Bcl6a function is required during optic cup formation to prevent p53-dependent apoptosis and colobomata

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Mutations in BCOR (Bcl6 corepressor) are found in patients with oculo-facio-cardio-dental (OFCD) syndrome, a congenital disorder affecting visual system development, and loss-of-function studies in zebrafish and Xenopus demonstrate a role for Bcor during normal optic cup development in preventing colobomata. The mechanism whereby BCOR functions during eye development to prevent colobomata is not known, but in other contexts it serves as a transcriptional corepressor that potentiates transcriptional repression by B cell leukemia/lymphoma 6 (BCL6). Here, we have explored the function of the zebrafish ortholog of Bcl6, Bcl6a, during eye development, and our results demonstrate that Bcl6a, like Bcor, is required to prevent colobomata during optic cup formation. Our data demonstrate that Bcl6a acts downstream of Vax1 and Vax2, known regulators of ventral optic cup formation and choroid fissure closure, and that bcl6a is a direct target of Vax2. Together, this regulatory network functions to repress p53 expression and thereby suppress apoptosis in the developing optic cup. Furthermore, our data demonstrate that Bcl6a functions cooperatively with Bcor, Rnf2 and Hdac1 in a common gene regulatory network that acts to repress p53 and prevent colobomata. Together, these data support a model in which p53-dependent apoptosis needs to be tightly regulated for normal optic cup formation and that Bcl6a, Bcor, Rnf2 and Hdac1 activities mediate this regulation.

INTRODUCTION

Colobomata are developmental defects of the eye which result in a cleft or absence of tissue in one or more ocular structures (1–3). Colobomata are also a common phenotypic manifestation in over 50 distinct human genetic disorders (OMIM: http://www.ncbi.nlm.nih.gov/), and they are estimated to be present in 3–10% of all blind children worldwide (4,5). During normal ocular morphogenesis, a region of the ventral optic cup called the choroid fissure must close in order to prevent colobomata. While choroid fissure closure is a critical aspect of ocular development that requires a precise interplay between growth, morphogenesis and regulated gene expression, the molecular and cellular mechanisms underlying this process have not yet been fully elucidated in any vertebrate organism (1).

Mutations in BCOR (Bcl6 Corepressor) have been found in patients with oculo-facio-cardio-dental (OFCD; OMIM 300166) syndrome, an X-linked condition in which afflicted patients display ocular abnormalities, cardiac septation defects and dental anomalies (6–9). Ocular defects in OFCD patients include microphthalmia, cataracts and higher risk of glaucoma (8). The mechanism whereby BCOR functions during eye development is not known, but in other contexts it serves as a transcriptional corepressor that potentiates transcriptional repression by B cell leukemia/lymphoma 6 (BCL6) (10). Knockdown of BCOR in zebrafish and Xenopus embryos results in OFCD-like ocular defects including microphthalmia and colobomata, as well as skeletal and central nervous system (CNS) abnormalities (8,11). BCOR is a core part of a transcriptional repression complex containing Ring finger protein 2 (RNF2), an E3 ubiquitin ligase that ubiquitinates histone H2A, and FBXL10/JHDM1B, an F-box protein with a JmjC domain that functions in H3K36 demethylation (12). H2A ubiquitination and H3K36 demethylation both lead to transcriptional repression (13,14), suggesting that BCOR function may be required to mediate epigenetic silencing during development (15).

The BCOR complex is recruited to target genes by BCL6, and target genes include p53 and cyclin D2 in B cells (15). BCL6 encodes a sequence-specific transcriptional repressor (16,17) that has been extensively studied for its role as a proto-oncogene and its involvement in certain types of lymphomas (18–20). BCL6 is required for germinal-center formation in mouse (21),...
where it functions to repress p53 expression in order to prevent apoptosis that would be otherwise triggered by DNA breaks occurring during immunoglobulin class switch recombination (22). While the involvement of BCL6 in B cell development and lymphomagenesis has been well studied in knockout mice (21,22) and cell culture (23), its functions during embryonic development remain poorly elucidated. Outside of the immune system, BCL6 is known to function in the development of the olfactory system, where it also serves as an anti-apoptotic factor during olfactory sensory neuron differentiation (24). Recently, BCL6 has been reported to be involved in the development of left-right asymmetry during Xenopus embryogenesis by inhibiting Notch target genes and thereby maintaining the expression of Pitx2 in the left lateral plate mesoderm (25). Interestingly, BCOR is also involved in the development of left–right asymmetry, suggesting a conserved developmental role for BCL6-mediated BCOR repression in this process (11).

