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The Bcl-2 family member BIM has multiple glaucoma-relevant functions in DBA/2J mice

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Axonal insult induces retinal ganglion cell (RGC) death through a BAX-dependent process. The pro-apoptotic Bcl-2 family member BIM is known to induce BAX activation. BIM expression increased in RGCs after axonal injury and its induction was dependent on JUN. Partial and complete *Bim* deficiency delayed RGC death after mechanical optic nerve injury. However, in a mouse model of glaucoma, DBA/2J mice, *Bim* deficiency did not prevent RGC death in eyes with severe optic nerve degeneration. In a subset of DBA/2J mice, *Bim* deficiency altered disease progression resulting in less severe nerve damage. *Bim* deficient mice exhibited altered optic nerve head morphology and significantly lessened intraocular pressure elevation. Thus, a decrease in axonal degeneration in *Bim* deficient DBA/2J mice may not be caused by a direct role of *Bim* in RGCs. These data suggest that BIM has multiple roles in glaucoma pathophysiology, potentially affecting susceptibility to glaucoma through several mechanisms.

BAX is a critical mediator of neuronal cell death. In the retina, *Bax* deficiency protects against developmental apoptosis of many retinal cells, including, retinal ganglion cells (RGCs)[1-5]. BAX is also an important mediator of RGC loss after axonal insult. BAX is up-regulated in response to optic nerve injury[6] and its suppression rescues RGC somas from death after mechanical optic nerve injury[2,3,7]. *Bax* was also shown to be required for RGC death in an animal model of glaucoma[3], a common neurodegeneration where elevated intraocular pressure (IOP) ultimately leads to an RGC axonal insult[8-11]. Importantly, other apoptotic or non-apoptotic pathways do not circumvent the long-term protection provided by *Bax* deficiency[3,7]. Thus, BAX is major mediator of RGC death in disease. The funneling of cell death pathway(s) to a central point in glaucoma–BAX activation–provides a powerful starting point for unraveling the complex process that determines RGC survival in glaucoma.

BAX is a member of the Bcl-2 family of proteins[12]. Direct interactions between different family members (both pro-survival and pro-death family members) determine the likelihood of BAX activation in a cell. BH3-only proteins are pro-death Bcl-2 family members that trigger cell death through BAX activation. After injury, activation of different BH3-only proteins occurs in an insult and context-specific manner[12]. For example, BBC3 is required for BAX activation in developing RGCs, but does not play a primary role in regulating RGC death after axonal injury[1]. In fact, it is unknown which BH3-only proteins, if any, are required for RGC death after axonal injury and in glaucoma. The BH3-only protein BIM is a good candidate for activating BAX in axonally-injured RGCs. After optic nerve injury, *Bim* transcription increases in the retina and BIM is detected in the RGC layer[6,13,14]. In addition, an *ex vivo* study demonstrates a pro-apoptotic function for BIM early after axonal insult[13]. However, it is unknown if BIM is required for RGC death after axonal injury *in vivo* or if its role varies based on the type of axonal insult. The importance of BIM was tested *in vivo* after optic nerve crush and in glaucoma and, unlike BAX, BIM is not required for RGC death in these models. Surprisingly, BIM was found to have multiple functions, both intrinsic and extrinsic to RGCs, relevant to glaucoma pathophysiology.

Results

BIM plays several roles in retinal morphogenesis. BIM is expressed in the developing retina[15], suggesting that it may regulate normal retinal developmental cell death. As previously reported[1,16,17], *Bim* deficiency does not grossly affect retinal neuronal patterning (Fig. 1A). BIM is known to be critical for retinal vasculature remodeling and deficiency in *Bim* does increase the amount of retinal vasculature ([17]and data not shown). In

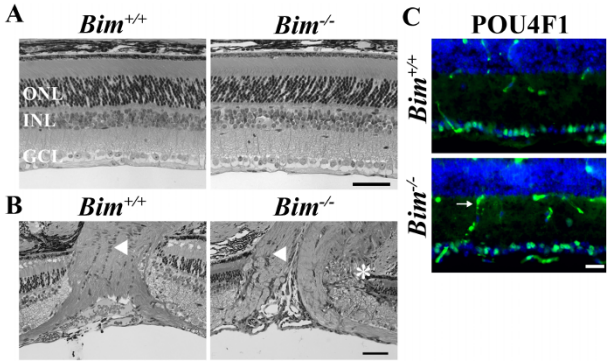

Figure 1 | *Bim* deficiency affects retinal development. (A) Retinal sections from *Bim*$^{+/+}$ and *Bim*$^{-/-}$ mice confirms previous reports[1,17] that *Bim* deficiency did not alter the gross organization of number of retinal neurons. (B) *Bim* deficiency caused dysmorphogenesis of the optic nerve. In *Bim* deficient mice the retinal-optic nerve head border is abnormal, with apparent retinal neuronal layers entering the optic nerve head (asterisk). Also the normal arrangement of glia cell bodies does not appear to be present in the area of the lamina cribrosa (arrowhead). (C) However, optic nerve head morphology changes did not affect the number of RGCs, as judged by POU4F1 (BRN3A, green) expression, which is specifically expressed in 80% of RGCs in the retina[19]. Note, the secondary antibody also detects retinal vasculature (arrow). DAPI, blue; ONL, outer nuclear layer; INL inner nuclear layer, GCL, ganglion cell layer; scale bar, **A**,**B**=50 μm, **C**= 25 μm.

contrast to the retina, the optic nerve head of B6.*Bim*$^{-/-}$ mice had significant morphological abnormalities (Fig. 1B). In 5 out of 5 B6.*Bim* deficient retinas there were clear abnormalities in the retinal-optic nerve head boundary. Also, the arrangement of glial cells just behind the retina in the optic nerve, the glial lamina cribrosa in mice, appeared less well organized than in wild-type mice. Thus, *Bim* deficiency alters morphology of the retina in several ways that could be important in retinal disease, increasing retinal vasculature and causing optic nerve head dysmorphogenesis.

