Proapoptotic *Bcl-2* Family Members, *Bax* and *Bak*, Are Essential for Developmental Photoreceptor Apoptosis

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Purpose. Apoptosis has been implicated in retinal development and degeneration, but the specific apoptotic pathways used are incompletely understood. The purpose of this study was to characterize the roles in retinal development of the proapoptotic *Bcl-2* family members *Bax* and *Bak*.

Methods. Eyes from mice at postnatal day (P)7, during the peak of developmental apoptosis in the retina, were processed for TdT-dUTP terminal nick-end labeling (TUNEL) to determine whether *Bax* knockout or double *Bax/Bak* knockout causes a defect in developmental apoptosis. Adult (>2-month-old) eyes from wild-type, $Bak^{-/-}$, $Bax^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ mice were analyzed by histology and immunocytochemistry to identify persistent retinal cells.

Results. Adult $Bax^{-/-}Bak^{-/-}$ eyes showed significant increases in the number of inner retinal cells, with an almost complete absence of TUNEL-positive cell death at P7. Some of these persistent cells in the inner retina notably included rod photoreceptors that normally undergo apoptosis after failure to migrate to the outer retina. These inner nuclear layer (INL) rods contained markers of early rod differentiation: rod opsin, arrestin, and recoverin. However, they did not form ectopic outer segments or contain the associated markers ROM-1, peripherin-2, and RP1.

Conclusions. *Bax* and *Bak* are important for retinal development and are the first apoptotic factors identified as essential for developmental photoreceptor apoptosis. Future studies will investigate the potential role of *Bax* and *Bak* in mediating pathologic photoreceptor death. (*Invest Ophthalmol Vis Sci.* 2003;44:3598–3605) DOI:10.1167/iovs.02-1113

Apoptosis is a process of programmed cell death without an ensuing inflammatory response. This neat packaging of cellular components allows for the precise removal of tissue during developmental remodeling, particularly in the retina. As the retina develops from a single layer of undifferentiated ventricular neuroectoderm to a mature retina containing three cell layers of postmitotic, fully differentiated cells—ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL)—extraneous cells unable to make functional neural connections are eliminated by apoptosis.[1,2] As cells in each layer become postmitotic and then differentiate, there are well-characterized, successive waves of apoptosis during the first 3 weeks after birth in the mouse,[1] eliminating as many as half of some cell types[3] in generation of the intricately organized, mature retina.

Beyond this involvement in ocular development, apoptosis rarely occurs in a normal, healthy retina but has been implicated in both inherited and acquired retinal degenerations.[4–6] The molecular pathogenesis of these retinal degenerations is still unclear, but apoptosis is the final common pathway in many retinal diseases, ranging from glaucoma to age-related macular degeneration, retinitis pigmentosa, and retinal detachment.[5,7–13]

The specific apoptotic factors involved in developmental apoptosis have only recently been investigated, demonstrating a role for *Bcl-2* family members in ocular development. Transgenic overexpression of antiapoptotic *Bcl-2* in the GCL and INL results in an adult retina with up to twice as many cells in these layers as in adult wild-type retinas.[14] In addition, retinas deficient in proapoptotic *Bax* contain fewer TUNEL-positive INL and GCL neurons than do wild-type retinas at P7, the peak of developmental apoptosis, resulting in a corresponding increase in the thickness of these layers by adulthood.[15] No study detected changes in photoreceptors.

It has been proposed that neurodegenerative disorders, including retinal degenerations, involve a pathologic reactivation of developmental apoptotic programs.[16] Apoptotic factors involved in developmental regression of neurons may also mediate apoptosis in neurodegenerative diseases. Indeed, *Bcl-2* overexpression, shown to substantially reduce the elimination of ganglion cells during development, rescues ganglion cell death induced by optic nerve axotomy.[17]

No apoptotic factor has yet been demonstrated to be involved in developmental photoreceptor apoptosis. In pathologic photoreceptor apoptosis, *Bcl-2* overexpression mediates transient protection in several models of photoreceptor degenerations,[18] suggesting that other *Bcl-2* family members may also influence photoreceptor survival. However, *Bax*-deficiency does not rescue photoreceptor degeneration induced by a cGMP-phosphodiesterase mutation in the *rd* mouse.[15] Clearly, there is a therapeutic need for improved understanding of the mechanisms underlying pathologic photoreceptor degenerations, an understanding likely to be advanced by the identification of apoptotic factors involved in developmental photoreceptor elimination.

We report that two proapoptotic *Bcl-2* family members, *Bax* and *Bak*, are essential mediators of inner retinal apoptosis without which virtually all developmental apoptosis at P7 is absent. $Bax^{-/-}Bak^{-/-}$ retinas retain inner retinal neurons, notably including rod photoreceptors in the INL. This is the

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Supported by National Eye Institute Grants EY00417 and EY01583; National Institutes of Health Grant MSTP T32-GM-07170; a Career Development Award from Research to Prevent Blindness; the International Retina Research Foundation; the F. M. Kirby Foundation; the Foundation Fighting Blindness; and the Paul and Evanina Bell Mackall Foundation Trust.

Submitted for publication October 31, 2002; revised December 20, 2002; accepted January 5, 2003.

Disclosure: **P. Hahn**, None; **T. Lindsten**, None; **G.-S. Ying**, None; **J. Bennett**, None; **A.H. Milam**, None; **C.B. Thompson**, None; **J.L. Dunaief**, None

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Investigative Ophthalmology & Visual Science, August 2003, Vol. 44, No. 8

first report of apoptotic factors involved in developmental photoreceptor regression.

Materials and Methods

Generation of Mice and Fixation of Eyes

C57BL/6 mice with targeted mutations in *Bax*, *Bak*, and *Bax/Bak* were generated along with wild-type littermates and genotyped as previously described.[19,20] For bromodeoxyuridine (BrdU) analysis, mice were labeled in vivo with BrdU.[21] Eight-week-old mice were injected intraperitoneally (IP) with BrdU (Sigma-Aldrich, St. Louis, MO) daily for 12 days at 50 mg/kg body weight. Mice were killed 12 days after final injection. In addition, 3-week-old mice were injected IP with BrdU at 50 mg/kg body weight 1 hour before death. Eyes were enucleated immediately after death and fixed overnight in 4% paraformaldehyde and 0.5% glutaraldehyde.

