Molecular Mechanisms Regulating Ocular Apoptosis in Zebrafish *gdf6a* Mutants

Sameer D. Pant,[1,2] Lindsey D. March,[3] Jakub K. Famulski,[3] Curtis R. French,[1] Ordan J. Lehmann,[1,4] and Andrew J. Waskiewicz[3]

[1]Department of Ophthalmology, University of Alberta, Edmonton, Canada
[2]Department of Veterinary Clinical and Animal Science, University of Copenhagen, Denmark
[3]Department of Biological Sciences, University of Alberta, Edmonton, Canada
[4]Department of Medical Genetics, University of Alberta, Edmonton, Canada

Correspondence: Andrew J. Waskiewicz, Department of Biological Sciences, CW405, Biological Sciences Building, University of Alberta, Edmonton, AB T6G 2E9, Canada; aw@ualberta.ca.

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Purpose. To characterize the molecular mechanisms underlying retinal apoptosis induced by loss of Gdf6, a TGFβ ligand.

Methods. The role of Gdf6 in regulating apoptosis was studied using a zebrafish *gdf6a*$^{-/-}$ mutant, which encodes a truncated, nonfunctional protein. To investigate whether intrinsic or extrinsic apoptotic mechanisms were involved, morpholino antisense oligonucleotides targeting *baxa*, *baxb*, and *p53* were employed. Caspase-3 immunohistochemistry (IHC) was performed to assay apoptosis. Pharmacologic inhibition (using SB203580) and IHC were used to investigate the role of p38 mitogen activated protein (MAP) kinase activation in *gdf6a*$^{-/-}$-induced apoptosis. To assess the role of Gdf6a in transcriptional regulation of TGFβ signal transducers, in situ hybridization (ISH) was performed using probes to *smad1*, *5*, 7, and *8*.

Results. Results indicate maximal ocular apoptosis occurs 28 hours post fertilization (hpf) in *gdf6a*$^{-/-}$ mutants that is mediated independently of p53 by intrinsic mechanisms involving Bax proteins. Also, *gdf6a*$^{-/-}$ mutants exhibit markedly increased p38 MAP kinase activation that can be inhibited to significantly reduce retinal apoptosis. A reduction in retinal *smad1* expression was also noted in *gdf6a*$^{-/-}$ mutants.

Conclusions. *gdf6a*$^{-/-}$-induced apoptosis is characterized by the involvement of intrinsic apoptotic pathways, p38 MAP kinases, and dysregulated *smad* expression. Modulation of key mediators can inhibit retinal apoptosis offering potential avenues of therapy. However, the efficacy of pharmacomodulation in improvement of visual function needs to be further examined.

Keywords: Gdf6, apoptosis, p38 MAP kinase, Bax, zebrafish

Retinal apoptosis is a major contributor to degenerative blinding disorders.[1,2] While apoptosis plays important physiologic roles in regulating cell numbers and removing damaged tissue, the level is tightly controlled to prevent excessive degeneration or uncontrolled proliferation. Stringent regulation is achieved via mediators acting at multiple levels within apoptotic pathways. One class of mediators, the bone morphogenetic proteins (BMPs) and closely-related growth differentiation factors (GDFs), together with activins, inhibins, nodals, and TGFβ proteins, comprise the TGFβ superfamily of multifunctional, secreted signaling ligands. Notably, BMPs have diverse roles, including control of cellular differentiation, proliferation, migration, pluripotency, and cell survival. Although their precise role in regulating programmed cell death (apoptosis) has been well studied during bone and limb remodeling, their functions in regulating ocular apoptosis remain largely unstudied.[3,4] Patients with mutations in *BMP4*,[5] *BMP7*,[6] *GDF3*,[7] and *GDF6*[8,9] exhibit a spectrum of ocular anomalies, and increased apoptosis has been described in animal models lacking *Bmp7*[10] and *Gdf6*.[11,12] Dissecting the molecular basis of BMP-mediated ocular apoptosis may reveal mechanisms relevant to TGFβ-dependent cell survival that could be therapeutically modulated to prevent vision loss.[13]

BMP ligands exhibit dose-dependent functions via Smad-dependent (canonical) and Smad-independent (noncanonical) pathways. At the cell surface, homo- or heterodimeric ligands bind to heteromeric complexes of transmembrane Type I (ALK2-7) and Type II (BMPRII, ActRIIA, ActRIIB) serine–threonine kinase receptors. Once phorphorylated, Type I receptors activate downstream signaling that is primarily driven via phosphorylation of Smad proteins.[14] The Smad family consists of eight members subdivided into three distinct groups: receptor activated Smads (Smad1, 2, 3, 5, and 8), inhibitory Smads (Smad6 and 7), and common mediator Smad4. BMPs primarily phosphorylate Smad1, 5, 8 (Smad8 also known as Smad9), which oligomerize with Smad4 and subsequently translocate to the nucleus to regulate gene expression by interacting with transcription cofactors such as mixer, OAZ (OE/EBF associated zinc-finger protein), FoxH, and FoxO.[15–18] Smad independent or noncanonical BMP signaling can also be induced by Type I receptors by activating either the Erk/Mitogen Activated Protein (MAP) kinase or LIM kinase 1 pathways.[19,20] While MAP kinases are key regulators of a broad range of cellular activities from apoptosis to cytokine production, LIM kinase 1 pathway is known to be particularly important for neural development.[21,22] Tight spatiotemporal

regulation of BMP signaling is achieved by precisely regulating expression of ligands and receptors, altering oligomerization of cell surface receptors that determine ligand binding affinity, varying levels of BMP antagonist expression, and post translational modification of Smad proteins.[23]

Noncanonical BMP signaling, especially mediated by MAP kinases, is particularly important in mediating apoptosis. BMP2 and BMP4 activate TGFβ-activated kinase 1 (TAK1), which in turn, induces apoptosis via activation of the MAP kinase kinase 3/6 (MKK3/6)–p38 MAP kinase pathway.[24,25] Similarly X-linked inhibitor of apoptosis protein (XIAP), which is known to interact with Type I receptors, causes apoptosis-related protein in the TGFβ signaling pathway (ARTS) to translocate from the mitochondria into the cytoplasm, where it binds and inactivates XIAP, thereby causing activation of caspase-3 in response to TGFβ signaling.[26–28] While these mechanisms have been demonstrated in vitro and in other tissues, their role in ocular apoptosis remains to be evaluated.