Using RGC layer thickness measurements Doonan and colleagues[16] reported that *Bim* deficiency delayed developmental RGC death, but had no effect on the final number of RGCs. There are several mouse mutants where there is a small, but significant increase in RGC number (e.g.[1,18]). It is unlikely that this level of increase would be detectable by measuring RGC layer thickness, which is generally a single layer of cells thick. Therefore to determine BIM's impact on the final number of RGCs in the mouse retina, POU4F1 (BRN3A) positive cells were counted in sections from the central retina. *Bim* deficiency did not alter the number of RGCs surviving in the adult (Fig 1C; as judged by POU4F1 expression, which is specifically expressed in 80% of RGCs in the retina[19]; POU4F1+ cells per mm ± SEM: *Bim*$^{+/+}$ 58±5, *Bim*$^{+/-}$ 61±4, *Bim*$^{-/-}$ 56±3; P=0.69; n=4 for each genotype).

BIM is expressed in RGCs after axonal injury. RGC death after axonal injury is an apoptotic and BAX-dependent process[1-3,7]. Unlike in BAX-dependent RGC developmental cell death[1], no specific BH3-only protein is known to be similarly required after axonal injury. BIM is a primary candidate to activate BAX after RGC axonal injury because the loss of BIM protects RGCs in retinal explant cultures for up to four days[13]. Therefore, BIM expression was examined following mechanical axonal injury, controlled optic nerve crush (CONC) and in glaucomatous DBA/2J mice. In unmanipulated retinas BIM was not detected in the RGC layer (Fig. 2). Using mice that express CFP predominately in RGCs[20,21], BIM was shown to be expressed in RGCs by the time RGCs begin to die after CONC. BIM was also expressed in RGCs in glaucomatous DBA/2J mice (10 months of age; Fig. 2). Thus, the expression pattern of BIM is consistent with a role in axonal-injury induced RGC death.

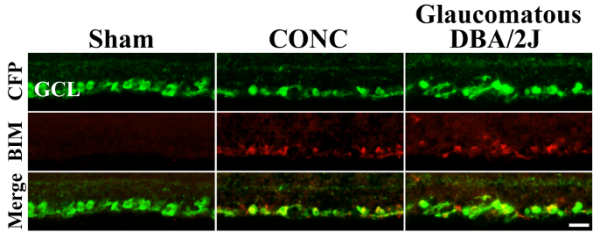

Figure 2 | BIM is expressed in RGCs after axonal injury. In young uninjured RGCs (Sham), BIM is not detected in RGCs. RGCs are marked genetically with CFP using B6.Thy1-CFP mice. In these mice 95% of RGCs express CFP and only a small percent of the other type of neurons in the ganglion cell layer, displaced amacrine cells, express the transgene[20,21]. At the beginning of RGC death after CONC (3 days after injury) BIM colocalizes with the vast majority of CFP+ cells. BIM continues to be expressed in the RGC layer at 5 days and 7 days indicating it is expressed throughout the time when RGC death peaks. In glaucomatous DBA/2J mice, BIM is also expressed in RGCs (RGCs are also marked with the Thy1-CFP transgene; backcrossed into DBA/2J mice >20 times). BIM expression was not detected in all sections, but was present in RGCs in 4 out of 6 retinas examined. This expression is consistent with the asynchrony of DBA/2J glaucoma, with only some 10 month old animals undergoing active RGC loss[29]. Also, in diseased retinas, BIM was not detected in every section likely reflecting the naturally-occurring sectorial pattern of RGC loss[8]. In addition, BIM expression was absent in 10 month old D2.*Gpnmb* mice (data not shown; D2.*Gpnmb* mice are a control strain of D2 mice that are wild-type for one of the genes that causes the iris disease and do not develop elevated IOP[40,44]). The lack of BIM expression in D2.*Gpnmb* RGCs indicates that change in BIM expression in DBA/2J RGCs is caused by the glaucomatous insult and not age or genetic background. These data show that BIM is expressed in axonally injured RGCs. Scale bar, 25 μm.

***Bim* deficiency reduces CASP3 activation after mechanical optic nerve injury.** McKernan et al.[13] showed that *Bim* deficiency could protect RGCs in explant cultures for at least 4 days (the longest time in culture examined). Explanting a retina likely involves numerous retinal injuries, but an important one for RGCs is axotomy. However only a small percentage of RGCs die 4 days after axonal injury *in vivo* and the loss of RGCs can continue for several weeks[1,22,23]; so whether BIM is critical for RGC death after optic nerve injury is unclear. If BIM is required for BAX-dependent RGC death, then axonally injured RGCs in *Bim* nulls should not undergo apoptosis and survive for months as observed in *Bax* deficient mice[2,3,7]. To determine the role of BIM in RGC death after axonal injury *in vivo*, CONC was performed on *Bim*$^{+/+}$, *Bim*$^{+/-}$ and *Bim*$^{-/-}$ mice (Fig 3). Counts of activated (cleaved) caspase 3 positive cells (cCASP3+) in the RGC layer of retinal flat mounts were used to identify apoptotic RGCs. At the beginning of RGC death after CONC, 3 days following injury, the complete absence of BIM drastically lessens cell death (Fig. 3; P<0.001). By 5 days, cCASP3 was observed in *Bim* null retinas, although the number of cCASP3+ cells was still significantly less than in wild-type retinas (P<0.001). Interestingly, at 14 days after CONC, *Bim*$^{-/-}$ mice have significantly more dying cells than *Bim*$^{+/+}$ suggesting that *Bim* deficiency only delays cell death (P=0.01). *Bim* transcription is known to be involved in its induction after cell death stimuli and heterozygosity for a *Bim* null allele can reduce death in neurons after injury[24,25]. Consistent with this effect, the loss of one allele of *Bim* also delays CASP3 activation both 3 and 5 days after CONC (Fig. 3, P<0.001). Thus, BIM is an important early pro-apoptotic factor in RGC death after axonal injury.

