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JUN regulates early transcriptional responses to axonal injury in retinal ganglion cells

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Abstract

The AP1 family transcription factor JUN is an important molecule in the neuronal response to injury. In retinal ganglion cells (RGCs), JUN is upregulated soon after axonal injury and disrupting JUN activity delays RGC death. JUN is known to participate in the control of many different injury response pathways in neurons, including pathways controlling cell death and axonal regeneration. The role of JUN in regulating genes involved in cell death, ER stress, and regeneration was tested to determine the overall importance of JUN in regulating RGC response to axonal injury. Genes from each of these pathways were transcriptionally controlled following axonal injury and *Jun* deficiency altered the expression of many of these genes. The differentially expressed genes included, *Atf3*, *Ddit3*, *Ecel1*, *Gadd45a*, *Gal*, *Hrk*, *Pten*, *Socs3*, and *Sprr1a*. Two of these genes, *Hrk* and *Atf3*, were tested for importance in RGC death using null alleles of each gene. Disruption of the prodeath Bcl2 family member *Hrk* did not affect the rate or amount of RGC death after axonal trauma. Deficiency in the ATF/CREB family transcription factor *Atf3* did lessen the amount of RGC death after injury, though it did not provide long term protection to RGCs. Since JUN's dimerization partner determines its transcriptional targets, the expression of several candidate AP1 family members were examined. Multiple AP1 family members were induced by axonal injury and had a different expression profile in *Jun* deficient retinas compared to wildtype retinas (*Fosl1*, *Fosl2* and *Jund*). Overall, JUN appears to play a multifaceted role in regulating RGC response to axonal injury.

Keywords

glaucoma; cell death; regeneration; ER stress; trauma; axonal injury; AP1

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Introduction

Numerous studies have shown that axonal injury is a critical insult to retinal ganglion cells (RGCs) in glaucoma (e.g. Anderson and Hendrickson, 1974; Buckingham et al., 2008; Howell et al., 2007; Howell et al., 2012; Li et al., 1999; Quigley et al., 1983; Schlamp et al., 2006). A major part of a neuron's response to axonal injury is the activation of transcription factors, which result in large changes to the cell's transcriptome (Hanz and Fainzilber, 2006; Michaelevski et al., 2010; Smith and Skene, 1997). These changes are critical for initiating regeneration and degeneration pathways in the injured cell (Leppa and Bohmann, 1999; Raivich, 2008). The ensuing gene expression changes ultimately decide whether a neuron will live or die following the axonal insult (Yang et al., 2007). The AP1 family member JUN (previously known as cJUN) is a transcription factor that is induced soon after neuronal injury and regulates diverse neuronal injury responses (Herdegen et al., 1997; Raivich and Behrens, 2006). Interestingly, JUN can promote both regenerative and degenerative states after axonal injury (Hull and Bahr, 1994; Isenmann and Bahr, 1997; Koistinaho et al., 1993; Levkovitch-Verbin et al., 2005) and has been proposed to be a central hub controlling the expression of axonal regeneration and cell death genes (Herdegen et al., 1997; Raivich and Behrens, 2006). JUN is upregulated in RGCs after several glaucoma-relevant insults such as excitotoxicity, mechanical optic nerve injury, and elevated intraocular pressure (Fernandes et al., 2012; Isenmann and Bahr, 1997; Levkovitch-Verbin et al., 2005; Munemasa et al., 2006). Importantly, inhibiting JUN expression or altering JUN activity significantly delays RGC death after axonal injury (Fernandes et al., 2012; Lingor et al., 2005; Yoshida et al., 2002). Given *Jun's* role in mediating RGC viability after axonal injury, JUN-regulated transcriptional programs are likely to be major factors controlling RGC fate. Surprisingly given the importance of JUN, the number of known direct transcriptional targets of JUN are limited (Hartl et al., 2003), especially in the central nervous system (Freeman et al., 2004).

To determine if JUN played an extensive role in regulating RGC response to axonal injury at the transcriptional level, the expression of multiple genes implicated in this response were analyzed in wildtype and *Jun* deficient retinas after controlled optic nerve crush (CONC). Specifically, the transcriptional profile of genes implicated in determining neuronal survival (Bcl2 family members, ER stress pathway members) and ones implicated in regeneration were examined. Furthermore, the expression of known targets of JUN and other members of the JUN and FOS transcription family (AP1 family) were examined. Gene expression was examined at two time points: prior to the onset of cell death (2 days after CONC) to identify genes that may have a role in initiating cell death and/or promote viability/regeneration pathways; and at the peak of cell death (5 days after CONC) to determine if a gene's expression is consistent with an involvement in RGC death. Furthermore, the importance of two of the genes found to be upregulated after CONC, *Hrk* and *Atf3*, were tested to determine if they were critical for RGC death after axonal injury. Overall, our results demonstrate that *Jun* regulates diverse signaling pathways that may ultimately influence RGC regeneration and survival following axonal injury.

2 Materials and Methods

2.1 Mice

Mice with conditional deletion of *Jun* in the retina were generated by crossing mice carrying a floxed allele of *Jun* (*Jun*^{fl} Behrens et al., 2002) with mice expressing cre recombinase under the control of an early retinal promoter, *Six3* (Furuta et al., 2000). These mice were on a mixed genetic background of C57BL/6J and 129 origin. Note, there are ~10% of RGCs cells that do not have Six3-cre mediated recombination of the *Jun*^{fl} allele (see results and Fernandes et al., 2012). Traditional germ line null alleles of *Atf3* (Hartman et al., 2004) and *Hrk* (Imaizumi et al., 2004) were also used. There were no differences observed between

wildtype and heterozygous mice for either *Atf3* or *Hrk* and the two genotypes were used as controls when studying these genes (referred to in the text as +/+). Mice were housed in a 12-hour light dark cycle and were fed chow and water ad libitum. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology's statement on the use of animals in ophthalmic research and were approved by the University of Rochester's University Committee on Animal Resources.

2.2 Optic nerve injury

Controlled optic nerve crush (CONC) was performed as previously described (Libby et al., 2005). Briefly, mice were anaesthetized and the optic nerve was exposed. The optic nerve was clamped for 4 seconds approximately 0.5 mm from the globe using self-closing forceps (Roboz RS-5027).

2.3 Realtime PCR

All dissections were performed in RNAse free conditions. Eyes were dissected and placed in RNAse-free ice cold PBS. The retina was dissected free from the eye and submerged in RNA-later (Qiagen 76106). Retinas were stored in RNA-later at 4°C until RNA extraction. RNA was extracted as per manufacturer's instructions using an RNAeasy Microkit (Qiagen 74004). The amount of RNA in each sample was estimated using Nanodrop. 500ng of RNA from each sample was reverse transcribed to cDNA using iScript (Biorad 170-8891). 20ul SYBR green (Biorad 170-8882) amplification reactions were prepared using 2ul of cDNA. Realtime PCR reactions were performed on a CFX Connect system (Biorad). Primer sequences for all genes tested are summarized in Table 1. Product sizes were validated on a gel. The geometric mean of C_T values of the reference genes (*Gapdh* and *Gad1*) was subtracted from the C_T value of the gene of interest to obtain the ΔC_T value. The expression level for each gene was calculated using the $\Delta\Delta C_T$ method as described previously (Livak and Schmittgen, 2001). The following genotypes: $Jun^{+/+}$ Six3-cre^{-}, $Jun^{+/+}$ Six3-cre^{+}, $Jun^{+/fl}$ Six3-cre^{-}, or $Jun^{fl/fl}$ Six3-cre^{-} were used as controls and are collectively referred to as $Jun^{+/+}$. Mice with retinal deletion of *Jun* ($Jun^{fl/fl}$ Six3-cre^{+}) are referred to as $Jun^{-/-}$. No mice heterozygous for *Jun* deletion ($Jun^{+/fl}$ Six3-cre^{+}) were used. At least 6 retinas of each genotype were assessed for gene expression at all time points examined (summarized in Table 2).