All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Histology and Morphometry

Fixed globes were rinsed in PBS, and eyecups were prepared by removing the anterior segment and lens, with careful attention to preservation of the vitreous. Eyecups were oriented and embedded in JB-4 plastic (Polysciences, Inc., Warrington, PA). Sections 1-μm-thick through the optic nerve head (ONH) were stained with 30% Richardson's methylene blue/azure II.

Digitized images of each section were acquired with a light microscope (Eclipse TE-300; Nikon, Inc., Melville, NY) and digital camera (Spot RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI; with ImagePro Plus software, ver. 4.1; Media Cybernetics, Silver Spring, MD) or with a second microscope (DMRE; Leica Microsystems, Inc., Bannockburn, IL) and camera (Orca C4742-95 Hamamatsu Corp., Bridgewater, NJ; with OpenLab software, ver. 2.2.0; Improvision, Inc., Boston, MA). The number of nuclei per column of each nuclear layer (ONL, INL, and GCL) were counted in triplicate at 200 μm intervals from 300 to 2000 μm from the ONH. The thickness of the outer plexiform layer (OPL) and inner plexiform layer (IPL) as well as total retinal thickness, from internal to external limiting membranes, were also measured in triplicate at the same intervals, using image analysis software (ImagePro Plus ver.4.1; Media Cybernetics) to calculate distances from manually set lengths.

Statistical Analysis

Analysis was performed by calculating means and standard errors (SEM) of each retinal layer from each genotype. The SEM was calculated from the generalized equation estimate, with adjustments for correlations among repeated measurements from the same eye.[22] To examine differences among different genotypes, linear mixed models were fit using methods to account for the correlation among repeated measurements from the same eye. The models also included the distance from the optic nerve head and its interaction with genotype to explore whether measurements vary with distance from the ONH and whether the effect of distance is the same in all genotypes. When the overall F-test for any difference among the four genotypes was found to be significant, pair-wise differences among genotypes and their 95% confidence intervals were estimated from the mixed model. The probabilities for the significance of pair-wise differences were calculated with the multiple tests taken into consideration by the method of Tukey.[23] All data analyses were performed on computer (SAS, ver. 8.0 (SAS Institute, Inc., Cary, NC).

TUNEL Analysis and Immunocytochemistry

Fixed globes were rinsed in PBS and prepared as eyecups, which were treated with 0.1% sodium borohydride, cryoprotected in 30% sucrose, and embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura Finetek, USA., Inc., Torrance, CA).[24] Cryosections 10 μm thick were cut through the optic nerve head and stored at -20°C.

Some fixed globes were rinsed in PBS, dehydrated, and embedded in paraffin. Sections 5 μm thick were cut through the optic nerve head and stored at room temperature.

TUNEL analysis was performed on cryosections and paraffin-embedded sections from eyes harvested at P7, during the peak of retinal developmental apoptosis,[1] using a commercial kit (Roche Diagnostics Corp., Indianapolis, IN). Nuclei were counterstained (blue) with 4′,6′-diamino-2-phenylindole (DAPI; 1.5 μg/mL)–supplemented mounting medium (Vectashield; Vector Laboratories, Inc.). Sections were analyzed by fluorescence microscopy, and digitized images were acquired.

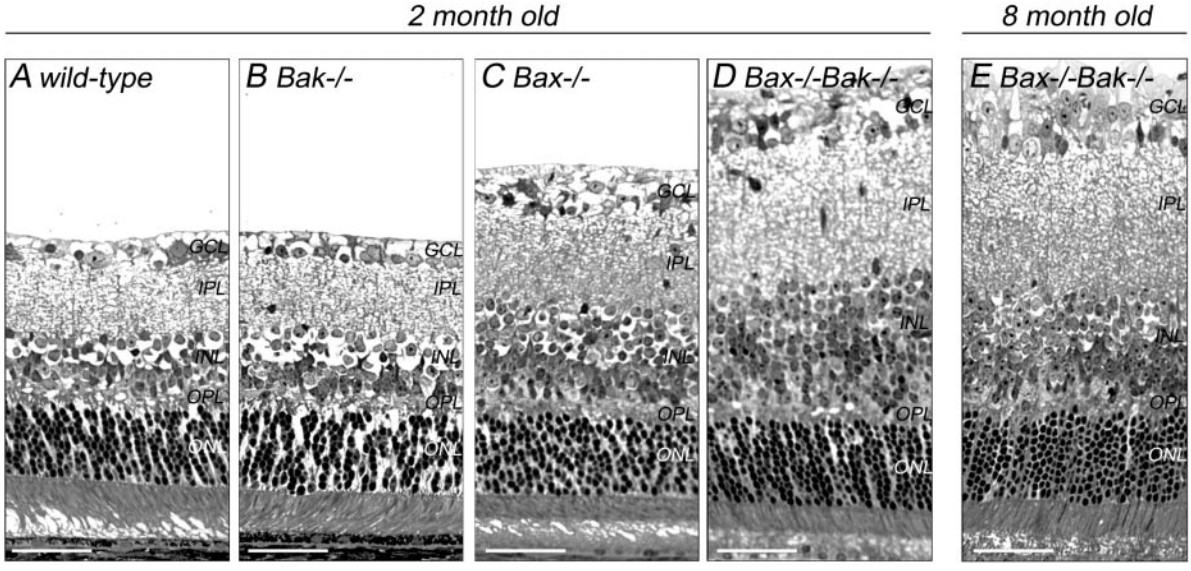

Figure 1. Photomicrographs of 1-μm plastic sections from adult retinas of indicated genotypes at 1000 μm from the ONH. (**A–D**) Two to 3-month-old retinas. The inner retinal layers (INL, GCL, IPL) were thickest in the $Bax^{-/-}Bak^{-/-}$ retina compared with the other genotypes. The outer retinal layers (ONL, OPL) appear unchanged among all genotypes. (**E**) Eight-month-old $Bax^{-/-}Bak^{-/-}$ retina. The inner retinal layers remained thickened, and there was no apparent degeneration of photoreceptors at 8 months. Bar, 50 μm.