***Bim* deficiency delays RGC death after mechanical optic nerve injury.** Nissl stained RGC layer neurons were counted at 14 and 35 days after CONC in order to assess long term RGC survival in *Bim*$^{+/+}$

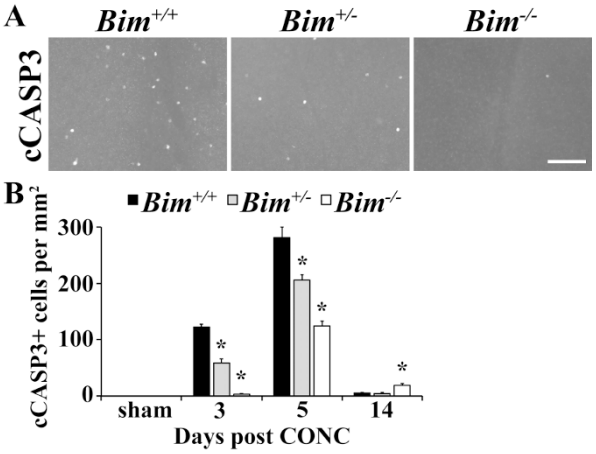

Figure 3 | ***Bim* deficiency delays RGC death after CONC.** (A) RGC death, as judged by presence of cleaved caspase 3 (cCASP3+), was clearly decreased in both *Bim*$^{+/-}$ and *Bim*$^{-/-}$ mice 3 days after injury (the onset of cell death). (B) Quantification of cCASP3+ cells shows that there is a significant decrease (*, $P<0.01$) in cell death in both *Bim*$^{+/-}$ and *Bim*$^{-/-}$ mice at 3 and 5 days after injury. Interestingly, 14 days after injury, when about 50% of RGCs are lost, cell death is increased in the *Bim*$^{-/-}$ mice compared to the other genotypes (cCASP3+ cells per mm^2: *Bim*$^{+/+}$ 6±1; *Bim*$^{-/-}$ 19±3; $P=0.01$). N≥6 for each genotype and time point; scale bar, 100 μm.

and *Bim*$^{-/-}$ mice (Fig. 4A,B). 14 days post injury all genotypes had lost a significant number of RGC layer neurons compared unmanipulated retinas (Fig 4B, $P<0.001$). Thus, BIM is not required for RGC death after axonal injury. However, *Bim*$^{-/-}$ retinas had significantly more surviving RGC layer neurons than injured wild-type retinas at 14 days post injury (Fig 4B; $P<0.001$). The protection of RGCs in *Bim*$^{-/-}$ mice at 14 days after injury was confirmed by counting RGCs using TUJ1[26] as a marker (Fig. 4C; $P<0.001$). By 35 days, when RGC death after CONC is nearly complete, the number of surviving RGC layer neurons is similar between *Bim*$^{+/+}$ and *Bim*$^{-/-}$ retinas (Fig. 4B). Although BIM plays an early role in axonal injury induced death of RGCs, the loss of RGCs still occurs in the absence of BIM.

JUN regulates BIM expression in RGCs after axonal injury. JUN has been shown to be a major regulator of RGC death after axonal injury[27] and is known to regulate *Bim*[28]. Prior to cell death, RGCs expressing JUN display perinuclear BIM expression (Fig 5A). To determine if JUN controls BIM expression in RGCs, BIM expression was assessed in *Jun* deficient retinas (*Jun*$^{fl/fl}$ Six3-cre^{+}) after CONC. In the absence of *Jun*, BIM could not be detected in RGCs (Fig. 5B) at 3 days or 7 days after injury, suggesting that in RGCs, *Bim* is a downstream target of activated JUN.

***Bim* deficiency alters glaucoma relevant morphology and ocular hypertension in DBA/2J mice.** DBA/2J mice develop an iris disease that leads to ocular hypertension and RGC death in most mice by one year of age[29-31]. To test the importance of BIM in glaucomatous neurodegeneration a null allele of *Bim* was backcrossed for 10 generations into the DBA/2J genetic background (D2.*Bim*$^{-}$; all mice used for the glaucoma studies, including D2.*Bim*$^{+/?}$ and D2.*Bim*$^{-/-}$ mice, were from this segregating line). Since the optic nerve head is thought to be a key initial site of injury[8-11], alteration in its morphology could have an effect on glaucoma progression. As with B6.*Bim*$^{-/-}$ mice (Fig. 1B), ocular nerve head morphology was abnormal in young D2.*Bim*$^{-/-}$ eyes (3-5 months of age; prior to pigment disease and IOP elevation). Similar to B6.*Bim*$^{-/-}$ optic nerve heads, in all 5 D2.*Bim*$^{-/-}$ eyes examined there was a disruption of the border between the retina and the optic nerve (Fig 6A). Also, the cellular arrangement of the glial lamina region appeared disrupted. Thus, the loss of BIM during development may alter optic nerve head dynamics during a glaucomatous insult.