Amongst the multiple BMPs with key roles in embryonic patterning[29] and eye development,[30] Gdf6 establishes the dorsoventral retinal axes during development.[12,31–33] Loss of Zebrafish Gdf6a[32] dramatically decreases dorsal retinal marker expression (*tbx2b*, *tbx5*, *aldh1a2*, *efnb2a*, *bambia*), whilst expanding that of ventral markers (*vax2, aldh1a3, epha4b*).[12,32] Consistent with this result, eye-specific transgenic overexpression of *gdf6a* causes an increase in dorsal retinal genes and a reduction in ventral markers.[12,32] Gdf6a and Bmp2b are expressed in extraocular tissues at the initiation of dorsal retinal marker gene expression and both are required to initiate dorsal identity.[33] It is likely, therefore, that heterodimeric Bmp2b:Gdf6a complexes activate dorsal identity in the adjacent retinal tissue.[33] In addition to its function in patterning the eye, Gdf6 appears to protect retinal cells from apoptosis. This is demonstrated by transiently increased caspase-3 activation and TUNEL immunolabeling consistently observed in murine, zebrafish, and xenopus models.[12,34,35]

Consistent with its key ocular developmental roles and observations from three model organisms, patients with *GDF6* mutations exhibit a spectrum of phenotypes including microphthalmia, anophthalmia, and coloboma (MAC),[8,11,12] as well as a wider range of retinal disorders including AMD and Leber Congenital Amaurosis.[36] Notwithstanding GDF6′s regulation of ocular development, the molecular mechanisms mediating these phenotypes are poorly understood. The objective of this study was to characterize Gdf6a-dependent cell survival, identify the molecular mechanisms involved in apoptosis in *gdf6a*$^{-/-}$ mutants, and evaluate the efficacy of apoptosis inhibition in restoring vision. The results demonstrate increased p38 MAP kinase activation in *gdf6a*$^{-/-}$ mutants, which can be inhibited to rescue ocular apoptosis. Similarly, morpholino knockdown of *baxa* and *baxb* also rescues ocular apoptosis that is indicative of an intrinsic apoptotic pathway involving B-cell Lymphoma-2 (BCL2) family proteins. Together, these results demonstrate that multiple signaling pathways likely mediate perturbed Gdf6a function. Although our efforts to restore vision using p38 inhibitors were not successful, targeting such apoptotic pathways might contribute to better therapeutic alternatives in future.

Materials and Methods

Animal Model and Genotyping

The zebrafish dark half (*gdf6a*^{s327}) strain, which encodes a S55X premature stop codon in the coding sequence of *gdf6a,* was used for all experiments.[37] Genotyping was performed using PCR amplification and sequencing/restriction digestion using SfaNI (NEB, Ipswich, MA),[12] or high-resolution melting curve analyses. For all experiments, embryos were raised in embryo medium, or dechorionated and raised in embryo medium containing DMSO/SB203580 (Invitrogen, Ontario, Canada), staged to 28 hours postfertilization (hpf), anaesthetized with tricaine, and fixed in 4% paraformaldehyde or 100% methanol. Animal care and experimentation protocols were approved by the Animal Care and Use Committee, Biosciences of the University of Alberta in accordance with the Canadian Council of Animal Care. Animal care protocols align with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Morpholino Inhibition

Morpholino antisense oligonucleotides (Gene Tools, Philomath, OR) targeting the translational start site of *baxa* (*baxa*^{MO}; 5′-TGAAAATAAGCGAACTGAAGAAGAC-3′), *baxb* (*baxb*^{MO}; 5′-ATTTTTCGGCTAAAACGTGTATGGG-3′), p53 (5′-GCGCCATTGCTTTGCAAGAATTG-3′); and a control morpholino (*Control*^{MO}; 5′-AGTTTTCTCTGGGCTGCTCGCCAT-3′) were used to perform knockdown assays in 1 to 4 cell stage *gdf6a*$^{-/-}$ embryos as previously described.[38,39]

In Situ Hybridization (ISH)

RNA probes to coding and 3′-untranslated region of *smad1*, *5*, *7*, and *8* were synthesized and labeled with Digoxigenin-UTP (Roche, Indianapolis, IN) using T7 RNA polymerase (Invitrogen). Whole mount RNA ISH were performed as previously described.[40] BM Purple (Roche) stained embryos were mounted in 70% glycerol and microdissected eyes were photographed using a Zeiss Axioimager Z1 and Axiocam HR camera (Zeiss, Jena, Germany).

Immunohistochemistry

To detect apoptosis, IHC was performed using antiactivated caspase 3 antisera (cat # 559565; BD Pharmingen, Missisauga, Ontario, Canada) as per manufacturer's instructions. Embryos staged to 28 hpf were fixed in 4% paraformaldehyde, permeabilized with Proteinase K (10 μg/mL; Sigma, St. Louis, MO) for 4 minutes, and blocked in 10% goat serum, 1% BSA, and 0.1% Triton X-100 prior to overnight incubation in primary antibody at 4°C. Embryos were then washed twice with PBS + 1% DMSO, 0.1% Tween-20, and 0.1% Triton X-100 (PBSDTT) and incubated in antirabbit Alexa Fluor 568 secondary antibodies (Invitrogen, Carlsbad, CA) for 2 hours at room temperature, and again washed in PBSDTT five times. Nuclear staining was performed by incubating embryos in 10 mg/mL Hoechst 332588 stain (Invitrogen, Carlsbad, CA) for 10 minutes at room temperature. For detecting p38 MAP kinase phosphorylation, a rabbit polyclonal antibody specific for phorphorylated p38 MAP kinase (Cell Signaling Technology, Beverly, MA) was used and IHC was performed as described above with the exception that embryos were fixed in methanol instead of 4% paraformaldehyde. In all cases, such whole-mount IHC procedures were followed by microdissection of eyes, which were subsequently mounted in ProLong Gold antifade (Invitrogen, Carlsbad, CA) and visualized using a confocal microscope (Zeiss LSM 700; Zeiss). Images shown represent Z-stack projections of the entire thickness of the retina for each eye.