2.4 Immunohistochemistry and Cell Counts

Eyes were processed as previously described (Harder and Libby, 2011; Libby et al., 2005). Briefly, following fixation in 4% paraformaldehyde (PFA), the anterior segment of each eye was removed and the posterior eye cup was processed for cryosectioning, whole mount immunostaining or whole mount Nissl staining. For immunohistochemistry rabbit anti-DDIT3 (CHOP; ABR, 1:250), rabbit anti-cCASP3 (RD, 1:1000), rabbit anti-JUN (Abcam, 1:250) and mouse anti-βIII tubulin (TUJ1; Covance, 1:1000) were used as primary antibodies. For cell counts, images were taken from eight 20x fields (for cCASP3+) or eight 40x fields (for TUJ1+ or Nissl+ cell counts) around the peripheral edge of whole mounted retinas. Each field was approximately 220 μm from the peripheral edge of the retina. Since Six3 mediated recombination varies with retinal eccentricity, for the JUN+ cell counts, images were obtained from four central 20x fields in addition to the eight 20x peripheral fields in order to obtain a fuller representation of the number of unrecombined cells throughout the retina. For Nissl counts, all ganglion cell layer cells within a field were counted with the exception of endothelial cells (which have an obvious elongated, non-neuronal morphology). The numbers of neurons immunolabeled with cCASP3, JUN or TUJ1 and the number of Nissl stained cells in each image were quantified using the cell-counter tool in ImageJ. Eyes from animals that underwent sham surgery were used as controls for all cell counts.

2.5 Statistical Analysis

P values < 0.05 were deemed to be significant for all experiments. Graphpad Prism was used for statistical analyses involving ANOVA. For experiments involving cell quantification the experimenter was masked to genotype and/or experimental group and a two way ANOVA was used to test for significance except for quantification of JUN positive cells which used a student's t-test. For analysis of realtime PCR experiments involving multiple genotypes ($Jun^{+/+}$ and $Jun^{-/-}$) and time points (naïve, 2, and 5 days after CONC), two way ANOVA's were performed followed by Bonferroni post hoc tests to determine whether gene expression changed within (*intra-genotype comparison*) and between (*inter-genotype comparison*) genotypes. A one way ANOVA followed by Bonferroni post hoc testing was used to determine if the expression of *Jun* changed in wildtype animals after CONC. ΔC_T values of a gene were used in the statistical analyses.

Results

3.1 *Jun* is transcriptionally regulated following axonal injury

Consistent with previous reports (e.g. Johnson et al., 1993; Koistinaho et al., 1993), at 2 and 5 days following axonal injury (controlled optic nerve crush; CONC), there was a significant increase in *Jun* transcript expression in wildtype retinas (Fig 1A; $P < 0.05$). To study the effect of *Jun* deficiency on the RGC injury response, a floxed allele of *Jun* (Jun^{fl}) was deleted using an early retinal deleter cre, Six3-cre, since germline deletion of *Jun* results in embryonic lethality (Hilberg et al., 1993; Johnson et al., 1993). No differences were observed between $Jun^{+/+}$ $Six3cre^{-}$, $Jun^{+/+}$ $Six3cre^{+}$, $Jun^{+/fl}$ $Six3cre^{-}$, or $Jun^{fl/fl}$ $Six3cre^{-}$ retinas. All of these genotypes were used as controls and collectively referred to as $Jun^{+/+}$ or wildtype (no mice heterozygous for *Jun* deletion were used). There was incomplete Six3-cre mediated recombination of the Jun^{fl} allele as a small number of cells still expressed JUN after CONC (10.3 ± 2.1% compared to wildtype; Fig. 1B,C). Consistent with high recombination efficiency, the level of *Jun* expression in $Jun^{fl/fl}$ $Six3cre^{+}$ retinas remained significantly below the level of *Jun* in $Jun^{+/+}$ retinas at all time points examined (fold reduction in $Jun^{-/-}$ compared to $Jun^{+/+}$: 0 days, 6.1; 2 days, 8.3; 5 days, 21.6). Therefore, JUN-dependent changes after CONC should be significantly attenuated in $Jun^{fl/fl}$ $Six3cre^{+}$ retinas (referred to as $Jun^{-/-}$ or *Jun* deficient retinas).

3.2 *Jun* deficiency alters the expression of an axonal injury response genes after CONC

To determine if *Jun* deficiency affected the pattern or level of a gene's expression after CONC two biologically important comparisons were made. 1) To evaluate gene expression changes after CONC, expression levels were compared to the naïve condition of the same genotype (*intra-genotype comparison*). 2) To determine whether gene expression was *Jun*-dependent, expression levels were compared between genotypes (*inter-genotype comparison*) at each time point assessed. Importantly this comparison includes assessing whether there is a difference between $Jun^{+/+}$ and $Jun^{-/-}$ retinas prior to injury (*naïve comparison*). The ΔC_T values for all the genes assessed along with the P values for each of the comparisons listed above are summarized in Table 2.

To test if axonal injury responsive genes were regulated by *Jun*, the expression of Endothelin-Converting Enzyme-Like 1 (*Ecel1*), a gene known to be regulated by *Jun* after axonal injury in other neurons (Kiryu-Seo et al., 2008; Kiryu-Seo et al., 2000) was characterized following CONC. Interestingly, *Jun* deficiency appeared to have a relatively minor, but significant effect on basal *Ecel1* expression. In unmanipulated eyes *Ecel1* was increased 2.80 fold in $Jun^{-/-}$ compared to $Jun^{+/+}$ retinas (Fig 2; Naïve comparison; P=0.016). This observation is consistent with basal JUN expression repressing the expression of some genes (Aguilera et al., 2011). Following CONC, the expression of *Ecel1*

progressively increased at 2 and 5 days in $Jun^{+/+}$ retinas (Fig 2). While *Ecel1* was upregulated in $Jun^{-/-}$ retinas, *Ecel1* expression was significantly attenuated at both 2 and 5 days following CONC in $Jun^{-/-}$ retinas (Fig. 2; $P < 0.001$ for both comparisons). These results indicate that *Jun* deficiency alters the expression of transcriptionally regulated genes associated with axonal injury.

3.3 JUN and FOS family member regulation after axonal-injury

Immediate early genes belonging to the JUN and FOS families have been shown to be induced following axonal injury (*e.g.* Guo et al., 2010; Howell et al., 2011a; Howell et al., 2011b; Hull and Bahr, 1994; Koistinaho et al., 1993; Takeda et al., 2000; Yang et al., 2007). JUN dimerizes with itself and various other members of the *Fos* and *Jun* family to form the AP1 complex. Importantly, the transcriptional targets of AP1 dimers containing JUN change depending on JUN's binding partner (Bakiri et al., 2002; Chinenov and Kerppola, 2001). The expression of several AP1 family members previously shown to be regulated in neurons following axonal injury was characterized following CONC. *Jund* was significantly upregulated in $Jun^{+/+}$ retinas ($P < 0.01$ for each time point) but not in $Jun^{-/-}$ retinas at both 2 and 5 days after CONC. At 5 days post CONC *Jund* expression was significantly attenuated in $Jun^{-/-}$ retinas compared to wildtype (Fig. 3A; $P = 0.016$). *Fos* expression did not appear to be regulated following CONC in either genotype (Fig. 3B). *Fosl1* expression increased significantly at both 2 and 5 days following CONC in wildtype retinas (Fig. 3C; $P< 0.002$ for each time point). In *Jun* deficient retinas, CONC did not lead to significant upregulation of *Fosl1* expression, though *Fosl1* expression levels were not significantly attenuated in $Jun^{-/-}$ mice compared to $Jun^{+/+}$ mice. In contrast to the *Jund* and *Fosl1* results where expression only increased in wildtype retinas, *Fosl2* expression did not change in $Jun^{+/+}$ retinas but did significantly increase in $Jun^{-/-}$ retinas at 2 days (Fig 3D; $P = 0.035$). Thus, it appears that JUN-dependent events are upstream of changes in expression of several AP1 family members after axonal injury. Since altered AP1 family member expression can alter the composition of AP1 dimers and thereby change transcriptional targets (Kaminska et al., 2000) it will be important to determine if different AP1 dimer combinations change with time and whether distinct AP1 dimers control cell death and cell regeneration programs.

3.4 JUN target *Atf3* is involved in axonal-injury induced RGC death

Atf3 is a stress induced member of the ATF/CREB family of transcription factors (Chen et al., 1996; Hai and Hartman, 2001) and can dimerize with JUN in neurons (Nakagomi et al., 2003; Pearson et al., 2003). In neurons *Atf3* is known to contribute to many aspects of response to injury and can be directly regulated by JUN (Mei et al., 2008; Nakagomi et al., 2003; Pearson et al., 2003). Consistent with previous studies *Atf3* expression increases in RGCs after axonal injury (e.g. Guo et al., 2010; Takeda et al., 2000). At both 2 and 5 days after CONC, *Atf3* was significantly increased in $Jun^{+/+}$ retinas (Fig 4A; $P<0.001$ for both time points). *Atf3* expression did not significantly change in $Jun^{-/-}$ retinas after CONC and the CONC-induced increase of *Atf3* was significantly attenuated in $Jun^{-/-}$ retinas (Fig 4A, $P < 0.005$ for both time points). Thus, the induction of *Atf3* expression after CONC appears to require JUN.