DBA/2J mice develop an iris pigment dispersion syndrome with age that eventually causes ocular hypertension. Consistent with other studies using DBA/2J mice, prior to the iris disease D2.*Bim*$^{+/+}$ mice had an average IOP of 13.0±0.3 mmHg (4 months of age; Fig 6B). The IOP of D2.*Bim*$^{+/-}$ mice were not significantly different than for wild-type DBA/2J mice at any time point examined, and D2.*Bim*$^{+/+}$ mice and D2.*Bim*$^{+/-}$ mice were used as controls (referred to as D2.*Bim*$^{+/?}$ mice). The IOP of D2.*Bim*$^{+/?}$ mice increased with age, similar to previous reports. IOP was significantly elevated at 9, 10.5 and 12 months of age (Fig. 6B; 9 months, 18.9±0.7 mmHg; 10.5 months, 20.4±0.7 mmHg; 12 months, 20.9±0.9 mmHg; $P<0.001$ for all genotypes). Prior to the onset of iris disease, D2.*Bim*$^{-/-}$ mice had normal IOPs (13.3±0.6 mmHg, $P=0.68$), suggesting BIM does not have a role in the regulation of IOP under normal physiological conditions. In contrast to D2.*Bim*$^{+/?}$, IOP was not significantly elevated in D2.*Bim*$^{-/-}$ at either 9 or 10.5 months of age (Fig. 6B; 9 months, 14.3±1.3 mmHg, $P=0.49$; 10.5 months,

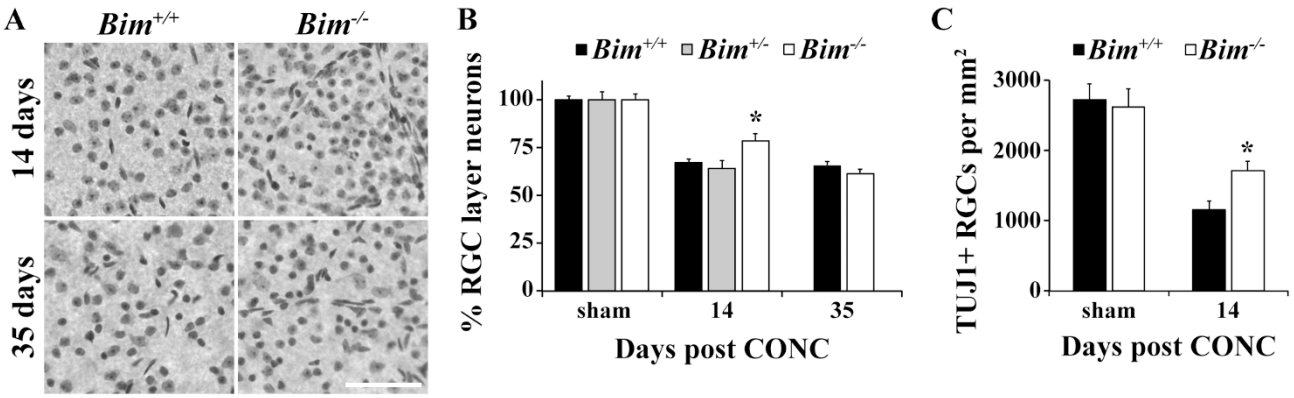

Figure 4 | ***Bim* deficiency increases RGC survival after CONC.** (A,B) To determine if *Bim* deficiency increases RGC survival after CONC, counts of Nissl stained ganglion cell layer neurons were performed. Note, only RGCs die after CONC and approximately half of RGC layer neurons are amacrine cells[32], so a loss of 50% of RGC layer neurons equals complete RGC loss. All genotypes had a significant cell loss compared to sham retinas at 14 days after CONC ($P<0.001$). Consistent with the decrease in cCASP3+ cell counts, there was a significantly increase in the number of RGC layer neurons at 14 days after injury in *Bim*$^{-/-}$ mice (*, $P<0.001$, compared to *Bim*$^{+/+}$ mice). However, by 35 days after injury, *Bim* deficiency did not provide protection. Note, since *Bim*$^{+/+}$ and *Bim*$^{+/-}$ mice had similar loss of RGC layer neurons at 14 days, *Bim*$^{+/-}$ retinas were not assessed at 35 days. (**C**) RGC counts, using the RGC marker TUJ1[26], confirmed the increased survival of RGCs 14 days after axonal injury (*, $P<0.001$). N≥5 for all genotypes and ages except n=3 for *Bim*$^{+/-}$; scale bar, 100 μm.

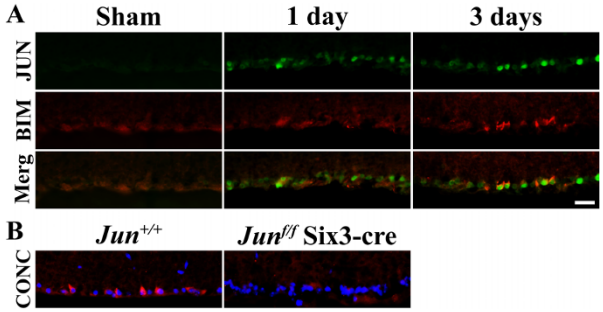

Figure 5 | JUN controls BIM expression after axonal injury. (A) JUN, which is known to control RGC death after CONC, is expressed by one day after injury, prior to BIM expression. By 3 days after CONC, RGCs coexpress JUN and BIM. (B) In contrast to the robust BIM expression (red) seen after CONC in RGCs of *Jun*$^{+/+}$ mice, there is no BIM expression in *Jun* deficient RGCs (*Jun*$^{f/f}$*; Six3-cre*). Thus, JUN appears to control BIM expression after axonal injury. Similar results were obtained from at least 3 different mice for each time point and genotype. DAPI, blue; Scale bar, 25 μm.

15.4±1.0 mmHg, P=0.10). However, individual D2.*Bim*$^{-/-}$ eyes did have elevated IOPs at both of these time points (Fig. 6B). By 12 months of age IOP was significantly elevated in D2.*Bim*$^{-/-}$ compared to young mice of the same genotype (20.3±1.3 mmHg, P<0.001).