Counting of Apoptotic Foci and Statistical Analysis

In order to count the number of apoptotic foci in each eye, Z-stacks for each eye were imported into ImageJ (National

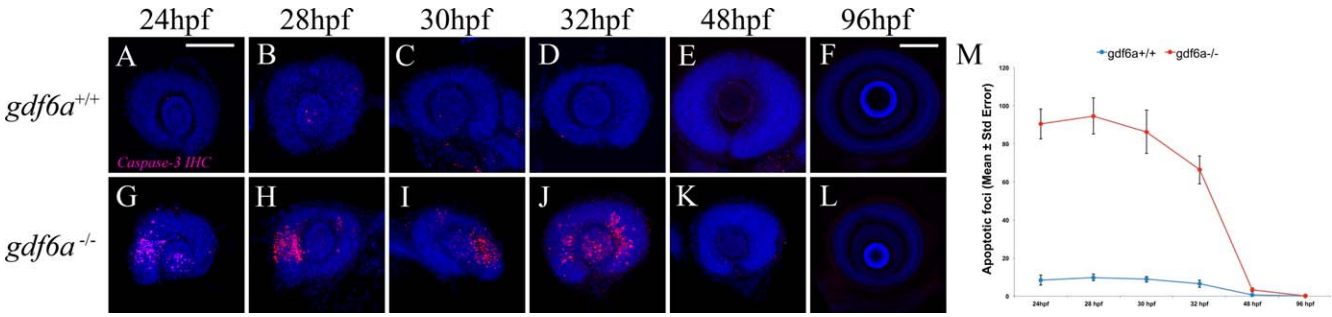

Figure 1. Analysis of apoptosis in *gdf6a*$^{-/-}$ zebrafish. Ocular immunolabeling of activated caspase-3 (*red*) in *gdf6a*$^{+/+}$ (**A–F**) and *gdf6a*$^{-/-}$ (**G–L**) zebrafish counterstained with DAPI (*blue*) illustrates maximal apoptosis at 28 hpf (**M**) spread throughout the retina and lens. After 28 hpf, the level of activated caspase-3 immunolabelling progressively declines and by 48 hpf, the reduced ocular size of *gdf6a*$^{-/-}$ mutants (**K**) compared with wild type (**E**) is evident. By 96 hpf, no differences in activated caspase-3 immunolabeling are observed (*gdf6a*$^{+/+}$ [**F**], *gdf6a*$^{-/-}$ [**L**]). *Scale bars*: 100 μm.

Institutes of Health, Bethesda, MD) using LOCI plugin (University of Wisconsin-Madison, Madison, WI); combined and transformed into an 8-bit image; and then individual foci were counted manually using a cell-counter plugin (Research Services Branch, National Institutes of Health). Data from Bax knockdown experiments was analyzed using one-way ANOVA with Tukey HSD post hoc tests. A two-tailed unpaired *t*-test was performed to analyze statistical significance in remaining experiments. Data from all experiments has been presented as means and SEs.

Results

Time Course of Apoptosis in *gdf6a*$^{-/-}$ Zebrafish

Zebrafish lacking Gdf6a (encoding S55X, a nonfunctional *gdf6a*$^{s327/s327}$ allele) display profound defects in eye size and visual function that are plausibly attributable to either increased levels of apoptosis,[8,12] or to deficiencies in patterning and dorsal marker expression.[32] To characterize the extent and timing of apoptosis occurring in *gdf6a*$^{-/-}$ mutants, whole-mount activated caspase-3 IHC was performed on embryos at 24, 28, 30, 32, 48, and 96 hpf. Compared with their wild-type siblings (Figs. 1A–F), *gdf6a*$^{-/-}$ mutants exhibit increased ocular immunolabeling (Figs. 1G–L) indicative of increased apoptosis. Maximal apoptosis is observed between 24 to 30 hpf (Fig. 1M), with levels gradually diminishing to 48 hpf (Figs. 1K, 1M), and barely detectable at subsequent stages (Fig. 1L). Taken together, these data are consistent with zebrafish Gdf6a maintaining retinal precursor cell survival from 24 to 48 hpf.