Immunocytochemistry was performed as published.[24] Cell-specific markers were anti-glial fibrillary acidic protein (Z0334, 1:500; Dako Corp., Carpinteria, CA); anti-γ aminobutyric acid (1:2000; Protos Biotech Corp., New York, NY); anti-glycine (1:100; Robert Marc, University of Utah, Salt Lake City); anti-calbindin-D (C8666, 1:250; Sigma-Aldrich Corp., St. Louis, MO); anti-cellular retinaldehyde binding protein (1:500; John Saari, University of Washington, Seattle); PNA-rhodamine (RL1072, 1:100; Vector Laboratories, Inc.); anti-BrdU (G3G4, 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA); anti-L7 (1:250, James Morgan, St. Jude's Children Research Hospital, Memphis, TN); mouse anti-rod opsin (4D2, 1:80; Robert Molday, University of British Columbia, Vancouver, Canada); sheep anti-rod opsin (1:1000; David Papermaster, University of Connecticut, Farmington, CT); anti-arrestin (A9C6, 1:1000; Larry Donoso, Wills Eye Institute, Philadelphia, PA); anti-recoverin (P26, 1:1000; Alexander Dizhoor, Kresge Eye Institute, Detroit, MI); anti-blue cone opsin (JH455, 1:5000; Jeremy Nathans, Johns Hopkins University, Baltimore, MD); anti-peripherin-2 (Per-5H2, 1:30; Robert Molday); anti-ROM-1 (1:20; Roderick McInnes, University of Toronto, Ontario, Canada), and anti-RP1 (1:1000; Qin Liu, Scheie Eye Institute, Philadelphia, PA). Control sections were treated identically but with omission of primary antibody. Secondary antibodies (donkey anti-rabbit, anti-mouse, anti-guinea pig, anti-chicken) were labeled with Cy-2 (green) or Cy-3 (red; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Nuclei were counterstained with DAPI (μg/mL)–supplemented mounting medium (Vectashield; Vector Laboratories, Inc.). Sections were analyzed by fluorescence microscopy, and digitized images were acquired as described earlier.

Results

Extra Cells in the INL and GCL of *Bax*$^{-/-}$*Bak*$^{-/-}$ and *Bax*$^{-/-}$ Retinas

To determine the effects of mutations in *Bax* and/or *Bak* on the retina, 1-μm sections through the optic nerve head (ONH) of 2- to 3-month-old retinas (four wild-type, four *Bak*$^{-/-}$, four *Bax*$^{-/-}$, and five *Bax*$^{-/-}$*Bak*$^{-/-}$) were compared. The retinas of each knockout eye had trilaminar retinal architecture as in wild-type eyes, with the three distinct nuclear layers (ONL, INL, and GCL) separated by the two plexiform layers (OPL, IPL; Fig. 1).

Morphometry of the retinas revealed quantitative differences in the relative thickness of the layers. Whereas outer retinas (ONL and OPL) appeared unchanged, inner retinas (INL, GCL, and IPL) of *Bax*$^{-/-}$*Bak*$^{-/-}$ mice were thicker than those of wild-type retinas, resulting in increased total retinal thickness. These changes persisted into adulthood, and even at 8 months, *Bax*$^{-/-}$*Bak*$^{-/-}$ inner retinas were thickened without any apparent degeneration of ONL photoreceptors (Fig. 1E). Similar increases in the INL, GCL, and IPL were also evident in the *Bax*$^{-/-}$ retinas, consistent with a previous report,[15] but these increases were smaller than those we observed in *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas. No changes were observed in *Bak*$^{-/-}$ retinas, which resembled the wild-type in all respects.

The thickness of each nuclear layer was measured as the number of nuclei per column, to avoid the confounding effects of variable fixation or glial expansion. The thickness of each plexiform layer and total retinal thickness were also measured. We confirmed statistically significant increases in *Bax*$^{-/-}$ inner retinas compared with wild-type[15] (Fig. 2). *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas exhibited further increases in inner retinal thickness compared with *Bax*$^{-/-}$ (GCL: $P < 0.005$; INL: $P < 0.001$; IPL: $P < 0.01$). There were no significant differences in the outer retina among all genotypes. In addition, *Bak*$^{-/-}$ retinas were not statistically different from wild-type in all measured parameters. As the results were not influenced by distance from the ONH, the thickness of each layer is represented by a single value, the mean of all measurements in each retina.

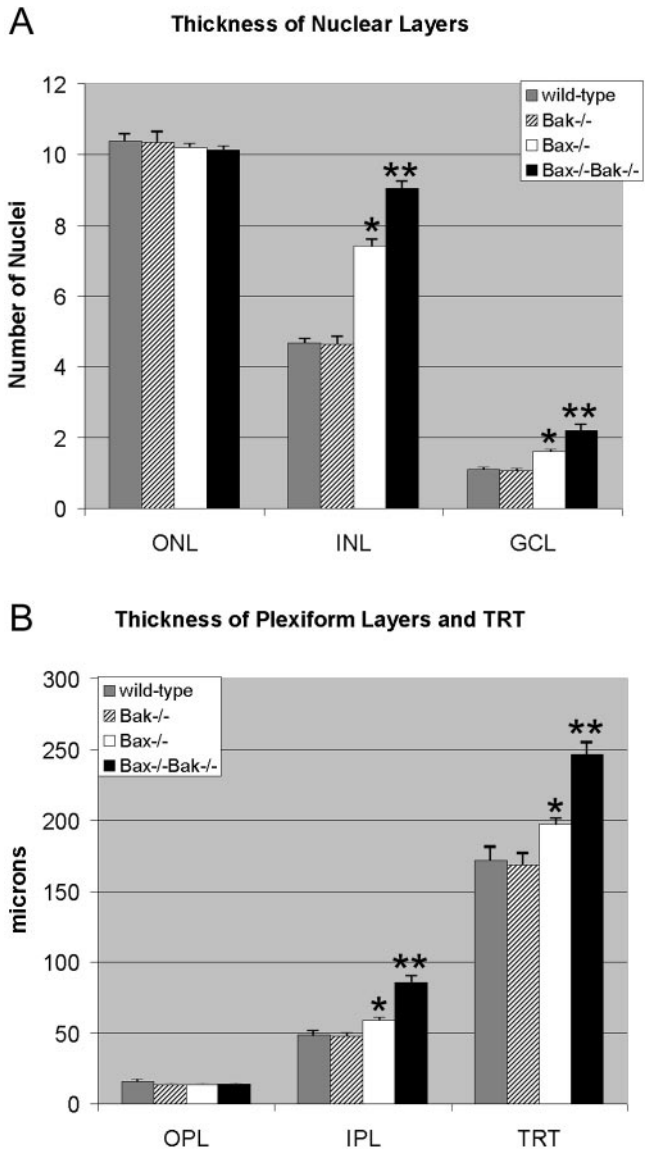

Figure 2. Histograms comparing retinal layer thicknesses from adult (2- to 3-month-old) mice of different genotypes (wild-type, $n = 4$; *Bak*$^{-/-}$, $n = 4$; *Bax*$^{-/-}$, $n = 4$; *Bax*$^{-/-}$*Bak*$^{-/-}$, $n = 5$). (**A**) Thickness of each nuclear layer measured by number of nuclei per column. (**B**) Thickness of plexiform layers and total retinal thickness (TRT) measured in micrometers. Shown is the mean (± SEM) of triplicate measurements at standard intervals in each retina. *Statistically significant difference between *Bax*$^{-/-}$ and wild-type for the measured parameter; **Statistically significant difference between *Bax*$^{-/-}$*Bak*$^{-/-}$ and *Bax*$^{-/-}$ (GCL: $P < 0.005$; INL: $P < 0.001$; IPL: $P < 0.01$).