In addition to BCOR, BCL6 is also known to require association with regulatory complexes containing histone deacetylases (HDACs), and other transcriptional co-repressors, in order to repress transcription of target genes (26–28). In zebrafish, a hdac1 mutation results in colobomata (29,30). Moreover, in retinal cell culture, pharmacological inhibition of Hdac function by trichostatin A (TSA) results in elevated expression of the pro-apoptotic protein, Apaf-1, via upregulation of p53, suggesting that Hdac activity may be required to prevent apoptosis in the retina (31,32).

Our interest is in determining the molecular and cellular mechanisms contributing to optic cup formation and choroid fissure closure. Our studies utilize the zebrafish, Danio rerio, as a model system for studying these processes. Many disrupted genes and pathways identified as integral to the formation of the zebrafish eye produce phenotypes that resemble disorders of the human visual system (33–35). Indeed, zebrafish have been a useful model for studying colobomata; loss-of-function phenotypes for aussicht, pax2a, vax1 and vax2, fgf19, n-cadherin, apc, laminin B1, laminin γ1, patched2, bcor, hdac1, nlz1 and nlz2 and tfap2 (8,29,30,36–48) each lead to colobomata in zebrafish, and these have been informative in furthering our understanding of choroid fissure closure in vivo. Of particular interest among this group of genes are vax1 and vax2. Vax1 and Vax2 are co-expressed in the optic stalk and ventral retina, and their expression overlaps with bcl6a (40,49–51). Knockout of either Vax1 or Vax2 leads to ventral optic cup defects and colobomata in mouse (52–55), and similarly, morpholino disruption of both vax1 and vax2 in zebrafish results in ventral optic cup defects and colobomata (40). Recently, Virgingipumanpeer et al. demonstrated that Vax2, along with Pax2, regulate the expression of the fas-associated death domain gene (fadd) in zebrafish (56). Deletions of FADD segregate with colobomata in human patients (57), and these have been modeled through morpholino knockdown in zebrafish (56). fadd-deficient embryos possess elevated levels of necroptotic cell death throughout the retina, and these correlate with colobomata (56), suggesting that Fadd-mediated suppression of necroptotic cell death may be a critical component of normal optic cup development in order to prevent colobomata.

Given that mutations in BCOR were found in OFCD patients (8), and knockdown of zebrafish and Xenopus orthologs of bcor resulted in ocular abnormalities that included colobomata, we were curious whether Bcl6 itself is required for normal eye development and, if so, whether it acts in conjunction with Bcor or other co-repressors in mediating these effects. Here, we demonstrate that loss of Bcl6a function leads to colobomata in zebrafish. Our data support a model in which Bcl6a expression is regulated by Vax1 and Vax2, and that Bcl6a functions in a genetic pathway that includes Bcor, Rnf2 and Hdad. These factors function to repress p53 expression, thereby preventing apoptotic cell death and colobomata during optic cup development.

**RESULTS**

*bcl6a* is expressed in the ventral optic cup and choroid fissure

*bcl6a* expression in zebrafish has been reported from a high-throughput expression screen (58), and here, we both confirmed these patterns and provide a more detailed spatiotemporal analysis of expression during eye development. *bcl6a* is expressed in the lens and myotome prior to 24 hpf (58; data not shown). In the retina, *bcl6a* begins to be expressed near the choroid fissure at 30 hpf, and this domain expands into the ventralnasal retina at 39 hpf (Fig. 1A–C). Transcripts expand both nasally and temporally in the outermost region of the ventral retina by 48 hpf, and *bcl6a* is detected in most of the ventral retina by 54 hpf (Fig. 1D–F). *bcl6a* is also expressed in the forebrain at 39 hpf (Fig. 1C) and the optic tectum from 39 to 54 hpf (Fig. 1G and H).