Gdf6a Induced Apoptosis Is Mediated by Bax Proteins Independent of p53

Activation of apoptotic cell death is mediated by either extracellular cues, such as growth factors or cytokines, or by intracellular stress events, such as heat, radiation, or hypoxia. The pro-apoptotic protein Bax (Bcl2 associated X protein) is expressed upon activation of intrinsic apoptotic pathways. In contrast, the *p53* gene is activated in response to extracellular apoptotic cues. Zebrafish contain a single *p53* gene and two *bax* genes (*baxa* and *baxb*) that are broadly expressed throughout early retinal development. Previous studies demonstrated that Baxa and Baxb are essential mediators of zebrafish intrinsic apoptosis.[38,41] Similarly, knock down of p53 using morpholino antisense oligonucleotides (MO) is sufficient to rescue zebrafish apoptosis models.[42,43] In the case of Gdf6a, mutant embryos (*gdf6a*$^{-/-}$) injected with *p53*^{MO} (Fig. 2A) demonstrated retinal caspase-3 immunolabeling at levels similar to those observed in uninjected *gdf6a*$^{-/-}$ embryos (Fig. 2B) (mean 162 ± 22 vs. 210 ± 28 apoptotic foci, $P = 0.19$, Figs. 2C, 2D). To further understand whether intrinsic or extrinsic apoptotic pathways are involved in mediating the increased caspase-3 activation observed in *gdf6a*$^{-/-}$ zebrafish, morpholinos were used to inhibit two pro-apoptotic genes, *baxa* or *baxb*. Injecting *gdf6a*$^{-/-}$ embryos with *baxa*^{MO} (mean 59 ± 14 apoptotic foci, $P = 0.011$), *baxb*^{MO} (mean 55 ± 13 apoptotic foci, $P = 0.016$), and combined *baxa*^{MO} *baxb*^{MO} (mean 55 ± 13 apoptotic foci, $P = 0.009$) significantly reduced apoptosis compared with *gdf6a*$^{-/-}$ embryos injected with *Control*^{MO} (mean 131 ± 20 apoptotic foci, Figs. 3A–F). Such experimental results are consistent with *gdf6a*$^{-/-}$ mutants displaying an increase in intrinsic apoptotic pathway activation.

Increased p38 Mapk Activation Mediates Apoptosis in *gdf6a*$^{-/-}$ Mutants

The MAP kinase p38 is activated by noncanonical BMP signaling, and also by cellular stress, with p38 activity providing a second readout of intrinsic and extrinsic function. If Gdf6a promotes apoptosis by increasing cell stress, upregulated p38 activity would be observed in mutants, while the converse would occur if p38 is a mediator of Gdf6a signaling. Compared with *gdf6a*$^{+/+}$ siblings (Fig. 4A), *gdf6a*$^{-/-}$ embryos exhibit markedly increased ocular p38 MAP kinase phosphorylation assessed with whole-mount IHC using phospho-specific (Thr180/Tyr182) p38 MAP kinase antibodies (Fig. 4B). If such increased levels of phosphorylated-p38 MAP kinases activate apoptosis, inhibition of this kinase would be expected to rescue the *gdf6a*$^{-/-}$ cell death phenotype. To test this, we applied a pharmacologic inhibitor of p38 MAP kinase (SB203580 [60 μM]) to zebrafish embryos and examined apoptosis using caspase-3 IHC. Treatment with SB203580 partially inhibits the increased ocular caspase-3 activation of *gdf6a*$^{-/-}$ embryos compared with DMSO-treated control *gdf6a*$^{-/-}$ mutants (Figs. 4C, 4D). The reduction in activated caspase-3 immunolabeling is statistically significant in *gdf6a*$^{-/-}$ embryos compared with DMSO-treated controls (mean 70 ± 7 vs. 162 ± 14 apoptotic foci, $P = 0.00001$) (Figs. 4E, 4F). Given the known role that p38 plays in mediating stress-based mechanisms of activating apoptosis, this provides further support for Gdf6a-mediated regulation of intrinsic apoptotic pathways. Further, to determine whether p38 MAPK activation was mediated by Smad independent pathways, Tak1 was inhibited using a pharmacologic inhibitor (5Z-7-Oxozeaenol)

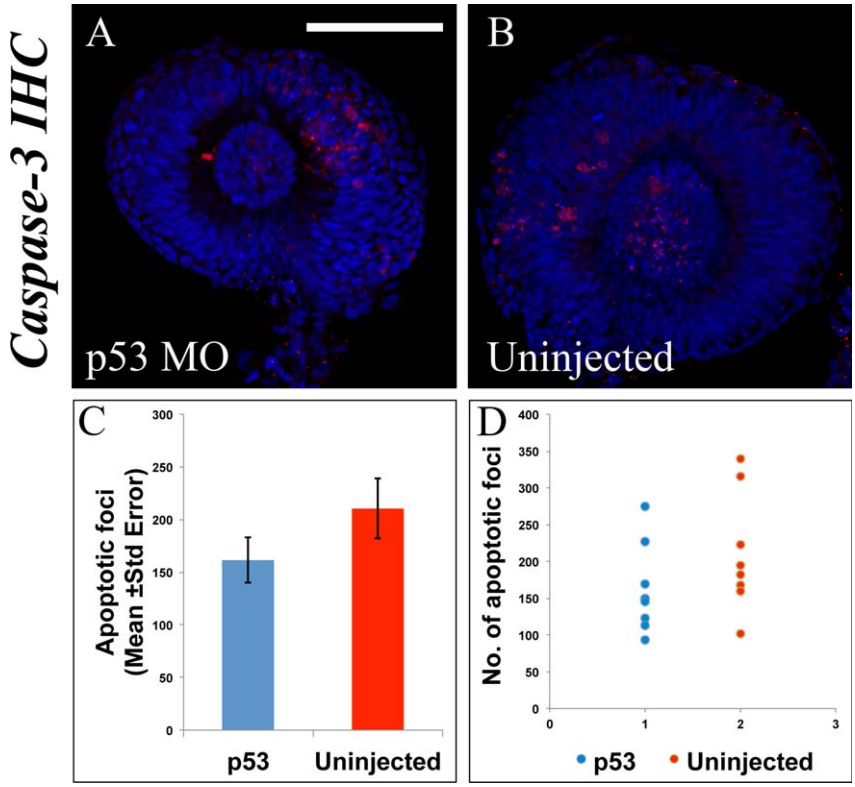

Figure 2. *gdf6a*$^{-/-}$-induced apoptosis is independent of p53 activation. Immunolabeling of activated caspase-3 (*red*) counterstained with Hoechst (*blue*) in *gdf6a*$^{-/-}$ mutants injected with *p53*^{MO} (**A**) demonstrate levels of retinal apoptosis comparable to those observed in uninjected *gdf6a*$^{-/-}$ mutants (**B**) ($P = 0.19$, [**C**, **D**]).