Effect of Decreased Developmental Cell Death on Number of Cells in *Bax*$^{-/-}$*Bak*$^{-/-}$ and *Bax*$^{-/-}$ Retinas

The increase in cells observed in the *Bax*$^{-/-}$*Bak*$^{-/-}$ and *Bax*$^{-/-}$ retinas probably resulted from decreased developmental apoptosis, given the apoptotic functions of these genes. To test this possibility, we compared TUNEL-labeled P7 retinas from wild-type, *Bax*$^{-/-}$, and *Bax*$^{-/-}$*Bak*$^{-/-}$ eyes. Morphometry of TUNEL-positive cells in P7 wild-type and *Bax*$^{-/-}$ retinas previously revealed a significant decrease in *Bax*$^{-/-}$ develop-

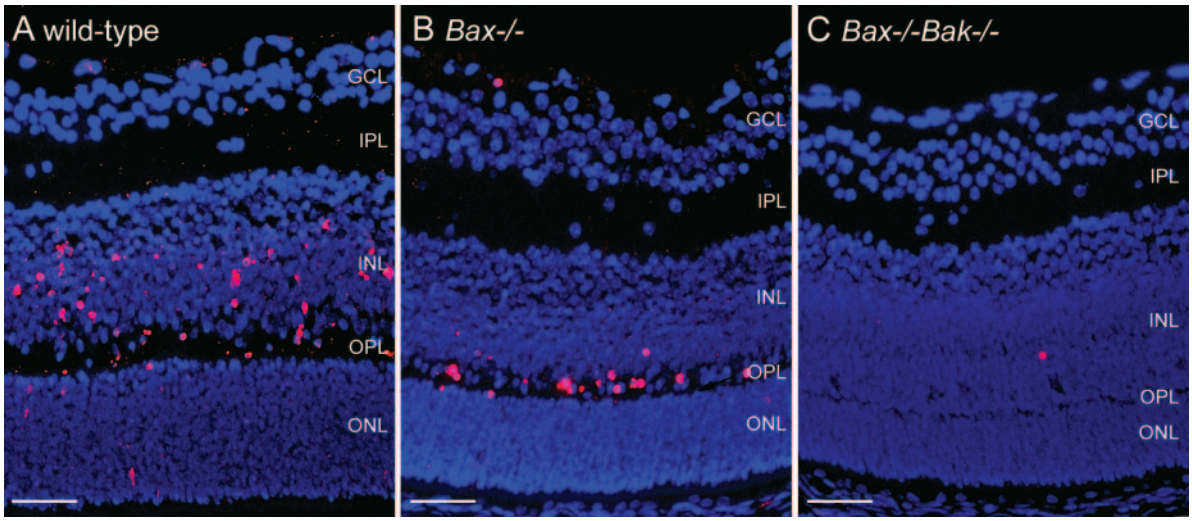

FIGURE 3. Fluorescence photomicrographs of TUNEL-stained (*red*) cells in wild-type (**A**), $Bax^{-/-}$ (**B**), and $Bax^{-/-}Bak^{-/-}$ (**C**) P7 retinas at 1000 μm from the ONH. Nuclei were stained with DAPI (*blue*). Scattered TUNEL-positive nuclei were present throughout the wild-type retina, mainly in the INL at this developmental stage. The $Bax^{-/-}$ retina had fewer TUNEL-positive cells, which were mainly in the outer INL. The $Bax^{-/-}Bak^{-/-}$ retinal section (**C**) was selected to show a rare TUNEL-positive cell. Bar, 50 μm.

mental cell death in P7 retinas.[15] Consistent with this previous report, we found decreased numbers of TUNEL-positive cells in the $Bax^{-/-}$ retinas compared with wild-type at P7 (Figs. 3A, 3B). Whereas P7 wild-type retinas contained numerous scattered TUNEL-positive nuclei throughout the retina, especially the INL, $Bax^{-/-}$ retinas had fewer TUNEL-positive cells, particularly in the inner INL, with more TUNEL-positive cells in the outer INL than the inner INL. $Bax^{-/-}Bak^{-/-}$ P7 retinas contained virtually no TUNEL-positive cells. In approximately a third of sections, a single TUNEL-positive cell per retina was found, usually in the mid- to inner INL (Fig. 3C). Developmental cell death was markedly deficient in $Bax^{-/-}Bak^{-/-}$ retinas, even compared with $Bax^{-/-}$ retinas, resulting in increased thickness in $Bax^{-/-}Bak^{-/-}$ adult retinas.

Photoreceptors in the INL of $Bax^{-/-}Bak^{-/-}$ Retinas

Having detected an increased number of inner retinal neurons in $Bax^{-/-}$ and $Bax^{-/-}Bak^{-/-}$ retinas, we identified these extra neurons by immunocytochemistry. The sensory retina normally contains nine identifiable cell classes or types (astrocytes, ganglion cells, amacrine cells, Müller cells, rod bipolar cells, cone bipolar cells, horizontal cells, rods, and cones). We labeled 2- to 3-month-old retinas of each genotype (four wild-type, two $Bak^{-/-}$, two $Bax^{-/-}$, and four $Bax^{-/-}Bak^{-/-}$) with cell-specific markers to identify the cell classes and types that increased in $Bax^{-/-}Bak^{-/-}$ and/or $Bax^{-/-}$ retinas compared with wild-type retinas.

Retinas were labeled with markers for rods and cones. PNA-rhodamine labeling for cones revealed no differences among genotypes (data not shown). Rods labeled with anti-rod opsin and anti-rod arrestin had positive outer segments and cell bodies in the ONL.[25,26] $Bax^{-/-}Bak^{-/-}$ retinas labeled with anti-rod opsin or anti-arrestin unexpectedly showed rods in the outer part of the INL (Figs. 4, 5). These rods were present in all $Bax^{-/-}Bak^{-/-}$ retinas, but not in retinas of any of the other genotypes. The INL rod cell bodies had no outer segments in the INL, but some had short processes (Figs. 4, 5, 7).