**Knockdown of Bcl6a results in colobomata**

To determine whether Bcl6a is required for normal ocular development, we utilized morpholino knockdown in zebrafish (59,60). Morpholinos were designed to the translation start site of *bcl6a* (bcl6a-ATGMO) and the exon2/intron2 splice junction (bcl6a-SPMO). Injection of either morpholino resulted in colobomata (Fig. 2 and Supplementary Material, Fig. S1); data are presented for bcl6a-SPMO since we could readily verify its efficiency in disrupting *bcl6a* splicing in each subsequent experiment (Fig. 2O). Injection of 7.5 ng of bcl6a-SPMO resulted in embryos with laterally curved bodies and colobomata at 3 dpf (Fig. 2B, E–H), while 5 bp mismatch morpholino (bcl6a-MM) injected embryos developed normally (Fig. 2A, C and D). By 5 dpf, colobomata in bcl6a-SPMO injected embryos were more severe, with Bcl6a-deficient embryos possessing obvious holes in the posterior of the eye through which retinal and RPE tissue were extruded into the forebrain (Fig. 2K–N). Histological analyses of the bcl6a-SPMO and bcl6a-MM injected embryos highlight the coloboma in Bcl6a-deficient embryos. While retinal lamination, lens and RPE formation appeared to be largely unaffected (Fig. 2J), all retinal cell types were present in the Bcl6-deficient embryos (data not shown), colobomata were obvious wherein a large region of the choroid fissure remained open (Fig. 2I and J).

Previous studies in Xenopus have demonstrated that BCL6 is required for left-right patterning during development (25); in this study, morpholino knockdown resulted in defects in heart positioning and gut rotation. To determine if Bcl6a functions similarly in zebrafish, cmlc2 expression was examined, which serves as a marker of left/right position of the developing heart. 100% of
bcl6a-MM injected embryos possessed a left-sided orientation of the heart (n = 41 embryos), whereas bcl6a-SPMO injected embryos possessed a randomized orientation of heart, with embryos presenting with left-sided (53.7%), right-sided (31.9%) or no orientation (14.4%) (n = 41 embryos; P < 0.01) (Supplementary Material, Fig. S2).

Morpholino injection can cause off-target effects in a sequence-specific manner (61). To determine whether colobomata in bcl6a-SPMO and bcl6a-ATGMO injected embryos were specific to the knockdown of Bcl6a, bcl6a-SPMO and bcl6a-ATGMO were co-injected at sub-threshold levels (i.e. those that do not lead to a coloboma when injected alone) and phenotypes assessed. When the injection amount of bcl6a-SPMO was reduced from 7.5 to 2.5 ng, no embryos showed colobomata. Similarly, when bcl6a-ATGMO was injected at 2.5 ng/embryo, development was normal (Supplementary Material, Fig. S3). However, co-injection of 2.5 ng bcl6a-SPMO and 2.5 ng bcl6a-ATGMO resulted in colobomata in all embryos (n = 23) (Supplementary Material, Fig. S3). Furthermore, co-injection of 250 pg bcl6a mRNA with 7.5 ng bcl6a-SPMO was highly effective in reducing the incidence of colobomata in bcl6a-SPMO injected embryos (Fig. 3A–D). In bcl6a-SPMO injected embryos, 65.2% possessed colobomata, and co-injection of bcl6a mRNA suppressed this to 11.3% (P < 0.01) (Fig. 3D). Taken together, these data support the notion that colobomata in bcl6a morpholino-injected embryos are a specific result of Bcl6a deficiency and not due to morpholino toxicity.