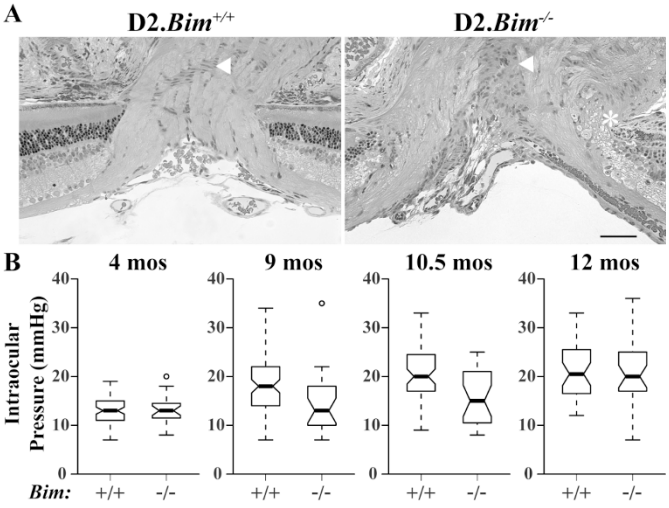

Figure 6 | *Bim* deficiency alters glaucomatous insult in DBA/2J mice. To test importance of BIM in glaucomatous RGC death, a null allele of *Bim* was backcrossed into DBA/2J for at least 10 generations (D2.*Bim*$^{-}$). The optic nerve head is the likely location of an important glaucomatous insult. (A) Since the optic nerve head was abnormal in B6.*Bim*$^{-/-}$ eyes we examined optic nerve head morphology in D2.*Bim*$^{-/-}$ mice. In 5 out of 5 D2.*Bim*$^{-/-}$ optic nerve heads examined there were clear abnormalities similar to those seen in B6.*Bim*$^{-/-}$. Most notably there were abnormal retinal optic nerve head borders (asterisk) and the gross arrangement of the glial cells in the area of the glial lamina (arrow) was poorly organized. Thus, it is possible that in D2.*Bim*$^{-/-}$ mice the changes in optic nerve head morphology alter the susceptibility of D2.*Bim*$^{-/-}$ to ocular hypertension induced neuronal injury. (B) The IOP profile of D2.*Bim*$^{+/+}$ mice was similar to previous reports[29]. At 9, 10.5 and 12 months of age IOP was significantly elevated compared to younger mice (P<0.001 for each age). The IOP in 4 month old D2.*Bim*$^{-/-}$ mice was not different to young D2.*Bim*$^{+/+}$ mice (P=0.68). There was also no significant increase in IOP in D2.*Bim*$^{-/-}$ mice at 9 or 10.5 months of age. IOP was significantly increased in D2.*Bim*$^{-/-}$ at 12 months of age (P<0.001). Thus, *Bim* deficiency appears to delay, but not prevent IOP elevation in DBA/2J mice. N≥24 for all ages and genotypes. Scale bar, 50 μm.

The IOP profile of D2.*Bim*$^{-/-}$ mice was similar to that observed in D2.*Bax*$^{-/-}$ mice[3]. Clinical examination of the anterior segment suggested the iris disease was not altered in D2.*Bax*$^{-/-}$ mice[3]. Gross examination of the anterior segment of D2.*Bim*$^{-/-}$ mice suggested there was no alteration of the iris pigment dispersion disease, though the anterior segment phenotype was not assessed in detail. Thus, there may be a loss of cells involved in IOP regulation in pigmentary glaucoma that occurs through an apoptotic process regulated by BIM and BAX.

BIM is not required for a glaucomatous neurodegeneration. Lower IOP delays but does not prevent optic nerve degeneration in *D2.Bax*$^{-/-}$ mice. In order to determine the extent of glaucoma in D2.*Bim*$^{-/-}$ mice, optic nerve degeneration was assessed using a validated optic nerve damage grading scale[3,8,29]. Optic nerve damage was categorized as no or early, moderate, or severe depending on the amount of axon loss and gliosis. Not all DBA/2J eyes develop glaucoma; however, by 12 months of age a majority of DBA/2J eyes will have severely degenerated optic nerves. As expected, in D2.*Bim*$^{+/?}$ 17 out of 28 (61%) nerves had severe degeneration (Fig. 7A). *Bim* deficiency did significantly lessen the amount of optic nerve damage at 12 months of age (P<0.001) with only 6 out of 41 (15%) D2.*Bim*$^{-/-}$ nerves graded as severe. There was a corresponding increase in moderate nerves in D2.*Bim*$^{-/-}$, suggesting that axonal injury in D2.*Bim*$^{-/-}$ mice is reduced, corresponding to the delay in IOP elevation.

In DBA/2J mice it appears that axonal degeneration precedes somal apoptosis[3,8,11]. RGC somal degeneration but not axonal degeneration is dependent on BAX in DBA/2J glaucoma[3]. Since some D2.*Bim*$^{-/-}$ mice did develop severe optic nerve degeneration, it was possible to determine if BIM is required to induce somal degeneration in DBA/2J glaucoma. Total RGC layer neurons were counted in 12 month D2.*Bim*$^{+/?}$ and D2.*Bim*$^{-/-}$ eyes either with severe optic nerve damage or no obvious glaucomatous damage (no or early nerves). There was significant loss of RGC layer neurons in D2.*Bim*$^{-/-}$ retinas when the optic nerves had severe degeneration (Fig. 7B; 72±6%; P<0.001). This loss in RGC layer neurons was slightly less than that observed in D2.*Bim*$^{+/?}$ eyes (64±3%), but this difference was not significant (P=0.21). Since the RGC layer consists of similar numbers of RGCs and amacrine cells[2,32-34] and only RGCs die in DBA/2J glaucoma[35], the glaucomatous eyes examined in D2.*Bim*$^{+/?}$ and D2.*Bim*$^{-/-}$ mice both had massive RGC loss.