To determine if ATF3 played a similar prodeath role in axonally injured RGCs as JUN, *Atf3* null mice were subjected to CONC. *Atf3* deficiency significantly reduced the number of dying cells (cleaved caspase 3+; cCASP3+) at 3 and 5 days after CONC by 45% and 27% respectively (Fig. 4B,C; $P < 0.001$ for both time points). However, this reduction of dying cells at the beginning of the CONC cell death window did not result in long term increase in the number of surviving RGCs, as judged by TUJ1+ cell counts 14 days post CONC (Fig 4D,E). Collectively, these results demonstrate that the JUN target, *Atf3*, has a minor proapoptotic role in RGCs following axonal injury.

3.5 *Jun* deficiency alters the expression of genes involved in RGC regeneration response

Although RGC axons do not naturally regenerate after injury in the mammalian retina, RGCs are responsive to numerous types of manipulations promoting regenerative outgrowth (de Lima et al., 2012; Fischer et al., 2004; Moore et al., 2009; Park et al., 2008; Sengottuvel et al., 2011; Smith et al., 2009; Yin et al., 2006). In combination these studies indicate that robust axon regeneration requires adding factors that promote regeneration and suppressing endogenous barriers to regeneration. JUN has been shown to promote axonal regeneration after injury (Raivich et al., 2004; Ruff et al., 2012; Smith and Skene, 1997). To test whether JUN could participate in controlling pro-regenerative genetic programs, the expression of two genes positively correlated with regenerative potential, *Gal* and *Sprr1a* (Holmes et al., 2000; Starkey et al., 2009), and known to be upregulated in an animal model of glaucoma (Howell et al., 2011a; Howell et al., 2011b) were assessed after CONC in wildtype and *Jun* deficient mice. At both 2 and 5 days after CONC, *Gal* and *Sprr1a* were significantly upregulated in both $Jun^{+/+}$ and $Jun^{-/-}$ retinas (Fig. 5A,B $P < 0.01$ for all comparison). However, the upregulation of both of these proregenerative genes was significantly attenuated in $Jun^{-/-}$ retinas after CONC. Axonal injury also results in concurrent activation of cell-intrinsic suppressors of regeneration in RGCs. Knockout of two such suppressors, *Klf4* and *Socs3*, has been shown to dramatically promote axon regeneration following optic nerve injury (Moore et al., 2009; Park et al., 2008; Smith et al., 2009). The expression of *Klf4* and *Socs3* were not significantly altered in wildtype retinas (Fig 5C,D) and *Klf4* was not significantly changed in $Jun^{-/-}$ retinas after CONC. However, the expression of *Socs3* significantly increased in *Jun* deficient retinas at 2 days following CONC (Fig 5D; $P = 0.029$), although the difference in expression between genotypes at this time point was not significant ($P = 0.143$). Collectively these data suggests that pro-regenerative pathways are suppressed in *Jun* deficient mice both through attenuation of pro-regenerative gene expression and through suppression of genes that inhibit regeneration.

Pten deficiency has been shown to promote RGC regeneration by rescuing the deficit in protein synthesis that is observed following axonal injury (Park et al., 2008). *Pten* was upregulated in $Jun^{+/+}$ retinas mice at 5 days after CONC (Fig. 5E, $P = 0.001$). This upregulation in expression was not observed in $Jun^{-/-}$ retinas and *Pten* expression was also significantly attenuated at 5 days following CONC in $Jun^{-/-}$ compared to $Jun^{+/+}$ retinas ($P < 0.003$). Thus, in contrast to the other regeneration-associated genes examined for which pro-regenerative changes were attenuated in $Jun^{-/-}$ retinas, the pattern of *Pten* expression in $Jun^{-/-}$ retinas may favor regeneration compared to $Jun^{+/+}$ retinas. However, the expression of *Pten* was significantly increased in $Jun^{-/-}$ compared to $Jun^{+/+}$ retinas before injury ($P = 0.008$) and therefore basal *Pten* expression in $Jun^{-/-}$ retinas may negatively impact an RGC's regenerative potential. Overall, these data suggest that JUN appears to prime RGCs for regeneration and/or may be important for making RGCs receptive to proregenerative manipulations.

3.6 *Jun* deficiency alters the expression of ER stress response genes after axonal injury

Axonal injury and ocular hypertension induce ER stress and the unfolded protein response (UPR) pathway in RGCs (Doh et al., 2010; Hu et al., 2012; Pernet et al., 2012). UPR activation has been shown promote apoptosis in RGCs (Hu et al., 2012). Specifically, the transcription of a key proapoptotic UPR target gene, *Ddit3* (also know as *Chop*) was shown to increase in RGCs following optic nerve crush and *Ddit3* deficiency reduced RGC death following axonal injury (Hu et al., 2012). Given the major role JUN-dependent pathways play in regulating RGC death following axonal injury, the possibility that JUN regulates key components of the ER stress and UPR activation pathways was tested (Lee et al., 2003; Schroder and Kaufman, 2005). *Atf6* was not significantly regulated at 2 days or 5 days in either wildtype or *Jun* deficient eyes following CONC (Fig 6A). The expression of the ER

stress marker, *Gadd45a* significantly increased in $Jun^{+/+}$ retinas at 2 days post CONC (Fig 6B, $P < 0.003$), but did not change in $Jun^{-/-}$ retinas. In $Jun^{+/+}$ retinas, the expression of *Ddit3* increased at both 2 and 5 days following CONC (Fig 6C, $P \leq 0.001$ for both comparisons). *Ddit3* expression significantly increased in $Jun^{-/-}$ retinas but only at 2 days following CONC ($P = 0.022$). Immunohistochemical staining for DDIT3 confirmed the induction of DDIT3 in $Jun^{+/+}$ and $Jun^{-/-}$ mice 3 days after CONC (Fig 6D). Collectively, these data demonstrate that upregulation of ER stress markers and the UPR pathway occurs retinas deficient in *Jun*, though JUN does appear to have a small role in transcriptionally regulating this pathway. Given that both of these pathways are ultimately prodeath, it will be interesting to determine if they interact to co-regulate downstream prodeath targets. In fact, DDIT3 has been shown to interact with JUN and other AP1 family members (Ubeda et al., 1999) and it is tempting to speculate that this dimer might control important prodeath pathways in RGCs. ER stress is also known to activate JNK signaling. Although upregulation of JUN in RGCs following axonal injury precedes the DDIT3 accumulation (data not shown), it is possible that ER stress signaling is required to sustain JNK activation in injured RGCs. Therefore, it will be important to determine whether JUN activation is sustained in mice where ER stress and/or UPR activation is altered.

3.7 *Jun* deficiency alters the expression of Bcl2 family members after axonal injury

The Bcl2 family is a major regulator of RGC apoptosis (Bahr, 2000; Nickells et al., 2008) and multiple family members, including BAX, BCL2L1 (BCL-X), BBC3, and BIM, significantly contribute to RGC death after axonal injury (Harder et al., 2012a; Harder and Libby, 2011, 2013; Li et al., 2000; Libby et al., 2005; Semaan et al., 2010). We previously showed that accumulation of the Bcl2 family member BIM in axonally injured RGCs requires JUN (Harder et al., 2012b); however, *Bim* deficiency does not provide robust long term protection as is observed in *Jun* deficient retinas (Fernandes et al., 2012), suggesting JUN controls other genes important for RGC death. JUN is known to regulate the expression of numerous other Bcl2 family members, thus we assessed the requirement of JUN for the expression several Bcl2 family genes that have been implicated in RGC death after CONC. BBC3 is a Bcl2 family prosurvival gene that has been shown to play a role in RGC death after axonal injury (Harder and Libby, 2011, 2013). *Bbc3* expression was not altered in $Jun^{+/+}$ retinas. However, *Bbc3* expression was significantly downregulated in $Jun^{-/-}$ retinas retinas 5 days after crush (Fig 7A; $P = 0.003$). Furthermore, at 5 days after CONC, *Bbc3* expression was attenuated in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas ($P = 0.007$). This change in *Bbc3* expression may have a role in RGC survival in $Jun^{-/-}$ retinas, however, it should be noted that BBC3 appears to play only a minor role in RGC death after axonal injury (Harder and Libby, 2011, 2013).