Previous studies have reported that Bcor deficiency in zebrafish and Xenopus laevis embryos resulted in colobomata, indicating a conserved function for Bcor among these vertebrates (8,11). Thus, we investigated whether Bcl6 function was also required during Xenopus laevis eye formation. Injection of a translation blocking morpholino (xbcl6-MO) targeting Xenopus bcl6 into one dorsal blastomere at the four-cell stage, whose cells are fated to give rise to CNS and eye field (62), resulted in colobomata in 76% of injected embryos (n = 25) when compared with control-injected embryos, and the contralateral uninjected side (Fig. 3E–H). Combined, these results strongly support a model in which Bcl6 function is required for normal eye development in both zebrafish and Xenopus embryos; loss of Bcl6 function in either system results in colobomata.

**Bcl6a prevents cell death in the retina by suppressing p53 expression**

BCL6 is required to prevent p53-dependent apoptosis that results from hypermutation and chromosome rearrangement in germinal center B cells (22). Thus, we reasoned that Bcl6a may play a similar role in the eye, and that elevated apoptosis may be the cellular mechanism leading to colobomata in Bcl6a-deficient embryos. To begin testing this hypothesis, TUNEL analyses were performed to detect apoptotic cells in bcl6a-MM and bcl6a-SPMO injected embryos at 48 hpf, a time at which there are typically no apoptotic cells in the retina of wild-type embryos (63). Indeed, bcl6a-MM injected embryos possessed no apoptotic cells (100%, n = 17), while there were numerous apoptotic cells in most bcl6a-SPMO injected embryos (76%, n = 21) (Fig. 4A and B). We next quantitatively assessed whether Bcl6a regulates p53 expression in zebrafish. Quantification of p53 expression at 48 hpf by qRT–PCR revealed that p53 levels were 2.3 times higher in bcl6a-SPMO injected embryos than in bcl6a-MM injected embryos (P < 0.0001) (Fig. 4C). To determine whether increased p53 levels were responsible for colobomata in bcl6a-SPMO injected embryos, we attempted to rescue the phenotype by reducing p53 levels through morpholino knockdown. Co-injection of 7.5 ng bcl6a-SPMO and 7.5 ng bcl6a-MM (as a ‘load’ control) resulted in colobomata, as in previous experiments (Fig. 4F and G). However, all embryos co-injected with 7.5 ng bcl6a-SPMO and 7.5 ng p53 morpholino (p53-MO) developed normally (n = 25; Fig. 4H and I), indicating that reduction of p53 rescued colobomata. These data support a model in which Bcl6a functions as a regulator of apoptosis by controlling p53 expression in the developing optic cup, and that Bcl6a-dependent repression of p53 is required to prevent apoptosis during normal eye development.
Overproliferation within the retinal neuroepithelium has been shown to be a cause of colobomata in mice (64), and reduced proliferation has been hypothesized to also lead to colobomata (1). Thus, we wanted to determine whether retinoblast proliferation was affected in Bcl6a-deficient embryos. To quantify retinoblast proliferation, we stained embryos for phosphohistone H3 (pH3), a marker of late G2/M phase and quantified the number of pH3+ cells/retinal area. No differences in proliferation were identified between bcl6a-SPMO and bcl6a-MM injected embryos at 52 hpf (Supplementary Material, Fig. S4), indicating that changes in retinoblast proliferation are not likely to underlie colobomata in Bcl6a-deficient embryos.