Retinas were also labeled with anti-recoverin (Fig. 6), which labels rods, cones, and cone bipolar cells.[27,28] In all genotypes, anti-recoverin labeled rods and cones in the ONL, as expected. All genotypes also had recoverin-positive cone bipolar cells with distinctly elongated cell bodies in the outer INL. Only $Bax^{-/-}Bak^{-/-}$ retinas, however, had a substantially increased number of INL recoverin-positive cells (Fig. 6B, white arrowheads).

To identify these additional INL recoverin-positive cells, $Bax^{-/-}Bak^{-/-}$ retinas were double-labeled with anti-recoverin and anti-rod opsin (Figs. 7A–C) or with anti-recoverin and anti-arrestin (Figs. 7D–F). Most of these INL cells coexpressed recoverin and rod opsin or recoverin and arrestin, consistent with their identification as INL rods. In addition, in $Bax^{-/-}Bak^{-/-}$ retinas labeled with anti-rod opsin and anti-arrestin (Figs. 7G–I), most cells coexpressed rod opsin and arrestin, further supporting the identification of these INL cells as rods.

A subset of these INL cells had elongated cell bodies and was positive for recoverin only (Figs. 7A–F, white arrowheads), corresponding to the normal number of cone bipolar cells, which do not express rod opsin or arrestin. We also identified arrestin-positive INL cells with undetectable levels of recoverin (Figs. 7D–F; yellow arrows) and rod-opsin (Figs. 7G–I; yellow arrows) as well as cells positive for rod opsin but not recoverin (not shown) or arrestin (Figs. 7G–I; white arrowhead).

Anti-arrestin has been reported to label blue cones in primate retinas,[29] but the INL cells positive only for arrestin were not blue cones, as labeling $Bax^{-/-}Bak^{-/-}$ retinas with anti-blue cone opsin did not reveal any blue cones in the INL (not shown). INL rods in $Bax^{-/-}Bak^{-/-}$ retinas were negative for ROM-1 or peripherin-2, markers of differentiated outer segments,[30,31] and for RP1, a protein in the photoreceptor connecting cilia[32] (not shown).

Effect of Ongoing Proliferation on the Number of Cells

To examine whether the increase in number of cells in the inner retina was due to deficient apoptosis or to an ongoing increase in proliferation beyond P11, the age by which all retinal neurons have become postmitotic,[33,34] $Bax^{-/-}Bak^{-/-}$ and control mice were pulsed with BrdU at 3 or 8 weeks of age. The brains of mice pulsed at 8 weeks showed increased BrdU-incorporation within cells in the periventricular zones of $Bax^{-/-}Bak^{-/-}$ brains compared with wild-type brains, as detected by anti-BrdU labeling (Lindsten T, Golden J, Thompson

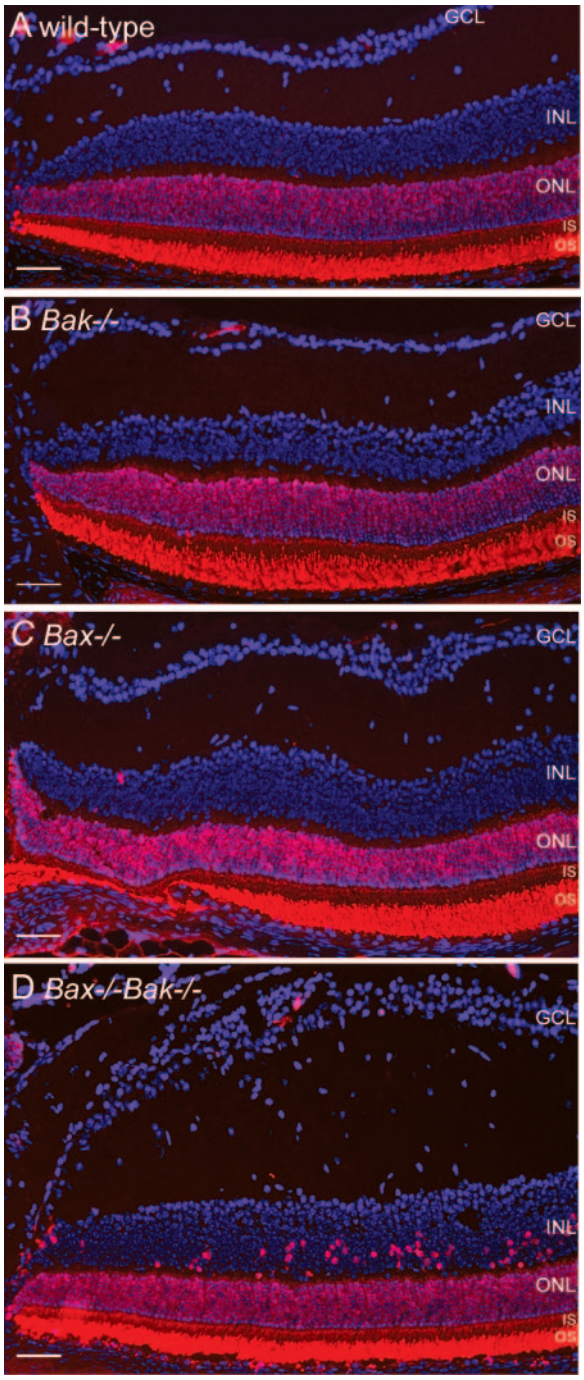

FIGURE 4. Fluorescence photomicrographs of anti-rod opsin–labeled retinas from adult (2- to 3-month-old) mice of the indicated genotypes. (**A**–**D**) Sections at the optic nerve head demonstrating perinuclear and outer segment (OS) rod opsin-labeling among all genotypes, as indicated, with ectopic rod opsin–positive cells in the INL of only the $Bax^{-/-}Bak^{-/-}$ retina. IS, inner segments. Bar, 50 μm.