BAX is required for RGC death after axonal injury (Li et al., 2000; Libby et al., 2005; Semaan et al., 2010). *Bax* expression was significantly increased 5 days after CONC in $Jun^{+/+}$ retinas (Fig 7B; $P = 0.008$). In $Jun^{-/-}$ retinas, *Bax* expression did not significantly change after axonal injury. The expression of the Bcl2 prosurvival family member *Bcl2l1*, which plays a major role in antagonizing RGC death after axonal injury (Harder et al., 2012a), was significantly increased in $Jun^{+/+}$ but not in $Jun^{-/-}$ retinas at both 2 and 5 days after CONC (Fig. 7C). Thus, in wildtype retinas major prodeath and prosurvival members of the Bcl2 family were both upregulated after CONC. Interestingly, both *Bax* and *Bcl2l1* were significantly upregulated in $Jun^{-/-}$ naïve retinas compared to $Jun^{+/+}$ retinas (2.95 and 3.26 fold respectively; $P < 0.05$ for both comparison). Thus, for two of the major Bcl2 family members regulating RGC death after axonal injury, *Bax* and *Bcl2l1*, it appears that in *Jun* deficient mice the basal level of expression of these genes is similar to expression levels wildtype mice after injury. Collectively, our data indicate that transcriptional changes of Bcl2 family members are unlikely to contribute to the near complete prevention of cell death

observed in *Jun* deficient retinas during the normal window of CONC induced RGC death (Fernandes et al., 2012). However, it is possible that *Jun* indirectly regulates the Bcl2 family by altering upstream events that initiate the cell death cascade and eventually funnel down to the Bcl2 family. Alternatively, *Jun* could also regulate the Bcl2 family translationally by altering expression of microRNAs that regulate expression of multiple BH3 only proteins (Kole et al., 2011).

3.8 HRK is not critical for RGC death after axonal injury

Despite numerous molecules being implicated in BAX activation in RGCs following axonal injury, the prodeath Bcl2 family members that activate BAX after axonal injury are not completely defined (Harder and Libby, 2013). The prodeath Bcl2 family member *Hrk* is known to kill neurons in a BAX-dependent manner (Harris and Johnson, 2001) and may have either a redundant or synergistic role with BIM in other neurons (Ghosh et al., 2011; Young et al., 2009). Therefore, *Hrk* may induce RGC death by complementing BIM (a JUN-dependent pathway). *Hrk* is known to be regulated by JUN and is important for injury induced cell death of some neurons (Besirli et al., 2005; Harder and Libby, 2011; Imaizumi et al., 2004; Ma et al., 2007). *Hrk* was significantly upregulated after CONC at both 2 and 5 days in $Jun^{+/+}$ retinas (Fig. 8A; $P < 0.05$ for both comparisons). In $Jun^{-/-}$ retinas *Hrk* was only upregulated 2 days after CONC ($P = 0.011$). At 5 days after CONC *Hrk* expression appeared to return to baseline expression levels in $Jun^{-/-}$ retinas and was significantly attenuated compared to $Jun^{+/+}$ retinas (Fig 8A; $P = 0.036$). Thus, *Hrk* expression was increased after axonal injury and *Jun* may participate in sustaining *Hrk* expression after axonal injury.

The importance HRK in axonal injury-induced RGC death was tested using *Hrk* deficient mice. RGC death was assessed by immunostaining for activated caspase 3 (cleaved CASP3, cCASP3) at the onset of RGC loss and at the peak of RGC death (3 and 5 days post CONC respectively; Harder et al., 2012b) and RGC survival was assessed by anti-βIII tubulin (TUJ1) immunostaining. Following axonal injury, the amount of RGC death did not significantly differ between wildtype and *Hrk* deficient mice. Similar to wildtype mice, in *Hrk* deficient mice substantial numbers of cCASP3+ cells were observed in the RGC layer at 3 and 5 days after injury (Fig 8B). In addition there was no increase in RGC survival at 14 days after injury (a time point when the majority of RGCs have died; Fernandes et al., 2012; Harder et al., 2012b) in *Hrk* deficient mice indicating that *Hrk* is not required for RGC death (Fig 8C). However, this result does not rule out the possibility that *Hrk* contributes to RGC death in this model, particularly given *Hrk*'s known involvement in neuronal cell death pathways involving *Bim* in neurons (Ghosh et al., 2011; Young et al., 2009). Therefore to further test whether *Hrk* plays a role in RGC death following axonal injury, RGC survival was assayed in *Bim Hrk* double knockout mice. Deleting *Hrk* and *Bim* together did not increase survival of RGCs beyond what was observed in *Bim* deficient mice (Fig. 8D). Thus, despite *Hrk* being a prodeath gene that is significantly upregulated early after axonal injury, it does not appear to play an important role role in RGC death.

3.9 Conclusion

While it is clear that JUN expression regulates prodeath pathways in RGCs (Fernandes et al., 2012; Lingor et al., 2005; Yoshida et al., 2002), JUN is also known to control various other aspects of injury response pathways in neurons (Herdegen et al., 1997). To determine the extent of JUN's role in regulating the transcriptional response of RGCs to axonal injury, the expression of genes in multiple injury response pathways were analyzed in wildtype and *Jun* deficient retinas after injury. A representative subset of genes in pathways known to be important in RGC injury response, ER stress, regeneration, and cell death, were analyzed. Furthermore, the expression of JUN dimerization partners, which are known to alter JUN's

transcriptional targets were characterized. While this study only examined a small percent of the genome, it did reveal that JUN directly or indirectly regulated the expression of important genes in all of these pathways. Thus, these data support the hypothesis that JUN is a central hub in controlling RGC viability to axonal injury. It is important to note, that this study does not test whether JUN directly regulates the expression of a gene, merely whether changes in expression of the genes tested are downstream of JUN. Similarly, it is unclear, particularly at the 5 day time point where inter-genotype differences in gene expression are observed, if altered gene expression is because of a direct role of JUN or because *Jun* deficiency prevents RGC death (RGC cell death begins approximately 3 days after CONC). Therefore, follow up studies are required to determine if JUN binds to the promoters of the differentially regulated genes and directly affects their expression.

Sustained JUN transactivation activity has been linked with driving cells towards death. Analysis of the prodeath and prosurvival pathways did not show a transition of JUN targets from prosurvival/proregenerative pathways (2 day time point) to prodeath pathways when death was occurring (5 day time point). Ultimately, it will be important to understand the molecular pathways that JUN controls (either directly or indirectly) after axonal injury and whether these pathways change with time. This may not be an easy task since JUN can control transcription in multiple ways. In addition to direct transcriptional regulation, the expression of the microRNAs mir221/222 have been shown to be regulated by JUN expression (Galardi et al., 2011; Zhou et al., 2012) and these microRNAs are known to regulate genes involved in neuronal death and regeneration (Terasawa et al., 2009; Zhou et al., 2012). However, since JUN appears to control both prosurvival and prodeath pathways, understanding how it controls the transcriptome of a cell may provide important information about how RGCs remain viable when chronically insulted by ocular hypertension as occurs in glaucoma patients.

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Highlights

- JUN appears to regulate axon regeneration pathways in RGCs after axonal injury.
- JUN only has a minor role in regulating Bcl2 family genes in injured RGCs.
- JUN appears to regulate other AP1 family members in axonally injured RGCs.
- ATF3 has a minor prodeath role in RGCs.
- Despite upregulation, HRK is not critical for RGC death after axonal injury.

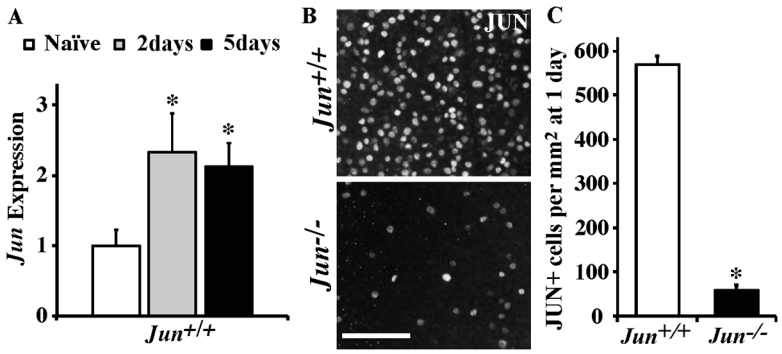

Figure 1. Axonal injury induces *Jun* upregulation

(A) The expression of *Jun* is significantly increased at both 2 and 5 days following CONC in $Jun^{+/+}$ mice (represented as normalized fold expression). (B,C) To determine the recombination efficiency of the *Jun* floxed ($Jun^{fl/fl}$) allele using Six3-cre, the number of JUN positive cells were counted in retinal flat mounts (RGC layer up) after CONC at a time when there is robust expression of JUN and before RGC cell death, 1 day after CONC. In $Jun^{fl/fl}$ $Six3cre^{+}$ ($Jun^{-/-}$) retinas, the number of JUN+ cells was significantly reduced. *, $P<0.05$. Scale bar, 20 μm.