Discussion

Glaucoma is a complex disease with the ultimate cause of blindness being the death of RGCs. Axonal injury in the lamina cribrosa, a specialized structure that RGC axons pass through as they exit the eye, is thought to be the critical insult for RGCs[8-11]. Axonal insult induced RGC somal degeneration is BAX dependent[2,3,7], but the molecules that control BAX activation are undefined. BH3-only proteins are pro-death Bcl-2 family members that help control BAX activation[12]. A likely candidate for activating BAX after axonal injury is the BH3-only protein BIM. Using *Bim* knockout mice we sought to determine if BIM was critical for BAX activation (RGC death) after axonal injury, including in a mouse glaucoma model.

Complete deficiency or heterozygosity for a null allele of *Bax* prevents RGC death even after extensive time after optic nerve crush injury and in glaucoma[3,7]. These data suggest that the levels of BAX, and subsequently activated BAX, are critical in determining RGC somal death. BAX activation is dependent on the level of BH3-only proteins, particularly those, like BIM, BID and BBC3, that can directly activate BAX[36,37]. A single member, BBC3, is required for the normal developmental death of RGCs, however, *Bbc3* deficiency only provided a minor delay in RGC death after axonal injury[1]. BIM has been shown to be involved in neuronal death during development and after injury[13,24,28,38]. In fact in a retinal explant model, where

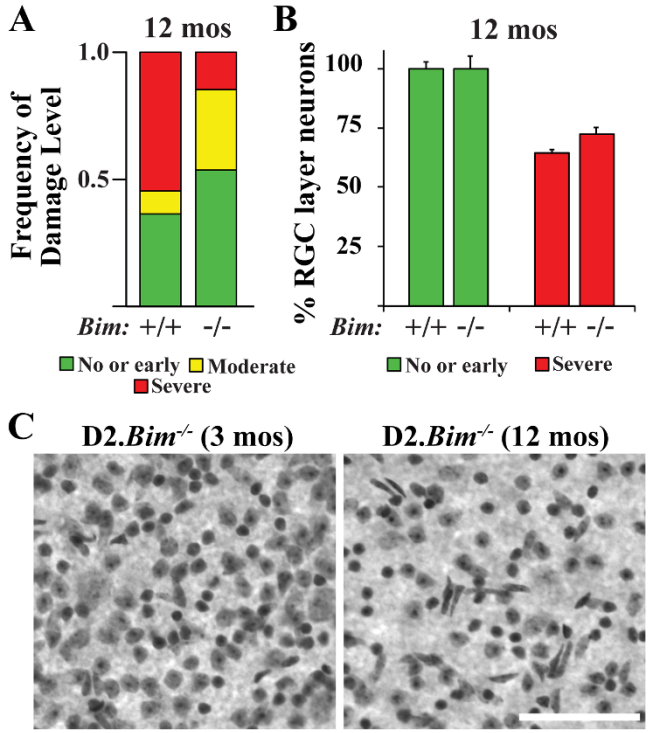

Figure 7 | BIM is not required for RGC death in glaucoma. (A) As judged by a validated grading scale of optic nerve damage[29], the distribution of optic nerve damage in D2.*Bim*$^{-/-}$ mice was significantly different to D2.*Bim*$^{+/+}$ ($P<0.001$) at 12 months of age. D2.*Bim*$^{+/?}$ (wild-type and heterozygous mice were included in the analysis; no difference in optic nerve degeneration was noted between the genotypes) had a similar profile of optic nerve damage to previous reports[3,8,29]: 33 nerves assessed with 36% no or early, 9% moderate, 55% severe. In contrast in D2.*Bim*$^{-/-}$ eyes there were far fewer nerves judged to have severe optic nerve damage (41 nerves assessed, 53% no or early, 32% moderate, 15% severe). (B) To determine if *Bim* deficiency protected RGC somas after glaucomatous injury ganglion cell layer neurons were counted in D2.*Bim*$^{+/+}$ and D2.*Bim*$^{-/-}$ eyes. For both genotypes, eyes with corresponding optic nerves judged either without damage (no or early) or severe were counted. Note, only RGCs die in DBA/2J glaucoma[35] and approximately half of RGC layer neurons are amacrine cells[32,34], so a loss of 50% of ganglion cell layer neurons equals complete RGC loss. *Bim* deficiency did not prevent somal loss in glaucoma. N≥5 for both genotypes and grades. Scale bar, 100 μm.

RGCs are injured by axotomy and likely other insults, *Bim* deficiency completely prevented RGC death for up to 4 days in culture[13]. This result implicates BIM as an important factor controlling RGC death after injury. Due to limitations of the explant model it can only assess the earliest time points of cell loss after axonal injury, which is just beginning *in vivo* at 3 days and occurs over at least 3 weeks[1,22]. *In vivo*, *Bim* deficiency significantly decreased RGC death after axonal injury at 3 days. However, by 5 days after injury there was substantial RGC death, though this death was also significantly reduced compared to *Bim*$^{+/+}$ mice. The loss of BIM did have a corresponding minor effect on RGC survival at later time points, but did not provide significant and complete protection as observed in *Bax* deficient mice after extensive time. Thus, it appears that the absence of BIM mainly affects the rate of death after axonal injury, but not the ultimate survival of RGCs.

