozygous for wild-type versions of Gpnmb and Tyrp1. *BMC Genet.* **8**, 45 (2007).
37. Silva, R. M. et al. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism. *J. Neurochem.* **95**, 974–986 (2005).
38. Harder, J. M., Fernandes, K. A. & Libby, R. T. The Bcl-2 family member BIM has multiple glaucoma-relevant functions in DBA/2J mice. *Sci. Rep.* **2**, 530 (2012).
39. Syc-Mazurek, S. B., Rausch, R. L., Fernandes, K. A., Wilson, M. P. & Libby, R. T. Mkk4 and Mkk7 are important for retinal development and axonal injury-induced retinal ganglion cell death. *Cell Death Dis.* **9**, 1095 (2018).
40. Harder, J. M. et al. BCL2L1 (BCL-X) promotes survival of adult and developing retinal ganglion cells. *Mol. Cell Neurosci.* **51**, 53–59 (2012).
41. Templeton, J. P. et al. Differential response of C57BL/6J mouse and DBA/2J mouse to optic nerve crush. *BMC Neurosci.* **10**, 90 (2009).
42. Wang, J., Li, Y., King, R., Struebing, F. L. & Geisert, E. E. Optic nerve regeneration in the mouse is a complex trait modulated by genetic background. *Mol. Vis.* **24**, 174–186 (2018).
43. Cui, Q., Yip, H. K., Zhao, R. C., So, K. F. & Harvey, A. R. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. *Mol. Cell Neurosci.* **22**, 49–61 (2003).
44. Kwong, J. M., Caprioli, J. & Piri, N. RNA binding protein with multiple splicing: a new marker for retinal ganglion cells. *Investig. Ophthalmol. Vis. Sci.* **51**, 1052–1058 (2010).
45. Zode, G. S. et al. Reduction of ER stress via a chemical chaperone prevents disease phenotypes in a mouse model of primary open angle glaucoma. *J. Clin. Investig.* **121**, 3542–3553 (2011).
46. Zode, G. S. et al. Ocular-specific ER stress reduction rescues glaucoma in murine glucocorticoid-induced glaucoma. *J. Clin. Investig.* **124**, 1956–1965 (2014).
47. Schuettauf, F., Quinto, K., Naskar, R. & Zurakowski, D. Effects of anti-glaucoma medications on ganglion cell survival: the DBA/2J mouse model. *Vis. Res.* **42**, 2333–2337 (2002).
48. Anderson, M. G. et al. Genetic context determines susceptibility to intraocular pressure elevation in a mouse pigmentary glaucoma. *BMC Biol.* **4**, 20 (2006).
49. Matsubara, A. et al. Investigating the effect of ciliary body photodynamic therapy in a glaucoma mouse model. *Investig. Ophthalmol. Vis. Sci.* **47**, 2498–2507 (2006).
50. Wong, A. A. & Brown, R. E. A neurobehavioral analysis of the prevention of visual impairment in the DBA/2J mouse model of glaucoma. *Investig. Ophthalmol. Vis. Sci.* **53**, 5956–5966 (2012).
51. Wong, A. A. & Brown, R. E. Prevention of vision loss protects against age-related impairment in learning and memory performance in DBA/2J mice. *Front. Aging Neurosci.* **5**, 52 (2013).
52. Hu, Y. Axon injury induced endoplasmic reticulum stress and neurodegeneration. *Neural Regen. Res.* **11**, 1557–1559 (2016).
53. Fernandes, K. A. et al. JNK2 and JNK3 are major regulators of axonal injury-induced retinal ganglion cell death. *Neurobiol. Dis.* **46**, 393–401 (2012).
54. Nickells, R. W., Semaan, S. J. & Schlamp, C. L. Involvement of the Bcl2 gene family in the signaling and control of retinal ganglion cell death. *Prog. Brain Res.* **173**, 423–435 (2008).