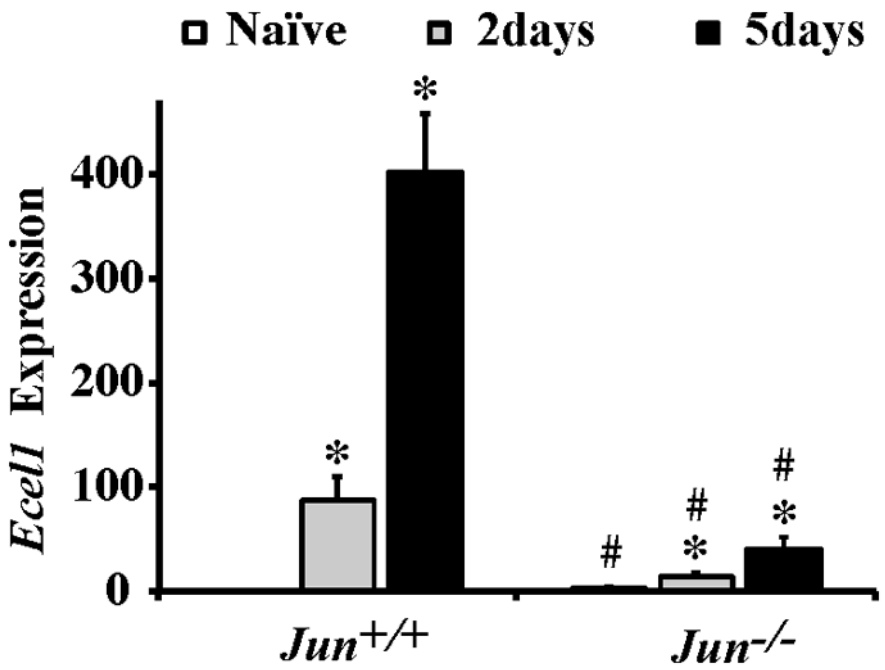

Figure 2. *Jun* deficiency alters the expression of an axon injury response gene, *Ecel1*, after CONC

Realtime PCR analysis for *Ecel1* from retinas of $Jun^{+/+}$ and $Jun^{-/-}$ mice at the indicated time-points following CONC shown as normalized fold change. Expression of the neuronal injury responsive gene *Ecel1* increased progressively in both $Jun^{+/+}$ and $Jun^{-/-}$ animals following CONC (*, intra-genotype comparison, $P < 0.001$). However, comparing the change in gene expression between $Jun^{+/+}$ and $Jun^{-/-}$ mice showed that in *Jun* deficient mice the CONC-induced expression of *Ecel1* was attenuated at both time points (#, inter-genotype comparison, $P < 0.05$). Also, *Ecel1* expression was significantly increased in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas prior to injury.

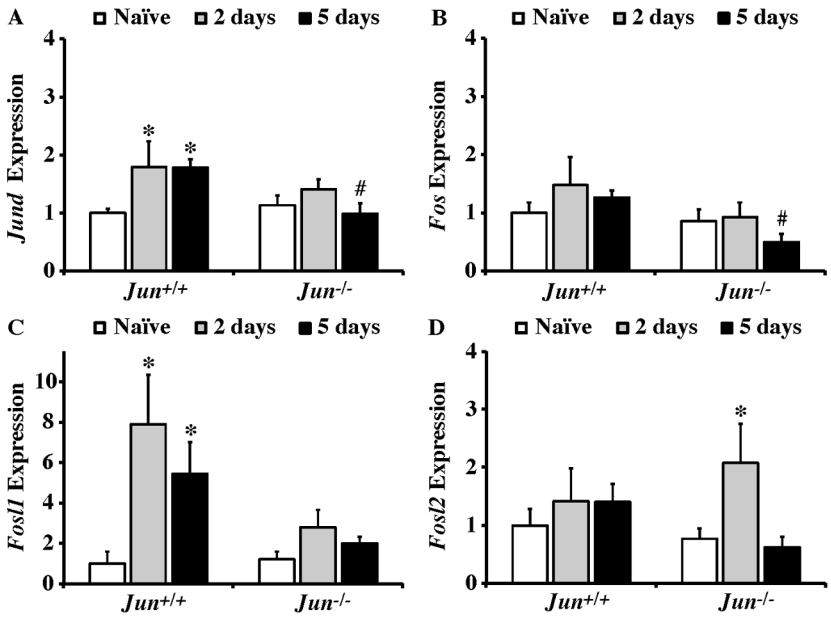

Figure 3. JUN and FOS transcription factor expression after axonal-injury

Realtime PCR analysis of JUN and FOS family members in $Jun^{+/+}$ and $Jun^{-/-}$ mice expressed as normalized fold change. (A) *Jund* expression significantly increased at both 2 and 5 days after CONC in $Jun^{+/+}$ mice (*, intra-genotype comparison, $P < 0.05$) and did not change in $Jun^{-/-}$ mice. Comparing expression level changes between genotypes showed that *Jund* expression was significantly attenuated in $Jun^{-/-}$ retinas compared to wildtype retinas at 5 days after CONC (#, inter-genotype comparison, $P < 0.05$) (B) *Fos* expression remained unchanged in $Jun^{+/+}$ and $Jun^{-/-}$ mice at both time points examined, however, *Fos* expression was attenuated in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas 5 days after CONC. (C) A significant increase in *Fosl1* expression was observed in $Jun^{+/+}$ mice at 2 and 5 days after CONC. *Fosl1* expression was not significantly changed at either time point examined in $Jun^{-/-}$ retinas. (D) *Fosl2* expression did not change after CONC in wildtype mice at 2 and 5 days compared to naïve retinas. However, in *Jun* deficient retinas *Fosl2* expression was significantly increased 2 days after CONC. *, $P < 0.05$ comparing 2 or 5 day time points to Naïve retinas of same genotype (Intra-genotype); #, $P < 0.05$, comparing same time points across genotypes (Inter-genotype).