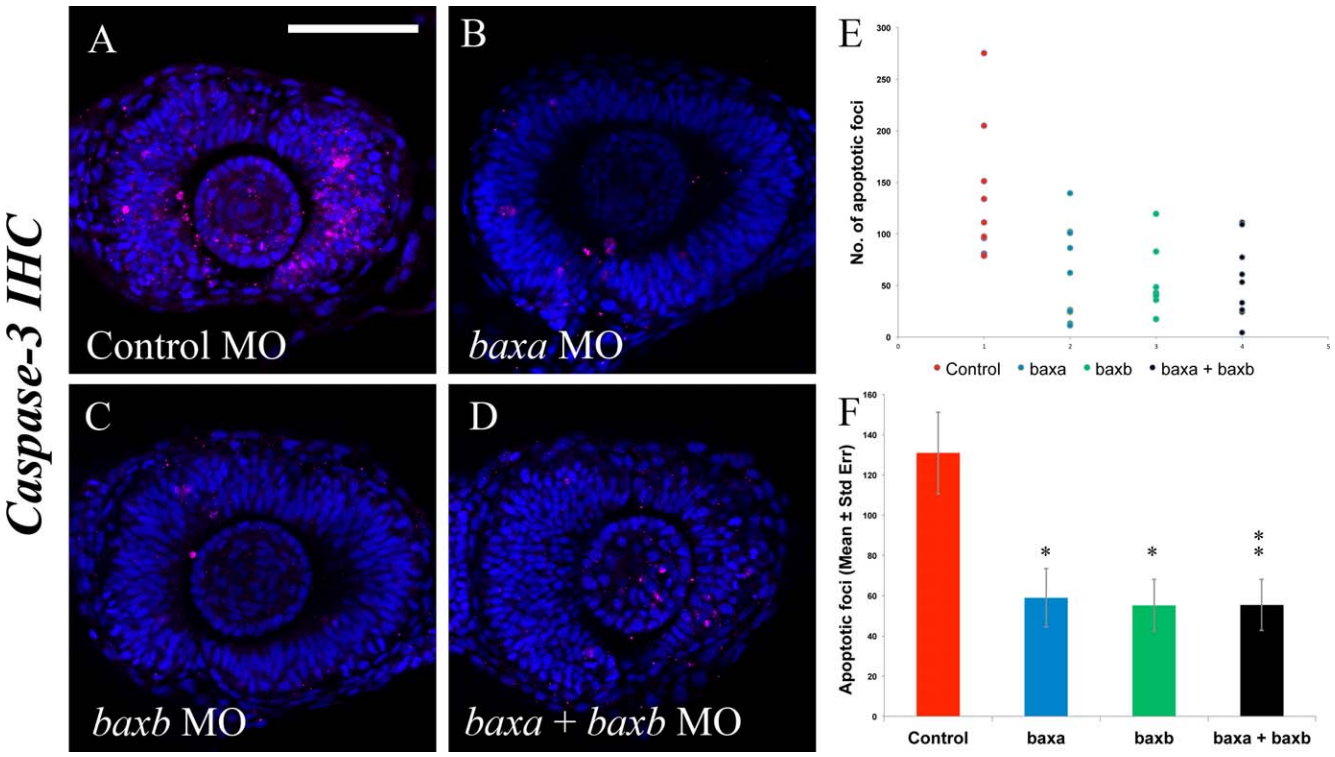

Figure 3. *gdf6a*$^{-/-}$-induced apoptosis is attributable to intrinsic pathways involving Bax proteins. Compared with *Control*^{MO}-injected *gdf6a*$^{-/-}$ embryos (**A**) immunolabeled with activated caspase-3 (*red*) and counterstained with Hoechst (*blue*); *baxa*^{MO} injections (**B**), *baxb*^{MO} injections (**C**), and combined *baxa*^{MO} *and baxb*^{MO} injections demonstrated significantly reduced activated caspase-3 signals ($P = 0.011$, 0.016, and 0.009, respectively, [**E**, **F**]).