The fact that single deficiency in *Bim* and to a far lesser extent *Bbc3*[1] only delay death suggests that other factors contribute to RGC death after axonal injury. The expression of BID, the other BH3-only protein that is capable of directly activating BAX, is consistent with a role in RGC death after axonal injury[39]. However, we have found that *Bid* deficiency does not delay or prevent RGC death after axonal injury (unpublished observation). It is unclear if these molecules work in combination or if indirect or non-canonical BAX activation can occur after axonal injury. Interestingly, we recently showed that the transcription factor JUN was a key mediator of RGC death after axonal injury[27]. JUN activation appears to be upstream of BIM (Fig. 5). *Jun* deficiency provided a far more extensive protection of RGCs than *Bim* deficiency[27], suggesting that other downstream targets of JUN activation are important mediators of BAX activation after axonal injury. Identifying additional targets of JUN will help to determine if BAX activation is completely dependent on pro-death Bcl-2 family members or whether alternative pathways are involved.

DBA/2J mice develop elevated IOP subsequent to an iris disease that is similar to pigment dispersion syndrome in humans[29,40,41]. It is possible that cell death may play a role in the iris disease or in the viability of trabecular meshwork cells (the cells primarily responsible for regulating IOP in the iridocorneal angle) in response to an insult. The significant lessening of IOP elevation (glaucomatous insult) in D2.*Bim*$^{-/-}$ mice was similar to that observed in D2.*Bax*$^{-/-}$ mice. Lessening IOP elevation is not seen in many of the other genetic or therapeutic manipulations that effect RGC neurodegeneration in DBA/2J mice (e.g.[42-47]). In D2.*Bax*$^{-/-}$ mice there were no changes in the clinical presentation of the iris disease, suggesting that *Bax* deficiency was affecting IOP regulation and not the iris disease. Loss of trabecular meshwork cells has been linked to IOP elevation in humans[48-51] and mice[52]. In humans, pigment dispersion syndrome only leads to pathological ocular hypertension (pigmentary glaucoma) and RGC loss in a subset of patients[41]. Why some patients are susceptible to IOP elevation is unknown. It is possible that this susceptibility results from the variability of the pigmentary insult causing death of trabecular meshwork cells. Our data suggests that a component of IOP elevation in pigment dispersion patients involves a BIM-BAX dependent cell death process. It will be important to test the role of BIM directly in trabecular meshwork cells and determine if a BIM dependent pathway can induce trabecular meshwork cell death. Manipulating this pathway may be a method of preventing IOP elevation in pigment dispersion syndrome and other ocular hypertensive diseases.

The lessening of IOP elevation in D2.*Bim*$^{-/-}$ mice may not completely explain the large protection from optic nerve degeneration conferred by *Bim* deficiency. The IOP profile of D2.*Bim*$^{-/-}$ mice was similar to that observed in D2.*Bax*$^{-/-}$ but the protection from glaucomatous optic nerve degeneration was far greater in D2.*Bim*$^{-/-}$ mice. *Bim* deficiency caused several abnormalities with retinal development that could contribute to the protection. Normal developmental retinal vasculature remodeling is disrupted in *Bim* deficient mice[17] leading to a significant increase in the amount of vasculature. Recently, work has implicated the vasculature as important unit in neurodegenerative disease, including glaucoma[45,53]. It is possible that extra retinal vasculature directly or indirectly alters the way the retina responds to IOP elevation. Finally, optic nerve morphogenesis was disrupted in *Bim* deficient mice. There was a clear lack of arrangement of glia in the area of the lamina cribrosa and abnormalities in the retina-optic nerve border and D2.*Bim*$^{-/-}$ mice. Interestingly, optic nerve morphology has been suggested to be an endophenotype for glaucoma[54,55]. This is perhaps not surprising since the lamina cribrosa is thought to be a key structure in many aspects of glaucoma and it is certainly plausible that alterations in its morphology change RGC susceptibility to IOP elevation. Thus, there are several roles for BIM in ocular development that may alter susceptibility to ocular hypertension.

Bim deficiency significantly reduced the number of eyes that had severe glaucoma, as judged by optic nerve degeneration. However, 15% of D2.*Bim*$^{-/-}$ eyes developed severe optic nerve degeneration. These degenerated nerves allowed us to test whether BIM was required for RGC death in glaucoma. Unlike in *Bax* deficient

mutants, RGCs were lost in D2.*Bim*$^{-/-}$ eyes with severe glaucomatous optic nerve degeneration. Even though BIM is expressed in glaucomatous RGCs and plays a role in axonal injury induced neuronal death, BIM is not required for BAX activation and RGC death in glaucoma.

It appears that BIM plays several roles in ocular development and disease that could directly affect glaucoma pathophysiology. During retinal development BIM is critical for normal retinal vasculature development[17] and optic nerve head morphogenesis, both of which have been implicated as endophenotypes in glaucoma. *Bim* deficiency also lessened/delayed IOP elevation in DBA/2J mice, suggesting BIM might regulate trabecular meshwork cell death after insult. Finally, *Bim* deficiency delayed RGC death after axonal injury, but did not prevent RGC death after a glaucomatous insult. In the future it will be important to uniquely manipulate BIM expression in each of the tissues where BIM has a role in ocular physiology and pathophysiology to gain a better understanding of its role in ocular hypertension and glaucomatous neurodegeneration.