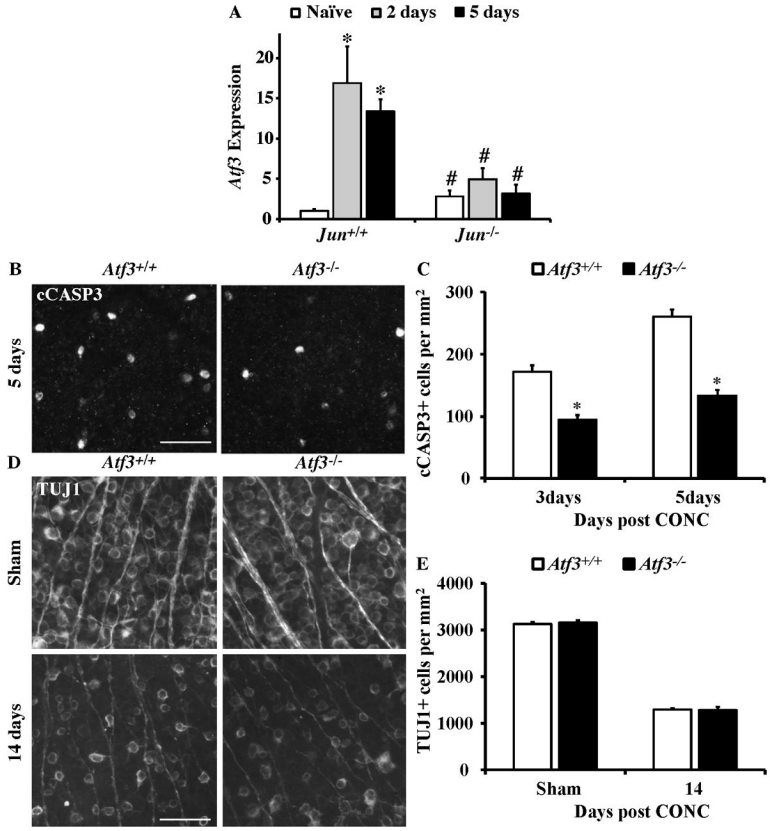

Figure 4. The *Jun* target, *Atf3*, suppresses RGC death following CONC

(A) The expression of *Atf3* significantly increased after CONC in $Jun^{+/+}$ retinas at 2 and 5 days (shown as normalized fold change; *, intra-genotype comparison, $P < 0.05$). *Atf3* expression was not significantly increased after CONC in $Jun^{-/-}$ retinas at 2 and 5 days. In fact, comparing the change in gene expression of *Atf3* between $Jun^{+/+}$ and $Jun^{-/-}$ mice showed that *Atf3* expression was significantly attenuated in $Jun^{-/-}$ at both time points after CONC (#, inter-genotype comparison, $P < 0.05$). Also, *Atf3* expression was significantly increased in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas prior to injury. (B) Representative images of cleaved caspase-3 (cCASP3) labeled, dying RGCs in $Atf3^{+/+}$ and $Atf3^{-/-}$ retinal whole mounts. (C) The number of cCASP3 labeled cells was significantly reduced in $Atf3^{-/-}$ retinas at both 3 days and 5 days after CONC (*, $P < 0.05$ for both time points; $N \geq 5$ for both genotypes and time points). (D) Representative images of anti-βIII tubulin (TUJ1) positive RGCs in retinal whole mounts from sham injured and CONC injured eyes 14 days following the insult. (E) Despite the small decrease in cell death in *Atf3* deficient mice, *Atf3* deficiency did not increase the number of surviving RGCs 14 days after CONC ($P = 0.82$, $N = 6$ for genotypes). Scalebar, 25 μm.

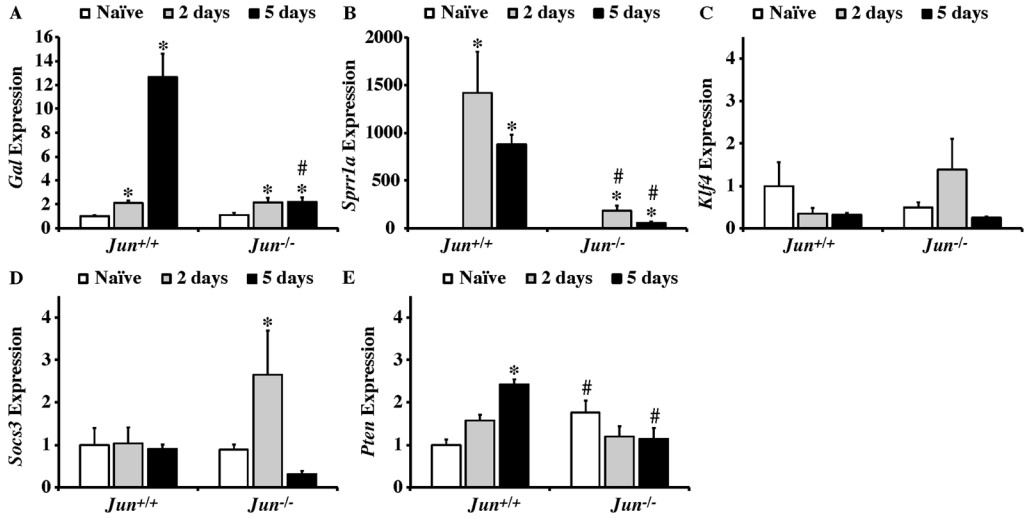

Figure 5. JUN contributes to transcriptional control of RGC regeneration potential

Realtime PCR analysis of genes involved in axon regeneration in $Jun^{+/+}$ and $Jun^{-/-}$ mice shown as normalized fold change. (A,B) The expression of both *Gal* and *Sprr1a* positively correlate with regeneration and both significantly increased in $Jun^{+/+}$ and $Jun^{-/-}$ mice after CONC (*, intra-genotype comparisons, $P < 0.05$). However, the increase in expression of both of these genes was significantly attenuated at least one time point in the *Jun* deficient mice (#, inter-genotype comparison, $P < 0.05$). (C–E) Genes that are known to suppress regeneration in injured RGCs were also examined. (C) The expression of *Klf4* was not altered following CONC in either genotype. (D) *Socs3* expression was not altered transcriptionally at 2 days or 5 days following CONC in $Jun^{+/+}$ retinas but was significantly increased in $Jun^{-/-}$ retinas 2 days post CONC. (E) *Pten* was significantly upregulated at 5 days following CONC in $Jun^{+/+}$ retinas. *Pten* expression did not change in $Jun^{+/+}$ retinas, however, *Pten* expression was significantly attenuated at 5 days in $Jun^{-/-}$ deficient retinas compared to wildtype. Also, *Pten* expression was significantly increased in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas prior to injury. *, $P < 0.05$ comparing 2 or 5 day time points to Naïve retinas of same genotype (Intra-genotype); #, $P < 0.05$, comparing same time points across genotypes (Inter-genotype).

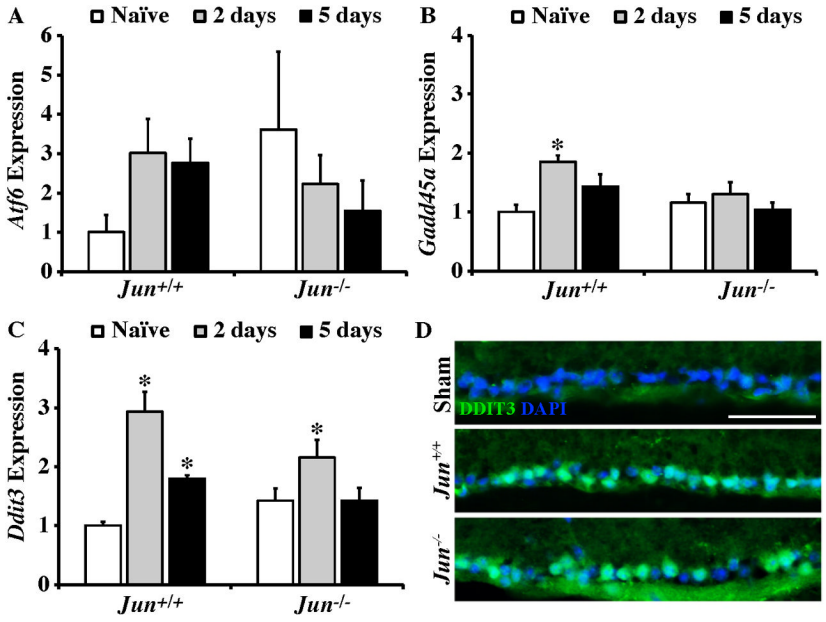

Figure 6. After axonal injury ER stress occurs in the absence of *Jun*

(A–C) Realtime PCR analysis of genes involved in ER stress response in $Jun^{+/+}$ and $Jun^{-/-}$ mice shown as normalized fold change. (A) The expression of ER stress sensor *Atf6* was not altered following CONC in either $Jun^{+/+}$ or $Jun^{-/-}$ retinas. (B) The expression of the ER stress marker *Gadd45a* significantly increased at 2 days following CONC in $Jun^{+/+}$ retinas (*, intra-genotype comparisons, $P < 0.05$). No other changes in *Gadd45a* expression were detected. (C) The expression of *Ddit3,* an ER stress target gene, significantly increased in $Jun^{+/+}$ at both 2 and 5 days after CONC. In $Jun^{-/-}$ retinas *Ddit3* expression was significantly increased only at 2 days following CONC. (D) Representative immunofluorescence staining for DDIT3 3 days following CONC confirms induction of DDIT3 in both $Jun^{+/+}$ and $Jun^{-/-}$ retinas (DDIT3, green; DAPI, blue, was used to stain nuclei; the experiment was performed on 3 different mice for each genotype and condition). *, $P < 0.05$ comparing 2 or 5 day time points to naïve retinas of same genotype (Intra-genotype).