CB, unpublished data, 2002). Sections of these brains were used as the positive control in parallel with retina sections from the same mice, and no BrdU incorporation was detected in the $Bax^{-/-}Bak^{-/-}$ retinas (not shown). The thymus of 3-week-old $Bax^{-/-}Bak^{-/-}$ mice incorporated BrdU (Lindsten T, Golden J, Thompson CB, unpublished data, 2002), and anti-BrdU labeling of whole eyes demonstrated BrdU-positive cells in the basal layer of the corneal epithelium, but not in the retinas (not shown). Absence of BrdU incorporation in $Bax^{-/-}Bak^{-/-}$ retinas at these two time points, both shortly after and significantly after retinal neurons become postmitotic, together with the absence of TUNEL label, indicates that the increased inner retinal cells are due to defective developmental apoptosis and not ongoing proliferation.

DISCUSSION

Apoptotic cell death mediates regression of tissue during normal development. The retina is one of the best examples of this phenomenon, in that the elimination of nonfunctional neurons during normal development has been extensively documented. Beyond this involvement in development, apoptosis is also the mechanism of pathologic cell death in retinal degenerations including glaucoma, retinitis pigmentosa, and age-related macular degeneration,[5,8,10–12] but the specific apoptotic factors involved have yet to be determined. *Bax*, *Bak*, and *Bok* are the only known multidomain proapoptotic members of the *Bcl-2* gene family,[35] previously shown to regulate apoptotic pathways. Because *Bok* expression is limited to reproductive tissues,[36] we examined the roles of both systemically expressed multidomain proapoptotic *Bcl-2* members, *Bax* and *Bak*, in retinal development.

In retinas of all genotypes examined, trilaminar retinal architecture characteristic of mature, wild-type retinas was preserved, although histologic comparison of retinas revealed increased numbers of neurons in the INL and GCL of $Bax^{-/-}Bak^{-/-}$ retinas compared with $Bax^{-/-}$, $Bak^{-/-}$, and wild-type retinas. Increased thickness of the IPL, which contains processes of cells with nuclei in the GCL and INL, was

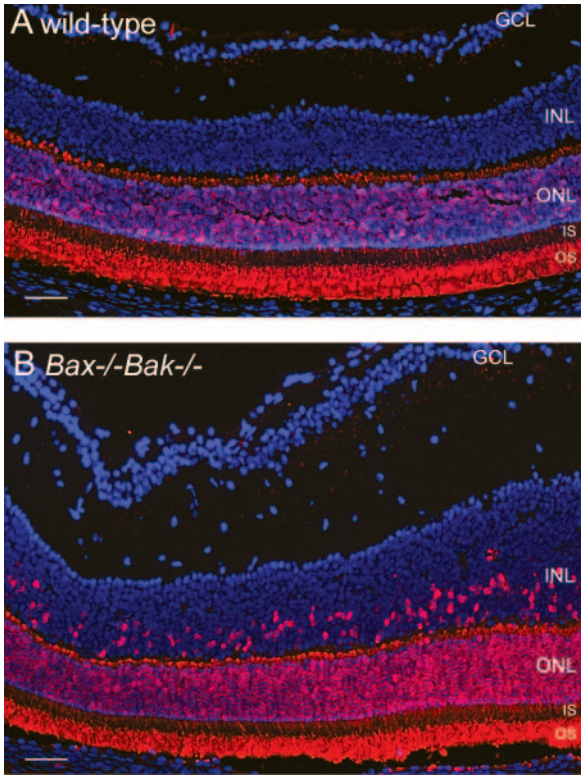

FIGURE 5. Fluorescence photomicrographs of anti-arrestin–labeled retinas from adult (2- to 3-month-old) mice of the indicated genotypes. (**A**, **B**) Sections near the optic nerve head demonstrating perinuclear and outer segment arrestin-labeling in wild-type (**A**) and $Bax^{-/-}Bak^{-/-}$ (**B**) retinas, with ectopic arrestin-positive cells in the INL of only the $Bax^{-/-}Bak^{-/-}$ retina. $Bax^{-/-}$ and $Bak^{-/-}$ retinas appeared similar to the wild-type retinas (data not shown). Bar, 50 μm.

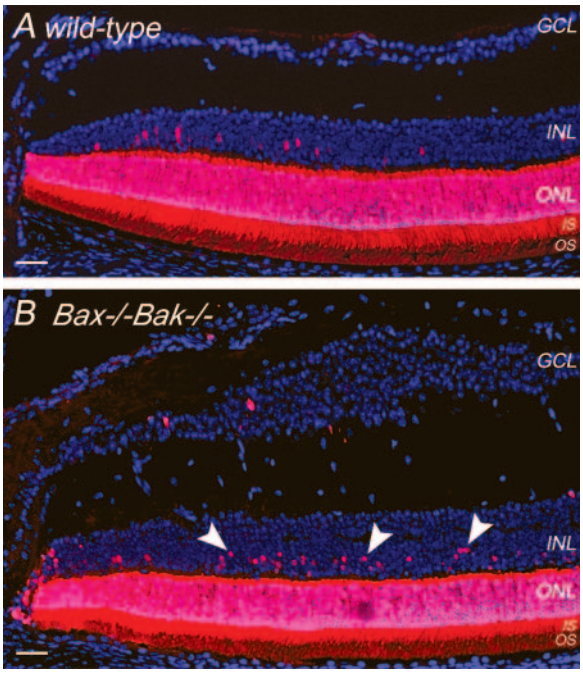

FIGURE 6. Fluorescence photomicrographs of anti-recoverin–labeled retinas from adult (2- to 3-months-old) mice of the indicated genotypes. (**A**, **B**) Sections at the ONH demonstrating a perinuclear labeling of rods and cones in both wild-type (**A**) and *Bax*$^{-/-}$*Bak*$^{-/-}$ (**B**) retinas, with an increase throughout the INL of recoverin-positive cells in the *Bax*$^{-/-}$*Bak*$^{-/-}$ retina compared with wild-type. Recoverin-positive cells in the INL of wild-type mice correspond to the normal complement of cone bipolar cells, which normally express recoverin. (**B**, *white arrowheads*) Subset of INL recoverin-positive cells in the *Bax*$^{-/-}$*Bak*$^{-/-}$ retina not present in wild-type. *Bax*$^{-/-}$ and *Bak*$^{-/-}$ retinas appeared similar to the wild-type retinas (not shown). Bar, 50 μm.

consistent with increased GCL and INL cells. No differences were found in the OPL or ONL of any genotype.

Consistent with an increased number of cells, *Bax*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas had fewer TUNEL-positive cells than did wild-type retinas at P7, the peak of developmental apoptosis in the rodent retina.[1] In *Bax*$^{-/-}$*Bak*$^{-/-}$ P7 retinas only, developmental cell death was almost completely absent, with fewer than one TUNEL-positive cell per retinal section, supporting a pivotal role for *Bax* and *Bak* in developmental elimination of retinal cells.