Methods

Animals. Mice were maintained in a 12-hour light dark cycle and 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 research and approved by the University of Rochester's University Committee on Animal Resources. A *Bim* null allele *B6.129S1-Bcl2l11*$^{tm1.1Ast}$*/J*[56] that had been backcrossed into C57BL/6J was obtained from Jackson Laboratory (B6.*Bim*). The B6.*Bim* colony was maintained by intercrossing. For glaucoma experiments, the null *Bim* allele was backcrossed into DBA/2J for 10 generations and then intercrossed (D2.*Bim*). Note, a null allele of *Hrk*[57], another BH3-only protein that is known to facilitate, but not add to, BIM function[58] was segregating in the D2 cross. Of the 22 D2.*Bim*$^{-/-}$ mice 41 eyes were assessed for optic nerve damage by PPD staining (described below; 3 either were not assessed or the histology was not of high enough quality to determine the level of glaucomatous damage). D2.*Bim*$^{-/-}$ eyes used in this study included the following *Hrk* genotypes: 3 *Hrk*$^{-/-}$, 26 *Hrk*$^{+/-}$ and 12 *Hrk*$^{+/+}$. *Hrk* deficiency did not appear to alter either the IOP profile or optic nerve damage in *Bim*$^{-/-}$ mice (not shown). In fact, one of the three double null mice had severe optic nerve degeneration and massive loss of RGC layer neurons. D2.*Gpnmb* mice were aged to 10 months and used as a control for DBA/2J mice. For the glaucoma experiments using the DBA/2J genetic background, male and female ratios were approximately equal between control and experimental eyes. The *Jun* and Six3 cre mice were crossed as previously described[27].

Histology and cell counting. For immunohistochemistry and retinal flat mounting, eyes were fixed in 4% paraformaldehyde in PBS at room temperature. The anterior segment was removed and eyes were processed for cryosectioning or whole mount staining as previously described[1]. POU4F1 (BRN3A, Santa-Cruz Biotechnology, 1:300) positive cells were counted in retinal cross sections in 40x fields located within 500 μm of the optic nerve head. In whole mounts, cleaved caspase-3 (activated caspase-3; R&D Systems, 1:1000) positive cells were counted in the retinal ganglion cell layer in eight 20x fields around the peripheral retina (specifically two fields from each quadrant approximately 220 μm from the peripheral edge). Also in the retinal ganglion cell layer, TUJ1 (Covance, 1:1000) positive cells were counted in eight 40x fields around the peripheral retina. For identifying RGCs in some experiments, B6.Cg-Tg(Thy1-CFP)23Jrs/J (Jax Stock Number 003710; referred to as B6.Thy1-CFP) mice were used (the allele was backcrossed into B6 >20 times). CFP was detected using a chicken anti-GFP antibody (Abcam, 1:500). BIM expression was detected using a rat anti-BIM antibody (A.G. Scientific, 1:300). BIM expression was checked in at least three areas of the retina from three retinas at each condition/time point examined. DBA/2J expression was examined in sections from six retinas. For RGC layer neuron counts, eyes were flat-mounted RGC layer up and stained with a modified Nissl stain as previously described[3]. All neurons in eight 40x fields (two fields per retinal quadrant) were counted for each eye. The fields were all equidistant from the retinal margin, centered approximately one 40X field from the margin. Nissl stains all retinal ganglion cell layer neurons and endothelial cells. Based on their obvious elongated, non-neuronal morphology, endothelial cells were excluded from the counts.

Mechanical injury of RGCs and Glaucoma. Controlled optic nerve crush (CONC) was performed as previously described[1,3]. Mice were anaesthetized with a mix of ketamine and xylazine. The optic nerve was crushed just behind the eye for approximately 4 seconds using self-closing forceps (Roboz RS-5027). Unmanipulated contralateral eyes or contralateral eyes that had a sham surgery performed (no crush of the optic nerve) were used as control eyes. All CONC experiments were performed on B6.*Bim* mice. DBA/2J mice were used as a glaucoma model. The null allele of *Bim* was backcrossed into DBA/2J mice for 10 generations and then intercrossed. The TonoLab (Colonial Medical Supply, Franconia, NH) was used to record IOP in D2.*Bim* mice. Mice were anaesthetized with a ketamine xylazine mix and IOP was recorded per manufacturers instructions between two and five minutes after administration of anesthetic. For determining the level of glaucomatous optic nerve damage, nerves were processed and stained with paraphenylenediamine (PPD) as previously described[3,8,29] except that nerves were embedded in Technovit 7100 and 2 μm sections were cut and stained. Nerves were graded using a validated grading scale as previously described[3,8,29]. The grading scale places eyes into three categories: no or early, less than 5% of the axons are thought to be damaged or lost, a number that is consistent with age-related damage; moderate, many damaged axons throughout the nerve averaging about 30% of the axons judged to be damaged or lost, often there is localized signs of gliosis; severe, greater than 50% of the axons are judged to be damaged or lost and often signs of large areas of glial scarring. For plastic sections of retinas, eyes were processed and cut as previously described[1].

Statistical Analysis. For RGC counts and cell death assessed by immunostaining, *Bim*$^{+/+}$, *Bim*$^{+/-}$ and *Bim*$^{-/-}$ were considered independent groups and comparisons were made using ANOVA. Upon finding statistically significant differences between groups, the Tukey-Kramer method was used post-hoc to perform multiple comparison tests. $P<0.05$ was considered significant. These analyses included counts of cells labeled by Nissl stain, POU4F1, TUJ1, and CASP3 performed by an experimenter blind to genotype and/or experimental group. Standard error of the mean (SEM) is used to define the error bars for all cell counts. The Student's *t*-test was performed to compare intraocular pressures grouped by age and genotype and counts of surviving GCL neurons grouped by optic nerve grade ($n\geq5$) and genotype. D2.*Bim*$^{-/-}$ significantly diminishes optic nerve damage compared to *Bim*$^{+/+}$ and *Bim*$^{+/-}$ littermates based on the chi-squared test.

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Author contributions:

JMH conducted the majority of experiments and KAF contributed to the experiments detailed in Figure 5. RTL and JMH designed experiments and analyzed data. JMH wrote the manuscript with assistance from RTL. All authors read and approved the manuscript.

Additional information

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