Vax1 and Vax2 regulate the expression of bcl6a in the ventral retina

vax1 and vax2 are expressed in the optic stalk and the ventral optic cup, and their expression domains overlap spatially and temporally with that of bcl6a (40). A loss of Vax1 and Vax2 function results in colobomata in mouse, combined morpholino knockdown in zebrafish also results in colobomata (40,52–55) and Vax2 has been recently shown to regulate fadd expression to prevent cell death and colobomata in zebrafish (56). Thus, we hypothesized that Vax1 and Vax2 may function as upstream regulators of bcl6a during eye development. To begin testing this hypothesis, in situ hybridization was utilized to determine whether bcl6a expression required Vax1 and Vax2 activity. While bcl6a was expressed in the ventral retina of control morpholino (conMO) injected embryos at 48 hpf, bcl6a was not detected in the retina of vax1 and vax2-MO injected embryos (Fig. 5A and B). To determine whether Vax2 directly regulates bcl6a expression, chromatin immunoprecipitation (ChIP) analyses were performed. A consensus zebrafish Vax2 binding sequence (TCATTA) was identified bioinformatically 214 bp upstream of the translation start site of bcl6a, and this region was utilized in qRT–PCR assays to quantify enrichment in ChIP samples. While a Vax2 antiserum that recognizes zebrafish protein has been reported in the literature (56), we were unable to validate a specific interaction when using this serum. Thus, we utilized an alternative approach, that is by generating a myc-tagged version of vax2 and injecting this into zebrafish embryos, we then performed ChIP using an anti-Myc antibody, an approach that has been successfully utilized in numerous other ChIP assays when cross-reactive antibodies are unavailable (65–67). Expression of myc-vax2 and anti-Myc ChIP resulted in a 2.23-fold enrichment by qRT–PCR of the Vax2 binding site containing region when compared with a ChIP sample isolated using a negative control antibody (Fig. 5C; Figure 2. Knockdown of Bcl6a causes colobomata in zebrafish. (A, C and D) bcl6a-MM and (B, E – H) bcl6a-SPMO injected embryos at 3 dpf imaged (A–C, E and G) laterally and (D, F and H) ventrally. Transverse histological sectioning of (I) bcl6a-MM and (J) bcl6a-SPMO injected embryos. 7.5 ng injection of bcl6a-SPMO targeting bcl6a transcript resulted in curved bodies and colobomata. (K and L) bcl6a-MM and (M and N) bcl6a-SPMO injected embryos at 5 dpf imaged (K and M) ventrally, and (L and N) laterally. By 5 dpf, (M and N) colobomata in bcl6a-SPMO injected embryos are more severe, with (M) Bcl6a-deficient embryos displaying extruded retina and RPE tissue into the forebrain and (N) an obvious non-pigmented ‘hole’ in the eye, when imaged through the lens. (O) Efficiency of bcl6a-SPMO in disrupting bcl6a splicing validated by RT–PCR in bcl6a-MM injected and bcl6a-SPMO injected embryos. Injection of bcl6a-SPMO resulted in the inclusion of intron2 (100 bp) into bcl6a transcripts, creating a frameshift and premature stop codon when translated. Dorsal is up in A–C, E, G, I, J, L and N. Anterior is up in D, F, H, K and M.)
Moreover, no enrichment was detected when a non-target region (∼1 kb upstream) lacking a Vax2 binding sequence was probed in the anti-Myc ChIP sample (Fig. 5C).

As previously reported, vax1 and vax2-MO injected embryos possessed coloboma (Fig. 5E–H). If Bcl6a mediates some of the downstream effects of Vax1 and Vax2 activity during eye development, we reasoned that Vax1 and Vax2 morpholino-injected embryos would also possess elevated apoptosis and p53 levels. TUNEL analysis at 48 hpf showed apoptotic cells in the retinas of 68% of the vax1 and vax2-MO injected embryos (n = 19), while no apoptotic cells were detected in the retinas of conMO injected embryos (n = 21) (Fig. 5I and J).

Quantitative analyses of p53 levels show a 2.3-fold increase in bcl6a-SPMO injected embryos over bcl6a-MM controls. (P < 0.0001). (D and E) Embryos injected with 15 ng of bcl6a-MM develop normally. (F and G) Embryos injected with bcl6a-SPMO (7.5 ng) and bcl6a-MM (7.5 ng) present with coloboma (arrows). (H and I) Co-injection of p53-MO (7.5 ng) with bcl6a-SPMO (7.5 ng) suppresses coloboma (arrows).