These findings are consistent with previous reports of preserved retinal architecture and increased inner retinal thickness in *Bax*$^{-/-}$ and *Bcl-2* transgenic mice, highlighting the importance of the *Bcl-2* family in late retinal development. Recently, quantitative analysis of murine retinas overexpressing *Bcl-2* driven by a neuron-specific enolase promoter[14] revealed neuron preservation similar to our *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas. Both have approximate doubling of inner retinal thickness with an approximately 50% increase in total retinal thickness.

Our histologic results suggest that only cells of the INL and GCL are affected by the absence of *Bax* and/or *Bak* expression. Using immunocytochemistry with cell type- or class-specific markers (not shown), we observed increases in the number of several inner retinal cell types, without a preponderance of any particular type, as previously described in the *Bax*$^{-/-}$ retina.[15] The presence of ectopic INL rods expressing rod opsin, arrestin, and recoverin in *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas was novel. In normal retinal development, as the OPL emerges at P5, it divides the population of developing rods approximately in half. During the ensuing 7 days, the 40% of rods situated in the inner aspect of the OPL migrate across the OPL into the ONL.[1] Those that are unable to migrate successfully into the ONL transiently remain in the INL until, in the normal retina, these ectopic INL rods undergo apoptosis, with maximum cell death at P7 and elimination of all INL rods by approximately 3 weeks after birth.[1] The absence of TUNEL-positive cells in the *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas at P7, the peak of INL rod apoptosis, suggests that the ectopic rods are present in the 2- to 3-month-old adult *Bax*$^{-/-}$*Bak*$^{-/-}$ INL because they fail to undergo developmental apoptosis. Because in *Bax*$^{-/-}$ single knockouts and *Bak*$^{-/-}$ single knockouts, but not in double knockouts, the INL rods are eliminated during development, either *Bax* or *Bak* is necessary for INL rod apoptosis.

It is possible that *Bax* and *Bak* do not act autonomously on these INL rods but rather that these ectopic rods survive in *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas for alternative reasons, perhaps because of altered trophic interactions resulting from increased numbers of other cell types. We believe this possibility is unlikely, given that *Bax*$^{-/-}$ retinas also retain many INL cells yet do not have ectopic rods. To determine whether INL rod survival is cell intrinsic or extrinsic, we will generate for future studies conditional *Bax/Bak* knockout mice lacking *Bax* and *Bak* only in rods.

Double labeling *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas with combinations of anti-rod opsin, -arrestin, and -recoverin revealed that most of the INL cells are rods coexpressing these three rod markers. A subpopulation of INL cells contained only recoverin and another subset contained only arrestin. A small number of cells contained rod opsin but not the other rod markers. Because recoverin is normally expressed in cone bipolar cells of the INL, the cells positive for only recoverin are most likely cone bipolar cells, consistent with their distinct morphologic features. The anti-arrestin antibody used in the current study has been reported to detect primate blue cones in addition to rods,[29] but labeling with a blue cone–specific antibody did not reveal ectopic INL blue cones in *Bax*$^{-/-}$*Bak*$^{-/-}$ retinas. In an in vitro culture system of dissociated primary rat retinal neurons cultured at P1 to P2, rod opsin- and arrestin-positive cells lacking recoverin were identified at P14 to P20[37] and believed to have lost recoverin expression. Perhaps, similarly, a subset of the ectopic INL rods loses expression of arrestin, rod opsin, and/or recoverin.

It is interesting that the INL rods, while persistent into adulthood in *Bax*$^{-/-}$*Bak*$^{-/-}$ mice, were less numerous at 8 months than at 2 to 3 months, as determined by the decreased number of 4D2-positive INL cells at 8 months (not shown) and dark INL nuclei characteristic of rods (Figs. 1D, 1E). Either these ectopic photoreceptors eventually die, or they differentiate into another cell class, such as bipolar cells, as previously documented in vitro.[38]

The INL rods appear unable to differentiate appropriately in their ectopic location and form outer segments, possibly as a result of inadequate proximity to the retinal pigment epithelium.[39] Although it is formally possible that these ectopic rods have normally positioned outer segments, their short processes do not appear to exit the INL. We did not detect other markers of differentiated rods—namely, the outer segment proteins ROM-1 and peripherin-2 and the connecting cilium protein RP1 in the INL rods. During development of ferret retina, rod opsin, arrestin, and recoverin are expressed early, whereas ROM-1 and peripherin-2 are expressed later, coincident with outer segment differentiation.[40] The *Bax*$^{-/-}$*Bak*$^{-/-}$ ectopic rods may represent a population of photoreceptors that have undergone initial but incomplete differentiation.

We have identified *Bax* and *Bak* as overlapping, essential mediators of retinal neuron regression, notably including at least a subset of rod photoreceptors. Most normal developmental rod elimination occurs within these INL rods, as very few