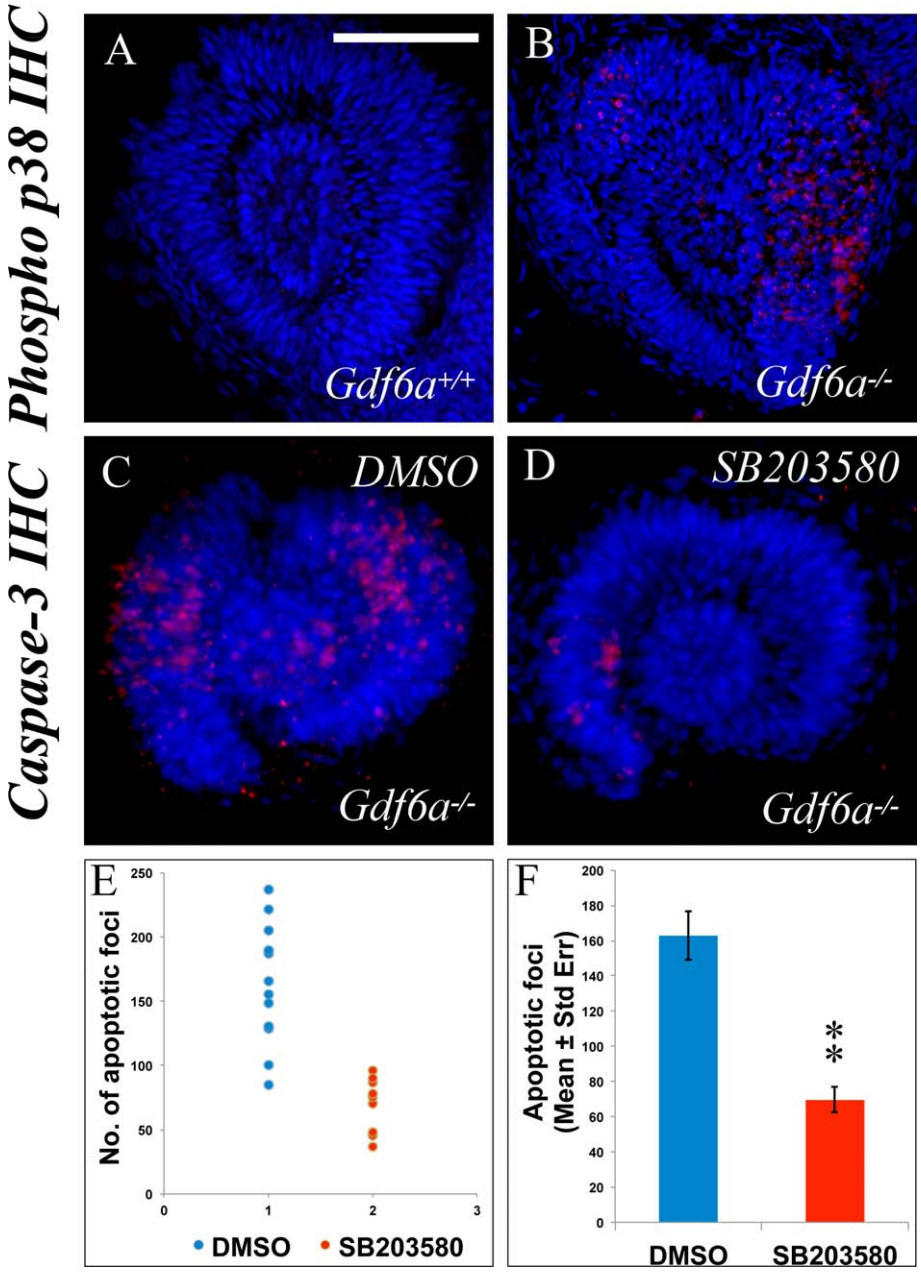

FIGURE 4. *gdf6a*$^{-/-}$-induced apoptosis is attributable to p38 MAPK activation. In contrast to their *gdf6*$^{+/+}$ siblings (**A**), immunolabeling of antiphospho p38 MAPK antibody (*red*) in embryos counterstained with Hoechst (*blue*) illustrates a marked increase of p38 MAPK phosphorylation in *gdf6a*$^{-/-}$ mutants (**B**) at 28hpf. Additionally, in contrast to DMSO-treated *gdf6a*$^{-/-}$ embryos (**C**), pharmacologic inhibition of p38 MAPK in *gdf6a*$^{-/-}$ using SB203580 demonstrates a significant reduction in ocular caspase-3 immunolabeling (*red*) observed in *gdf6a*$^{-/-}$ embryos (**D**) at 28 hpf.

and an antisense morpholino blocking its translation (*tak1*^{MO}). In both cases, embryos with reduced Tak1 function did not demonstrate levels of caspase-3 activation that were significantly different from DMSO- or *p53*^{MO}-injected controls (data not shown). These results are consistent with Gdf6a-dependent apoptosis occurring via Tak1-independent mechanisms.

Dysregulation of Ocular *smad* Expression in *gdf6a*$^{-/-}$ Mutants

BMP signaling classically functions by regulating phosphorylation of Smads 1, 5, and 8. Recent studies on Meis1 have demonstrated that it activates Smad1 transcription, thereby facilitating BMP signaling.[44] To determine whether loss of functional *gdf6a* affects *smad* expression, whole-mount ISH was performed. Compared with *gdf6a*$^{+/+}$ siblings (Figs. 5A–D), ocular *smad1* expression is reduced in *gdf6a*$^{-/-}$ embryos (Fig. 5E). No significant differences in ocular expression of *smad5, smad7*, and *smad8* (also known as *smad9*) (Figs. 5F–H) are observed. It is possible that our findings are indicative of a delay instead of reduction in *smad1* expression. However, this is typically an issue when measuring gene expression at a development stage at or near the onset of gene expression. Since ocular *smad1* expression was analyzed at 28 hpf, which is much later than the onset of ocular *smad* expression in zebrafish (11 hpf), these data most likely reflect reduced