Figure 4. Loss of Bcl6a function results in increased apoptosis and p53 expression, and this is responsible for colobomata. TUNEL analysis in (A) bcl6a-MM and (B) bcl6a-SPMO injected embryos at 48 hpf. Bcl6a-deficient embryos possess elevated apoptosis in the retina. Apoptotic cells are detected throughout the retina, surrounding the chorioid fissure, and in the lens. (C) Quantitative analyses of p53 levels show a 2.3-fold increase in bcl6a-SPMO injected embryos over bcl6a-MM controls. (P < 0.0001). (D and E) Embryos injected with 15 ng of bcl6a-MM develop normally. (F and G) Embryos injected with bcl6a-SPMO (7.5 ng) and bcl6a-MM (7.5 ng) present with coloboma (arrows). (H and I) Co-injection of p53-MO (7.5 ng) with bcl6a-SPMO (7.5 ng) suppresses coloboma (arrows).

qRT–PCR data revealed that p53 expression in vax1 and 2-MO injected embryos was 4.4 times higher than that in conMO injected embryos (P < 0.0001) (Fig. 5D). To determine whether elevated p53 levels in Vax1 and Vax2-deficient embryos contribute to increased apoptosis and coloboma, we determined whether these defects could be rescued by reducing p53 levels through morpholino knockdown, as was successful for Bcl6a morphants. Indeed, 86% of vax1 and 2-MO injected embryos co-injected with p53-MO developed normally, with no coloboma (Fig. 5K and L) and no detectable apoptotic cells (n = 22; Fig. 5M). These results support a model in which Vax1 and Vax2 function as upstream regulators of bcl6a, with Vax2 directly binding to the upstream regulatory elements of bcl6a, and that this regulatory network functions to repress p53 expression and thereby block apoptosis in the developing optic cup.
Bcor and Bcl6a functionally interact to prevent coloboma

Ng et al. reported coloboma in bcor-MO injected embryos (8), and we verified this observation at 3 dpf after injection of 1 ng of bcor-MO (Fig. 6A and B). The eyes from Bcor-deficient embryos showed severe coloboma, with many displaying medial displacement of the eye, as well as extruded retina/RPE tissue into the forebrain, making many appear as if they entirely lacked ventral retina (Fig. 6B and D). We hypothesized that Bcor deficiency would also lead to elevated p53 levels and apoptosis, given its known functional interactions with Bcl6a in mediating Bcl6a-dependent transcriptional repression. TUNEL analysis at 48 hpf demonstrated elevated apoptosis in the retina of bcor-MO injected embryos, while no apoptotic cells were detected in the retina of conMO injected embryos (Fig. 6E and F). qRT–PCR revealed a 5.4-fold increase of p53 expression in bcor-MO injected embryos over that in conMO injected embryos (P < 0.0001) (Fig. 6G). These results demonstrate that Bcor deficiency results in similar molecular phenotypes to Bcl6a deficiency, and suggest that Bcor may function in vivo as a cofactor for Bcl6a during normal eye development.

In vitro studies have demonstrated that Bcor coinmunoprecipitates with Bcl6 in B cells, suggesting that Bcor is found in a physical complex along with Bcl6 (15). We tested several polyclonal antibodies derived to Bcl6a and Bcor but could not find any that cross-reacted with the zebrafish proteins that would allow us to test this interaction biochemically. Thus, to determine whether Bcor functionally interacts with Bcl6a during eye development, we performed a synergistic interaction analysis. In these experiments, sub-threshold levels of different transcript-targeting morpholinos are injected to generate sensitized conditions, but no phenotypes. Then, embryos co-injected with both morpholinos at these sub-threshold levels are assayed for phenotypic defects; if defects are observed, a functional interaction between the two gene products in vivo is supported. To generate sensitized conditions, the amount of injected bcl6a-SPMO was reduced from 7.5 to 1.5 ng, and the amount of injected bcor-MO was reduced from 1 to 0.5 ng. These sub-threshold levels did not result in any observable phenotypes (Fig. 6H–M). Co-injection of these sub-threshold levels of bcl6a-SPMO and bcor-MO, however, resulted in coloboma in 68% of injected embryos (n = 31) (Fig. 6N and O). Thus, synergistic interaction data support a model in which Bcl6a and Bcor functionally interact during normal eye development to prevent coloboma.

