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 cribrosa1–5 (termed the glial lamina in mice5). 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 injury6–10. 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 injury6,11,12. Thus, the mechanism triggered by ocular hypertension that regulates glaucomatous neurodegeneration must ultimately converge upon BAX induction.
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. 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, Bim, Gadd34, Dr5, and Ero1α genes and inhibited transcription of the pro-survival gene Bcl2. DDIT3 was also shown to be important for the translocation of activated BAX from the cytosol to the mitochondria 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. DDIT3 was expressed by RGCs after glaucoma-relevant insults, including optic nerve crush and the microbead model of acute ocular hypertension. 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. Ddit3 deficiency or silencing was protective to RGC somas after mechanical axonal injury (optic nerve crush) and the microbead model of acutely induced ocular hypertension. Interestingly, despite not appearing to have a major role in RGC axonal degeneration after optic nerve crush, DDIT3 deficiency lessened axonal degeneration in an acute ocular hypertension model. 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.

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.

**Materials and methods**

**Mice**

DBA/2J (D2) mice and mice with a null allele of Ddit3 (B6.129S(Cg)-Ddit3 tm2.1Dron /) 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 litters 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, 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 PO4, 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 1 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. 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
masked observer used a validated grading scale to assess the level of glaucomatous damage of each optic nerve. As previously described\(^3\),\(^6\),\(^9\),\(^{38}\),\(^{39}\), 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\(^3\),\(^6\),\(^9\),\(^{38}\),\(^{39}\). 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 exposed but not crushed. Antibiotic ointment was applied on the contralateral eye, where the optic nerve was self-closing forceps for 5 s. Sham surgery was performed exposed and crushed immediately behind the eye with subcutaneously prior to surgery. The optic nerve was 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 exposed but not crushed. Antibiotic ointment was applied on the contralateral eye, where the optic nerve was self-closing forceps for 5 s.

**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\(^3\),\(^8\),\(^{39}\), 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 Fluoro-gel in TRIS buffer (Electron Microscopy Sciences, 17985-11).

**Cell quantification**

As previously described\(^3\),\(^8\),\(^{39}\), 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 Imagel 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\(^4\),\(^17\),\(^33\). 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\(^38\). 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

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**Table 1 Summary of antibodies**

| Antibody          | Concentration | Company                  |
|-------------------|---------------|--------------------------|
| Rabbit anti-cCASP3 (AF835) | 1:1000        | R&D Systems              |
| Rabbit anti-RBPMs (GTX1118619) | 1:250          | GeneTex                  |
| Guinea pig anti-RBPMs (1832-RBPM) | 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

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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 significantly more surviving RGCs post-CONC in D2 mice compared to D2.Ddit3++/+ controls (RBPMs+ cells/mm² ± SEM: D2.Ddit3−/−, 3647.0 ± 166.2; n = 6 per genotype; P = 0.001, 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 RGCs 14 days post-CONC stained for RBPMS (green) and cleaved caspase 3 (cCASP3, red). There were significantly fewer cCASP3 + RGCs (cCASP3+ RBPMs+ cells/mm² ± SEM: D2.Ddit3−/−, 139.7 ± 7.5, D2.Ddit3−/−, 100.2 ± 13.8; n = 6 per genotype, P = 0.031, two-tailed t test).

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 the site of an early critical axonal injury4,5. To ensure Ddit3 deletion did not cause any developmental ONH or retinal abnormalities in D2 mice, 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 glaucoma45,46. Since RGC degeneration in D2 mice depends on age-related IOP elevation36,47–51, 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 injury52 and
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, 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 were assessed for RGC somal survival. While D2. Ddit3+/− mice had no/early (left), moderate (middle), and severe (right) axonal damage, D2. Ddit3+/+ optic nerves had no/no/early, moderate, and severe axonal damage at 3 M and 12 M. 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 ≥ 60 per genotype, P = 0.474, Pearson Chi-square test). Scale bar, 100 µm.

Acute ocular hypertension. 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. 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. (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.

**Materials and methods**

Age: 3M, 12M

**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 ≥ 76), 7.5 M (n ≥ 72), 9 M (n ≥ 60), 10.5 M (n ≥ 56), and 12 M (n ≥ 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.
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 cribrosa1–5. Axonal injury is thought to trigger both RGC somal and axonal degeneration pathways6–10. ER stress, specifically DDIT3, has been implicated as a driver of RGC death after glaucoma-relevant injuries13–15,17,33. Importantly, DDIT3 was shown to regulate both RGC axonal degeneration and somal apoptosis in models of mechanical axonal injury and acutely induced ocular hypertension33. 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 CONC6,54 and in ocular hypertensive D2 mice6; however, BAX did not regulate RGC axonal degeneration in these models6. DDIT3 is important in the translocation of BAX from the cytosol to the mitochondria during prolonged ER stress25,26. 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 degeneration9. 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.

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 CONC33. 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/6 mice17, unlike manipulation of molecules known to protect axons from degeneration (WldS and Sarmpi10). 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 weeks33. 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. 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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