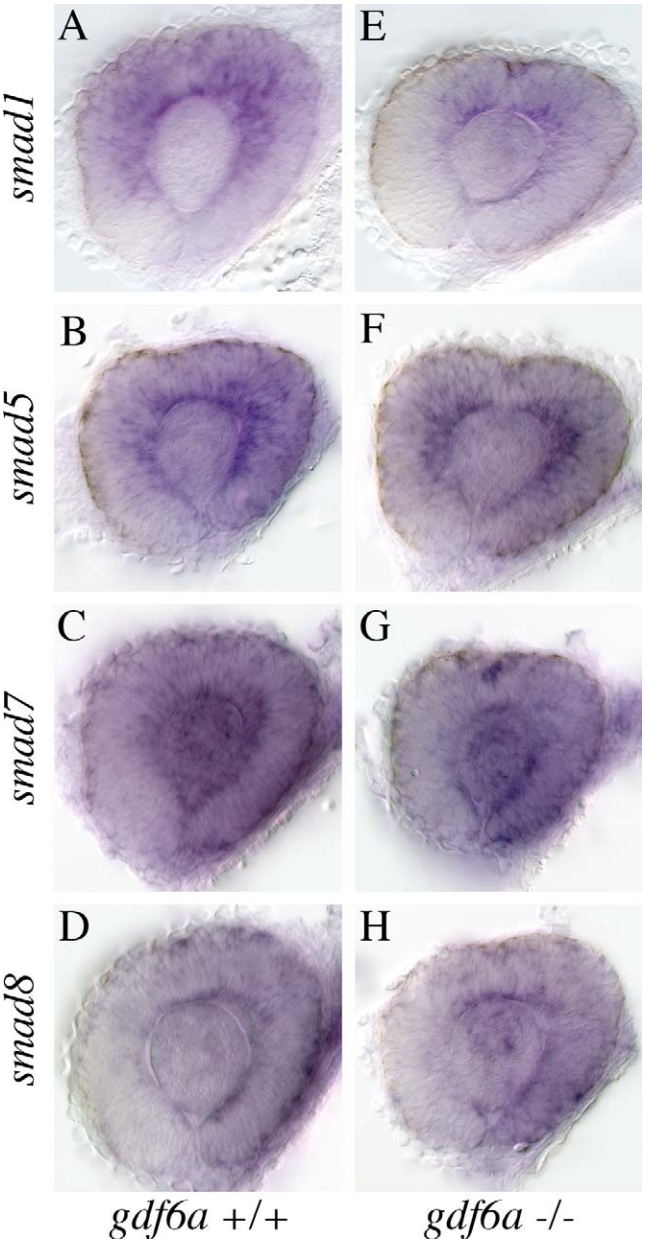

Figure 5. Ocular *smad1* expression is reduced in *gdf6a*$^{-/-}$ mutants. Compared with *gdf6a*$^{+/+}$ siblings (**A–D**), *gdf6a*$^{-/-}$ mutants display reduced ocular expression of *smad1* (**E**); while expression levels of *smad5* (**F**), *smad7* (**G**), and *smad8* (**H**) are unchanged at 28 hpf.

expression rather than a delay in expression. While it is known that Smad phosphorylation is lost in *gdf6a* morphants,[32] these results demonstrate that Gdf6a function is also crucial for *smad1* expression.

Discussion

The strong connection between BMP signaling and pathways regulating programmed cell death is illustrated by avian forelimb development. In the chick, extensive apoptotic cell death removes cells located in spaces that separate the digits,[4] whereas the webbed foot of the duck develops by interdigitally expressing the BMP antagonist gremlin.[4] This exemplifies a system whereby BMP signaling instructs cells to undergo apoptosis. In contrast, studies of retinal BMP signaling have revealed increased retina cell death with loss of Gdf6.[11,12] Clearly, given the survival-promoting function of BMP signaling in the retina, the molecular mechanisms mediating this phenotype must be distinct from those during digit specification. The present study characterizes the nature and extent of ocular apoptosis in a zebrafish model, providing evidence of the involvement of p38 MAP kinases and Bax proteins.

Growth Differentiation Factor 6 (Gdf6) is expressed extraocularly just after evagination of retinal precursor cells, with subsequent retinal expression demarcating the presumptive dorsal zebrafish retina. Our initial question was to ascertain the timing of apoptotic cell death in zebrafish embryos lacking functional zygotic *gdf6a* mRNA, and to determine whether there was a spatial link between such apoptosis and the localization of BMP signaling during early retinal development. We performed a time course analysis of activated Caspase-3 demonstrating that maximal apoptosis occurs between 24 to 30 hpf (Fig. 1M), that accords with data from other models.[11,12] Notably, even though *gdf6a* expression is restricted to the dorsal retina, the apoptosis induced in *gdf6a*$^{-/-}$ mutants extends throughout the developing retina and lens and is not localized to a specific region. This indicates that *gdf6a* induces the expression of survival mediators with broad ocular expression.

Apoptosis is commonly mediated either by intrinsic mechanisms involving the BCL2 proteins that regulate mitochondrial outer membrane integrity, or via death receptor-induced extrinsic mechanisms, both of which converge to activate intracellular proteases known as caspases.[45] Results from previous experiments in our laboratory indicated that a synthetic proneurogenic aminopropyl carbazole (P7C3) can significantly reduce caspase-3 activation observed in *gdf6a*$^{-/-}$ embryos at 28 hpf.[35] Experiments have shown that P7C3 preserves murine mitochondrial outer membrane (MOM) integrity, thereby inhibiting the release of factors that activate downstream apoptotic signaling.[46] Since, BCL2 pro- and antiapoptotic members are key regulators of mitochondrial outer membrane stability, we hypothesized that knocking down pro-apoptotic *baxa* and *baxb* (homologous to mammalian *Bax*) upstream of the mitochondria would phenocopy the effect of P7C3 in *gdf6a*$^{-/-}$ embryos. Since, Bax knockdown inhibited (Figs. 3A, 3C), and p53 knockdown did not affect retinal apoptosis (Figs. 2A, 2B), we concluded this apoptosis associated with loss of Gdf6a must be mediated by an intrinsic apoptotic pathway involving Bax. While the absence of antibodies specific for zebrafish Baxa and Baxb precludes determining the level of Bax protein knockdown, the reduced ocular apoptosis with *baxa*^{MO} and *baxb*^{MO} knockdown, and its absence in *Control*^{MO}-injected embryos; coupled with the lack of other overt phenotypes, indicate that Bax proteins are involved in mediating Gdf6 associated apoptosis.

In order to assess visual function improvement, we used a visual background adaptation (VBA) assay.[47] VBA refers to a neuroendocrine reflex that enables zebrafish to contract their melanophores in response to light stimuli. Thus, if retinal function is intact, embryos exposed to light contract their melanophores appearing light in color; and conversely, if retinal function is lost, embryos are unable to perceive the light stimulus and appear darker in color. However, VBA assays performed at 7 days postfertilization (dpf) after *baxa* and *baxb* knockdown did not reveal any improvement in visual function of *gdf6a*$^{-/-}$ mutants. These data accord with observations made in *bax*-deficient mice homozygous for a mutated *rd* (retinal degeneration) allele, where even though Bax deficiency leads to increased survival of retinal ganglion cells during development, it does not prevent photoreceptor degenera-

tion.[48] This demonstrates that inhibiting *bax*-mediated apoptosis, by itself, is insufficient in preserving retinal function. While it is possible that Gdf6a deficiency perturbs multiple aspects of cellular function (e.g., proliferation, cell cycle control), P7C3-mediated inhibition of caspase-3 activation leads to improved VBA responses in *gdf6a*$^{-/-}$ mutants.[35] Therefore it is likely that Gdf6a deficiency triggers multiple apoptotic pathways in the eye and inhibiting a single apoptotic pathway is insufficient to effect an improvement in vision.