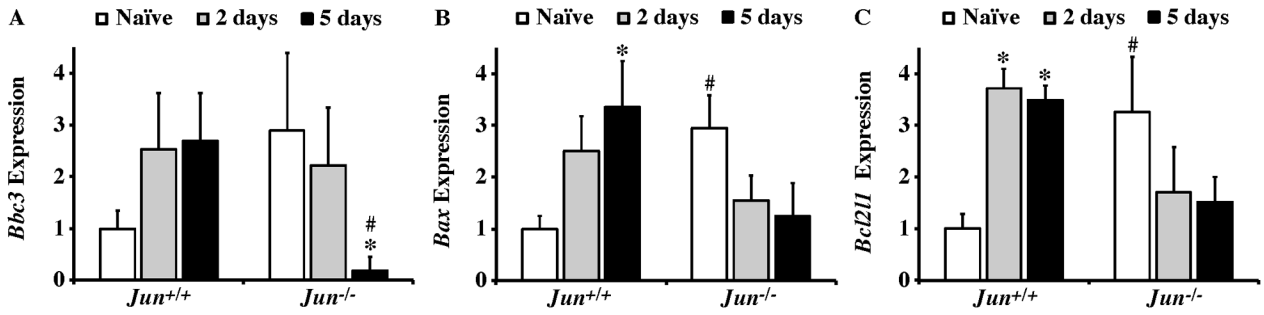

Figure 7. Bcl2 family expression after axonal-injury

Results of realtime PCR analysis showing the normalized fold expression of a subset of Bcl2 family members that have been implicated in axonal injury induced RGC death in $Jun^{+/+}$ and $Jun^{-/-}$ mice. (A) *Bbc3* expression was not significantly altered after CONC in either genotype, though the expression was significantly attenuated in $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ retinas 5 days after CONC (#, inter-genotype comparison, $P < 0.05$). (B) *Bax* was significantly increased 5 days after CONC in $Jun^{+/+}$ (*, intra-genotype comparisons, $P < 0.05$). There were no differences in Bax expression in $Jun^{-/-}$ animals after CONC. Also, *Bax* levels did not differ between $Jun^{+/+}$ and $Jun^{-/-}$ retinas after CONC, but *Bax* levels were significantly higher in naïve $Jun^{-/-}$ retinas compared to $Jun^{+/+}$ naïve retinas. (C) Interestingly, *Bcl2l1* had a similar expression pattern to *Bax* after CONC even though they have opposite effects on the probability of a cell undergoing apoptosis. The expression of *Bcl2l1* was significantly increased after CONC in $Jun^{+/+}$ but not $Jun^{-/-}$ retinas. Though as with *Bax*, *Bcl2l1* expression is significantly higher to begin with in $Jun^{-/-}$ retinas compared to controls. *, $P < 0.05$ comparing 2 or 5 day time points to Naïve retinas of same genotype (intra-genotype); #, $P < 0.05$, comparing same time points across genotypes (inter-genotype).

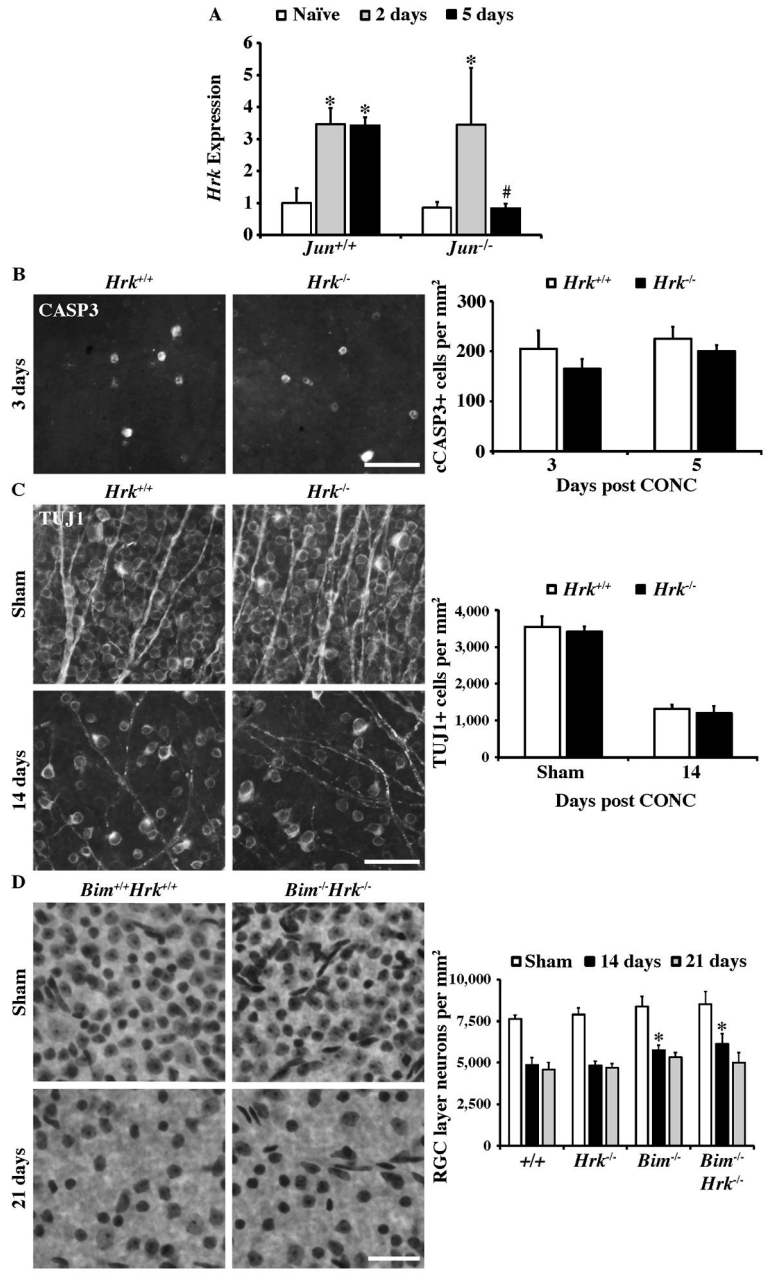

Figure 8. The proapoptotic gene *Hrk* is not required for RGC death after axonal injury

(A) Expression of *Hrk* (a proapoptotic gene previously identified as a target of *Jun*) was significantly increased at both 2 days and 5 days following CONC (*, intra-genotype comparisons, $P < 0.05$). However, in $Jun^{-/-}$ retinas, *Hrk* expression was only significantly increased at 2 days following CONC and appeared to return to baseline levels of expression by 5 days after CONC. In fact, *Hrk* expression was significantly attenuated in $Jun^{-/-}$ retinas 5 days after CONC compared to $Jun^{-/-}$ retinas (#, inter-genotype comparison, $P < 0.05$). (B) Representative images of cleaved caspase 3 positive cells in retinal whole mounts from $Hrk^{+/+}$ and $Hrk^{-/-}$ animals. At both 3 days and 5 days following CONC, the number of cleaved caspase-3 positive cells was unchanged by *Hrk* deficiency (N=4 for each genotype and time point; $P \geq 0.3$). (C) Counts of anti-βIII tubulin (TUJ1) labeled RGCs in retinal whole mounts from sham injured and CONC injured eyes. *Hrk* deficiency does not alter the

number of RGCs surviving 14 days following CONC (N=4 for each genotype; P = 0.37). (D) Counts of Nissl stained neurons in retinal whole mounts confirm the previous reported short term protection observed in *Bim* deficient mice compared to wildtype mice after CONC (*, P<0.01; Harder et al., 2012b). Combined deficiency of *Bim* and *Hrk* did not enhance RGC survival following CONC in comparison to single deficiency of *Bim* alone (P > 0.28 for both time points). Note, approximately 50% of RGC layer neurons are amacrine cells in mice (Jeon et al., 1998; Li et al., 1999; Li et al., 2007; Quigley et al., 2011) and do not die after CONC injury (Kielczewski et al., 2005), therefore a loss of 50% of RGC layer neurons reflects complete RGC loss. At least 4 retinas were examined at each time point for each genotype for the Nissl counts. Scale bar, 25 μm.