Rnf2 and Bcor functionally interact to prevent coloboma

As discussed above, Rnf2 is a component of the Bcor repression complex (15). Rnf2 possesses E3 ubiquitin ligase activity and is thought to function as an effector protein of the Bcor complex wherein it helps to maintain chromatin in a transcriptionally repressed state by monoubiquitinating H2A. Interestingly, enriched monoubiquitinated H2A has been detected at the BCL6 target genes Cyclin D2 and p53 in B cells. rnf2 is expressed in the zebrafish retina during development (68). Injection of 4 ng of an rnf2-MO targeting the 5′UTR of rnf2 mRNA resulted in coloboma, as well as heart edema, and an absence of pectoral fins, defects previously reported in rnf2 mutants and morphants (68) (Fig. 7A and B). Given the similarity in the phenotype to Bcl6a- and Bcor-deficient embryos, and its association with Bcor in the Bcor repression complex, we hypothesized that Rnf2 deficiency would also lead to elevated p53 levels and apoptosis in the developing retina. Indeed, TUNEL analysis at 48 hpf demonstrated elevated apoptosis in the retinas of rnf2-MO injected embryos, while no apoptotic cells were detected in the retina of control embryos (Fig. 7C and D).
qRT–PCR revealed a 1.8-fold increase of p53 expression in \textit{rnf2}-MO injected embryos over that in conMO injected embryos ($P < 0.0001$) (Fig. 7E).

To determine whether Rnf2 functionally interacts with Bcor during eye development, we performed synergistic interaction analyses, as above. To generate sensitized conditions, the amount of injected \textit{rnf2}-MO was reduced from 4 to 2 ng, and the amount of injected \textit{bcor}-MO was reduced from 1 to 0.5 ng. These sub-threshold levels did not result in any observable phenotypes (Fig. 7F – K). Co-injection of these sub-threshold levels of \textit{rnf2}-MO and \textit{bcor}-MO, however, resulted in colobomata in 86% of injected embryos ($n = 22$) (Fig. 7L and M). These data support a model in which Rnf2 functions \textit{in vivo} as an effector of the Bcl6a-recruited Bcor complex during normal eye development to prevent apoptosis and colobomata.

**Bcl6a and Bcor functionally interact with Hdacs to prevent colobomata**

As discussed above, the transcriptional repressive function of Bcl6 requires Hdac activity in cell culture (69), and mutations in zebrafish\textit{hdac1} result in colobomata (Fig. 8A and B).
Moreover, Hdac1 is found associated with the Bcor repressive complex, along with Bcl6 (10). Thus, we hypothesized that Hdac function might also act as a mediator of Bcl6a and Bcor activity in the developing eye. To test this hypothesis, we first determined whether a mutation in \textit{hdac1} results in elevated \textit{p53} expression and apoptosis in the developing retina. Indeed, \textit{p53} levels were significantly higher in \textit{hdac1} knockout embryos than in wild-type controls at 48 hpf (\( P < 0.0001 \)). (E) Quantitative analysis by qRT–PCR shows a 1.8-fold increase of \textit{p53} levels in \textit{rnf2-MO} injected embryos (\( P < 0.0001 \)). (F and G) Embryos with wild-type levels of \textit{Rnf2} and Bcor develop normally. (H and I) Reducing the levels of Bcor by injecting sub-threshold amounts of \textit{bcor-MO} (0.5 ng) has no effect on normal development. (J and K) Reducing the activity levels of \textit{Rnf2} by injecting sub-threshold amounts of \textit{rnf2-MO} (2 ng) has no effect on normal development. (L and M) Co-injection of sub-threshold amounts of \textit{rnf2-MO} (2 ng) and \textit{bcor-MO} (0.5 ng) results in colobomata (arrow in M). Dorsal is up in all panels.