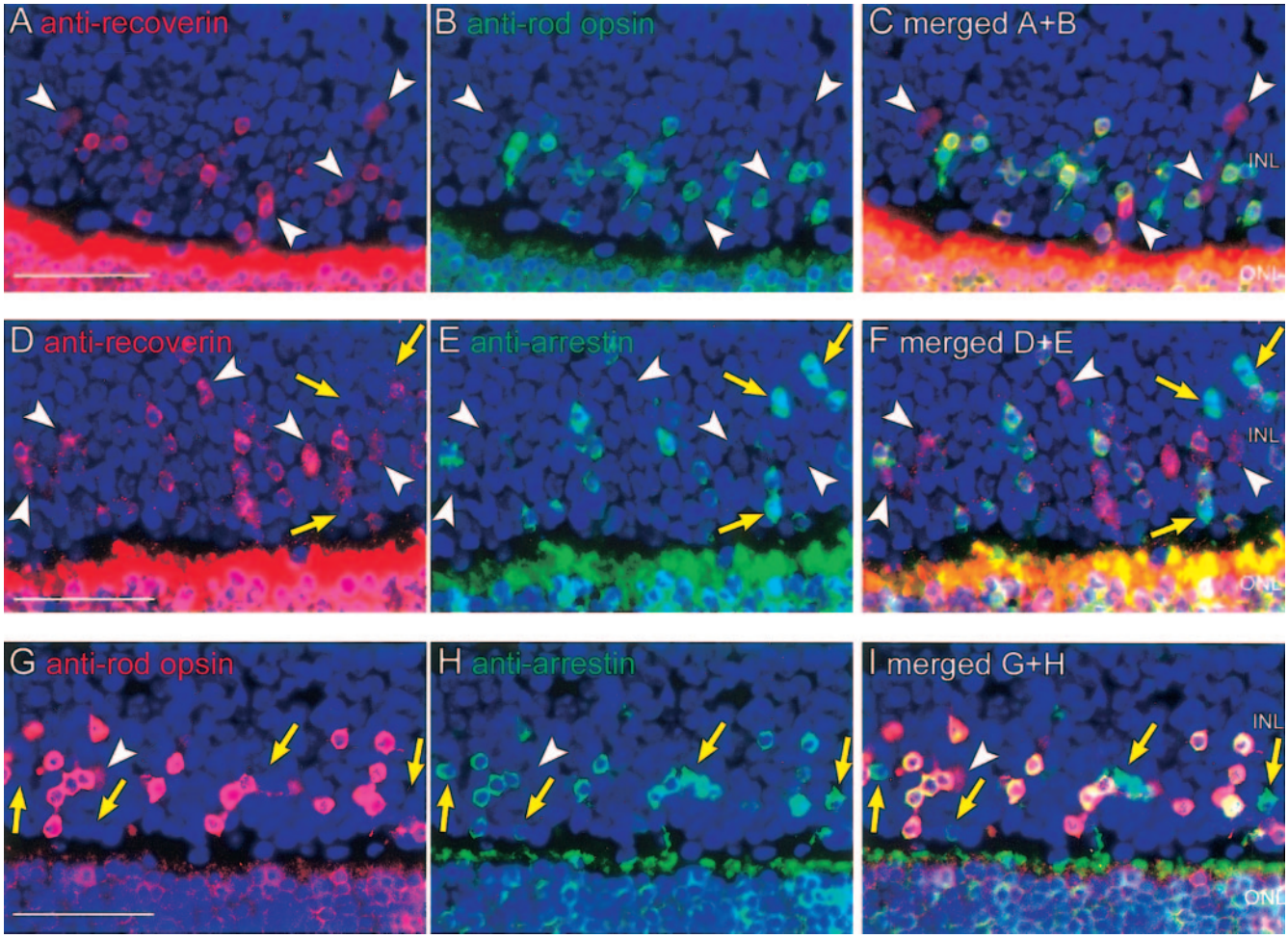

FIGURE 7. Fluorescence photomicrographs of adult (2- to 3-month-old) $Bax^{-/-}Bak^{-/-}$ retinas double-labeled with subsets of anti-rod opsin, anti-arrestin, and anti-recoverin. *Arrowheads/arrows*: cells expressing a single rod marker. (**A–C**) Section colabeled with anti-recoverin (**A**) and anti-rod opsin (**B**). Most ectopic INL cells had the same pattern of perinuclear labeling as normal ONL rods. These ectopic cells had short processes, but no ectopic outer segments. The merged image of both labels (**C**) indicates that most INL cells contained recoverin and rod opsin, consistent with their identity as rods. A subset of cells contained only recoverin (*white arrowheads*), corresponding to cone bipolar cells, and another subset of cells contains rod opsin with no detectable recoverin (not shown). (**D–F**) Section colabeled with anti-recoverin (**D**) and anti-arrestin (**E**). The merged image of both labels (**F**) indicates that most INL cells contained recoverin and arrestin. A subset of cells contained only recoverin, corresponding to the normal complement of cone bipolar cells (*white arrowheads*), and another subset of cells contained only arrestin (*yellow arrows*). (**G–I**) Section colabeled with anti-rod opsin (**G**) and anti-arrestin (**H**). The merged image of both labels (**I**) indicates that most INL cells contained rod opsin and arrestin. A subset of cells expressed only arrestin (*yellow arrows*) and another subset of cells expressed only rod opsin (*white arrowhead*). Bar, 50 μm.

ONL rods undergo apoptosis[1]; blocked apoptosis in this population is unlikely to result in a measurable excess of cells in the ONL. It is thus possible that elimination of both *Bax* and *Bak* preserves ONL rods as well, but beyond detectable levels. *Bax* and *Bak*, therefore, may be involved in developmental regression of retinal cells of all layers, including ONL rods, consistent with a previous finding of Bax immunolocalization in wild-type P16 retinas in the GCL, INL, and rod inner segments.[41]

Bax and *Bak* are the first apoptotic factors shown to be involved in developmental photoreceptor apoptosis. If degenerative diseases represent pathologic reactivation of developmental apoptosis, as previously proposed for Alzheimer's and Parkinson's diseases and retinal degenerations,[16,42] *Bax* and *Bak* may also mediate photoreceptor degeneration.

Although transgenic overexpression of *Bax* in photoreceptors results in extensive photoreceptor cell death,[41] *Bax* deficiency did not rescue photoreceptor degeneration in cGMP-phosphodiesterase–deficient *rd* mice.[15] $Bax^{-/-}$ retinas, however, did not have altered photoreceptor apoptosis during development. The finding that $Bax^{-/-}Bak^{-/-}$ retinas, but not $Bax^{-/-}$ or $Bak^{-/-}$ retinas, retained photoreceptors normally fated to die during development suggests that *Bak* can compensate for *Bax*-deficiency. Deficiency in both *Bax* and *Bak* may rescue photoreceptors, where *Bax*-deficiency alone is inadequate.

Bcl-2 overexpression, in contrast, partially protects photoreceptors from pathologic degeneration.[18] *Bcl-2* protection, however, is variable and most important, even in the presence of transient rescue, *Bcl-2* overexpression alone can be toxic to ONL photoreceptors.[43] Targeting *Bax*/*Bak* rather than *Bcl-2* is attractive because, like *Bcl-2*, *Bax* and *Bak* regulate mitochondrial membrane potential, which is disrupted in most apoptotic programs. However, unlike *Bcl-2* overexpression, deficiency of *Bax* and *Bak* is not toxic to ONL photoreceptors, even at 8 months (Fig. 1E). Future studies will test the hypothesis that *Bax* and *Bak* are essential for pathologic reactivation of apoptosis in photoreceptor and other retinal degenerations such that elimination of *Bax* and *Bak* functions may constitute a

therapeutic strategy to protect photoreceptors and retain vision.

Acknowledgments

The authors thank Maureen Maguire, Jeff Golden, Tzvete Dentchev, Waixing Tang, Nisha Gupta, Gulraiz Malik, and Nasreen Syed for advice and/or technical assistance.

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