Three major mammalian MAP kinase pathways play important roles in cell survival: the pro-apoptotic p38, the JNK, and the antiapoptotic p44/42 pathways.[49] Our research focused on p38 MAP kinase, as it has been tightly linked to both BMP signaling and apoptotic cell death. BMP2- and BMP4-dependent activation of p38 has been shown to induce apoptosis in vitro in murine hybridoma cells and in vivo in avian retina, respectively.[50,51] Furthermore, stress-dependent activation of p38 induces apoptosis in a range of tissues.[52] We predicted therefore, that p38 activity would be diagnostic of whether apoptotic cell death in the retina was regulated directly by BMP signaling.

Our results clearly demonstrate increased ocular p38 MAP kinase phosphorylation in *gdf6a*$^{-/-}$ embryos. Consistent with the key role of this kinase in regulating apoptosis, inhibition of p38 MAP kinase signaling by SB203580 rescues retinal apoptosis in *gdf6a*$^{-/-}$ mutants (Fig. 4). This finding is compatible with several possibilities including p38 MAP kinase activation occurring within the intrinsic apoptotic mechanisms involving BCL2 proteins described previously, or the involvement of multiple parallel apoptotic pathways. Moreover, the extent and distribution of antiphospho p38 MAPK immunolabelling in *gdf6a*$^{-/-}$ mutants appears similar to activated caspase-3 immunolabelling at 28 hpf, which raises the possibility that the underlying cells are the same. Given that p38 activation is mediated by Tak1 in response to TGFβ signals, we tested if it was possible to rescue retinal apoptosis by inhibiting Tak1 using a pharmacologic inhibitor 5Z-7-Oxozeaenol,[53] and antisense *tak1*^{MO}. However, these parallel approaches did not demonstrate any reduction in retinal apoptosis indicating that increased p38 MAP kinase activation in *gdf6a*$^{-/-}$ mutants likely occurs via mechanisms independent of Tak1.

In the case of both *baxa* and *baxb* knockdown; and inhibition of p38 MAP kinase downstream signaling using SB203580, rescuing apoptosis at 28 hpf neither rescued microphthalmia, nor led to improved VBA responses in *gdf6a*$^{-/-}$ mutants. There is some evidence in the literature to support these observations. For example, SB203580-mediated p38 MAP kinase inhibition is able to inhibit apoptosis of motor neurons observed in *Sod1* mutant mice without improving survival.[54] The lack of functional rescue in all these cases raises two possibilities; either partial inhibition of apoptosis is insufficient to cause functional rescue, or that other cell cycle or cell proliferation defects are involved. Indeed, an analysis of cell proliferation defects between 48 and 96 hpf in the *gdf6a*$^{-/-}$ retina has shown considerable defects,[55] with notable downregulation of proliferative genes *rx1*, *wnt2*, *myca*, *cnot7*, *ubrf1*, and *mycn*. Furthermore, defects were noted in the number of cells contributing to the optic vesicles, implying that the eyes may initially have a smaller population of cells.[55] Given these results, we can conclude that Gdf6-dependent regulation of eye size occurs via multiple mechanisms, likely including retinal cell proliferation, apoptosis, and precuror cell evagination.

It is unclear whether apoptotic or proliferative defects are independently sufficient to cause the visual deficit observed in *gdf6a*$^{-/-}$ mutants. While our data demonstrates that Bax or p38 MAPK inhibition rescues apoptosis without improving VBA responses, P7C3 is able to both rescue apoptosis and improve VBA responses in *gdf6a*$^{-/-}$ mutants. This indicates that either P7C3 acts downstream of multiple apoptotic pathways including those involving p38 MAPK and Bax proteins; or that P7C3 is also able to correct the proliferation defects that occur subsequent to the elevated apoptosis observed in *gdf6a*$^{-/-}$ mutants. Further examination of the mechanism of action of P7C3 would be required to answer these questions.

Loss of Gdf6a function leads to the loss of *bmp4* expression and also abolishes Smad phosphorylation in the eye.[32] While *bmp4* is genetically downstream to *gdf6a*, overexpressing *bmp4* in *gdf6a*$^{-/-}$ mutants neither causes Smad phosphorylation nor rescues *gdf6a*$^{-/-}$-associated phenotypes.[12] These results accord with our findings that indicate that *gdf6a*$^{-/-}$ mutants have reduced *smad1* expression (Fig. 5B). Since BMP4 also signals via Smads, it is possible that in the absence of *smad1* expression, overexpression of *bmp4* is ineffective in rescuing *gdf6a*$^{-/-}$ associated phenotypes. It would also be interesting to test if p38 MAP kinase activation observed in *gdf6a*$^{-/-}$ mutants is mediated by the loss of Smad1. However, since a zebrafish model with eye-specific reduction in Smad1 function is not available, this remains to be evaluated.

In summary, our results demonstrate, for the first time, that perturbed Gdf6a signaling leads to activation of intrinsic apoptotic mechanisms mediated by Bax proteins and also activate p38 MAP kinases, both of which can be modulated to rescue the apoptotic phenotype. We also demonstrate that loss of Gdf6a leads to dysregulated *smad* expression. Together, these results indicate that multiple apoptotic pathways are involved in mediated increased ocular cell death in *gdf6a*$^{-/-}$ mutants. We have also shown that although modulating either proapototic BCL2 proteins or p38 MAP kinase signaling partially inhibits ocular cell death, it does not improve visual function (Supplementary Fig. S1). Further investigation is required to determine why there is dissociation between survival of retinal cells and visual function upon modulation of these pathways.

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