Table 1

Primers used for qPCR

Gene Name	Common Name	Forward Primer	Reverse Primer
Atf3	*Atf3*	CTGGAGTCAGTTACCGTCAACA	CAGGCACTCTGTCTTCTCCTTT
Atf6	*Atf6*	TGGGAGTGAGCTGCAAGTGT	ATAAGGGGGAACCGAGGAG
Bax	*Bax*	GGAGATGAACTGGACAGCAATATG	GATCAGCTCGGGCACTTTAG
Bcl2l1	*Bcl-x*	GGAGAGCGTTCAGTGATCTAACAT	ACTTGCAATCCGACTCACCAATA
Ddit3	*Chop*	CTGCCTTTCACCTTGGAGAC	CGTTTCCTGGGGATGAGATA
Ecel1	*Dine*	ATGCCTACTATCTGCCCAACAA	GTCATAGCCATGGGTCAGTTC
Fos	*cFos*	CCTGTGAGCAGTCAGAGAAGG	TGGAAGAGGTGAGGACTGG
Fosl1	*Fra1*	GGAGACCGACAAGTTGGAGGAT	TGCAGTGCTTCCGGTTCAA
Fosl2	*Fra2*	TGCAGTCCTTGCGCGGTACGGG	GACAAGGTTTGAAGTGCCGGGAGTG
Gadd45a	*Gadd45α*	GAAGAAGGAAGCTGCGAGAAAA	CCTGGCCATCCTAAATTAGCAGT
Gad1	*Gad67*	TCTTCCACTCCTTCGCCTGC	GGAGAAGTCGGTCTCTGTGC
Gal	*Gal*	CAGTTTCTTGCACCTTAAAGAGG	GGTCTCAGGACTTCTCTAGGTCTTC
Gapdh	*Gapdh*	CAGGTTGTCTCCTGCGACTT	ATGTAGGCCATGAGGTCCAC
Hrk	*Dp5*	AATTGTAAAGAGCTGATGGTGGA	AGTCTCAGAGTTCACATCGCAAG
Jun	*cJun*	CTGATCATCCAGTCCAGCAA	GACACTGGGAAGCGTGTTCT
Jund	*JunD*	GTCAAGACCCTCAAAAGCCAGA	TGTTGACGTGGCTGAGGACTT
Klf4	*Klf4*	GATTGCAAGTTCCGCCACTGAACA	AATTTCCACCCACAGCCGT
Pten	*Pten*	AATTCCCAGTCAGAGGCGCTATGT	GATTGCAAGTTCCGCCACTGAACA
Socs3	*Socs3*	GTTGAGCGTCAAGACCCAGT	ACAGTCGAAGCGGGGAACT
Sprr1a	*Sprr1a*	CCTGCTCTTCTCTGAGTATTAGGAC	GCTGCTTCACCTGCTGCT

Table 2

Delta Ct values and comparisons.

	Jun$^{+/+}$					*Jun*$^{-/-}$					*Jun*$^{+/+}$ vs *Jun*$^{-/-}$		
	ΔC_T value ± SD (n)			Intra-geno P		ΔC_T value ± SD (n)			Intra-geno P		Inter-Geno P		
Gene	**Naïve**	**2 day**	**5 day**	**N vs 2**	**N vs 5**	**Naïve**	**2 day**	**5 day**	**N vs 2**	**N vs 5**	**N vs N**	**2 vs 2**	**5 vs 5**
Atf3	8.4 ± 1.1 (11)	4.3 ± 1.0 (7)	4.6 ± 0.5 (8)	**0.001**	**0.001**	6.9 ± 1.1 (9)	6.0 ± 1.3 (11)	6.7 ± 1.3 (7)	0.213	0.999	**0.011**	**0.005**	**0.002**
Atf6	7.2 ± 1.9 (9)	5.6 ± 1.1 (7)	5.7 ± 0.9 (8)	0.129	0.153	5.3 ± 2.3 (9)	6.0 ± 1.5 (11)	6.5 ± 1.9 (7)	0.723	0.301	0.069	0.999	0.991
Bax	7.6 ± 1.1 (10)	6.2 ± 1.0 (7)	5.8 ± 1.1 (8)	0.065	**0.008**	6.0 ± 0.9 (9)	6.9 ± 1.4 (10)	7.2 ± 1.7 (6)	0.208	0.122	**0.024**	0.757	0.109
Bbc3	10.1 ± 1.6 (11)	8.7 ± 1.6 (7)	8.6 ± 1.4 (8)	0.428	0.334	8.5 ± 2.1 (9)	8.9 ± 2.2 (10)	12.5 ± 4.1 (6)	0.999	**0.003**	0.385	0.999	**0.007**
Bcl2l1	7.8 ± 1.3 (11)	5.9 ± 0.4 (7)	6.0 ± 0.3 (8)	**0.013**	**0.014**	6.1 ± 1.3 (8)	7.0 ± 2.2 (10)	7.2 ± 1.2 (7)	0.323	0.258	**0.032**	0.321	0.299
Ddit3	3.7 ± 0.3 (11)	2.1 ± 0.4 (7)	2.8 ± 0.1 (8)	**0.001**	**0.001**	3.1 ± 0.6 (9)	2.5 ± 0.6 (10)	3.1 ± 0.6 (7)	**0.022**	0.999	0.081	0.224	0.610
Ecel1	12.1 ± 1.3 (11)	5.7 ± 1.0 (7)	3.5 ± 0.6 (8)	**0.001**	**0.001**	10.7 ± 1.2 (9)	8.3 ± 1.4 (11)	6.8 ± 1.0 (7)	**0.001**	**0.001**	**0.016**	**0.001**	**0.001**
Fos	5.7 ± 0.9 (11)	5.1 ± 1.2 (6)	5.3 ± 0.4 (8)	0.550	0.962	5.9 ±1.0 (8)	5.8 ± 1.2 (10)	6.7 ± 1.1 (7)	0.999	0.254	0.999	0.580	**0.033**
Fosl1	14.6 ± 2.2 (7)	11.6 ± 1.1 (6)	12.1 ± 1.2 (8)	**0.001**	**0.002**	14.3 ± 1.1 (6)	13.1 ± 1.3 (8)	13.6 ± 0.6 (7)	0.190	0.650	0.999	0.130	0.122
Fosl2	9.1 ± 1.4 (11)	8.5 ± 1.4 (6)	8.5 ± 0.9 (8)	0.850	0.784	9.4 ± 1.0 (8)	8.0 ± 1.5 (10)	9.7 ± 1.1 (7)	**0.035**	0.999	0.999	0.999	0.221
Gadd45a	3.1 ± 0.6 (11)	2.2 ± 0.2 (7)	2.5 ± 0.6 (8)	**0.003**	0.091	2.8 ± 0.5 (9)	2.7 ± 0.7 (10)	3.0 ± 0.4 (7)	0.999	0.999	0.999	0.192	0.324
Gal	9.8 ± 0.6 (11)	8.8 ± 0.4 (6)	6.2 ± 0.6 (7)	**0.004**	**0.001**	9.7 ± 0.8 (8)	8.7 ± 0.8 (10)	8.7 ± 0.6 (7)	**0.005**	**0.008**	0.999	0.999	**0.001**
Hrk	11.2 ± 2.2 (11)	9.4 ± 0.6 (7)	9.4 ± 0.3 (8)	**0.035**	**0.028**	11.4 ± 0.8 (9)	9.4 ± 2.3 (10)	11.5 ± 0.7 (7)	**0.011**	0.998	0.999	0.999	**0.036**
Jund	4.7 ± 0.4 (11)	3.8 ± 0.9 (6)	3.8 ± 0.3 (8)	**0.009**	**0.004**	4.5 ± 0.6 (8)	4.2 ± 0.6 (10)	4.7 ± 0.75 (7)	0.471	0.999	0.999	0.711	**0.016**
Klf4	6.2 ± 2.5 (11)	7.7 ± 1.5 (7)	7.8 ± 0.5 (8)	0.168	0.110	7.2 ± 1.1 (9)	5.7 ± 2.4 (11)	8.2 ± 0.5 (6)	0.134	0.623	0.615	0.075	0.999
Pten	2.4 ± 0.6 (11)	1.8 ± 0.3 (7)	1.1 ± 0.2 (8)	0.096	**0.001**	1.6 ± 0.7 (9)	2.2 ± 0.9 (10)	2.2 ± 0.9 (7)	0.140	0.127	**0.008**	0.237	**0.003**
Socs3	7.6 ± 1.9 (11)	7.6 ± 1.4 (7)	7.8 ± 0.5 (8)	0.999	0.999	7.8 ± 0.6 (9)	6.2 ± 1.8 (11)	9.4 ± 1.0 (7)	**0.029**	0.065	0.999	0.143	0.105
Sprr1a	16.4 ± 1.1 (8)	6.0 ± 1.1 (6)	6.7 ± 0.4 (7)	**0.001**	**0.001**	15.4 ± 1.1 (7)	8.9 ± 1.4 (10)	10.5 ± 0.8 (7)	**0.001**	**0.001**	0.238	**0.001**	**0.001**

N, Naïve; n, number of samples; Bold, P<0.05; P values listed as 0.001 are actually ≤0.001 and those listed as 0.999 are actually ≥0.999.