Figure 7. Knockdown of \textit{Rnf2} results in colobomata, increased apoptosis and elevated \textit{p53} levels, and \textit{Rnf2} and Bcor functionally interact in the eye. (A and B) Knockdown of \textit{Rnf2} results in colobomata (arrow in B). TUNEL analysis in (C) conMO and (D) \textit{rnf2-MO} injected embryos at 48 hpf. \textit{Rnf2}-deficient embryos present with an increased number of apoptotic cells in the retina. (E) Quantitative analysis by qRT–PCR shows a 1.8-fold increase of \textit{p53} levels in \textit{rnf2-MO} injected embryos (\( P < 0.0001 \)). (F and G) Embryos with wild-type levels of \textit{Rnf2} and Bcor develop normally. (H and I) Reducing the levels of Bcor by injecting sub-threshold amounts of \textit{bcor-MO} (0.5 ng) has no effect on normal development. (J and K) Reducing the activity levels of \textit{Rnf2} by injecting sub-threshold amounts of \textit{rnf2-MO} (2 ng) has no effect on normal development. (L and M) Co-injection of sub-threshold amounts of \textit{rnf2-MO} (2 ng) and \textit{bcor-MO} (0.5 ng) results in colobomata (arrow in M). Dorsal is up in all panels.

(29,30). Moreover, Hdac1 is found associated with the Bcor repressive complex, along with Bcl6 (10). Thus, we hypothesized that Hdac function might also act as a mediator of Bcl6a and Bcor activity in the developing eye. To test this hypothesis, we first determined whether a mutation in \textit{hdac1} results in elevated \textit{p53} expression and apoptosis in the developing retina. Indeed, \textit{p53} levels were significantly higher in \textit{hdac1} knockout embryos than in wild-type controls at 48 hpf (\( P < 0.0001 \)) (Fig. 8E), and \textit{hdac1} knockout embryos possessed numerous apoptotic cells in their retinas (Fig. 8C and D). TSA is a chemical inhibitor of histone deacetylase activity, and previous studies have shown that TSA treatment (1200 nM, 14 hpf–48 hpf) phenocopies coloboma observed in \textit{hdac1} mutants (30). To determine whether functional interactions existed between Hdacs, Bcl6a and Bcor, we utilized subthreshold TSA exposures and performed synergistic interaction analyses, as above. Embryos treated with 700 nM TSA from 24 to 72 hpf (sub-threshold condition) developed normally and did not show colobomata (Fig. 8F–I; 9A–D). Embryos injected with 2 ng of \textit{bcl6a-SPMO} also developed normally (Fig. 8J and K). However, treatment of 2 ng \textit{bcl6a-SPMO} injected embryos with 700 nM TSA from 24 to 72 hpf resulted in embryos resembling \textit{hdac1} knockout embryos, and their defects included obvious colobomata (81%, \( n = 27 \)) (Fig. 8L and M). Similarly, embryos injected with sub-threshold...
amounts of bcor-MO (0.5 ng) developed normally (Fig. 9E and F). When 0.5 ng bcor-MO injected embryos were treated with 700 nm TSA from 24 to 72 hpf, they also possessed colobomata (86%, n = 29) (Fig. 9G and H). TSA is a broad-spectrum Hdac inhibitor so we next sought to determine whether Hdac1 specifically was required along with Bcor for normal eye development. To achieve this, we utilized hdac1+/− embryos as a sensitized condition, as these do not possess colobomata. Injection of sub-threshold bcor-MO into embryos from an hdac1+/− × WT cross resulted in 47.7% of the injected embryos (n = 44) possessing colobomata (Fig. 9I and J). Genotyping of five embryos with colobomata confirmed that all of them were genotypically hdac1+/−. These data strongly support a model in which Hdac1 functionally interacts with Bcor during eye development and that, along with Bcl6a, act together to mediate normal optic cup formation by preventing colobomata.

**DISCUSSION**

A number of factors have been identified that lead to colobomata when mutated in human patients or from mutants, knockouts or loss-of-function experiments in a variety of animal model systems, but the molecular and cellular mechanisms underlying the colobomata in most of these cases have not been resolved (1,35). Here, we add another gene to the growing list of those
required to prevent colobomata—bcl6a—and we demonstrate that it acts downstream of Vax1 and Vax2, well-known regulators of ventral optic cup development (40,52–55). Our data support a model in which bcl6a is a direct target of Vax2, where it functions to repress p53 expression and prevent apoptosis in the developing optic cup. Furthermore, our data demonstrate that Bcl6a acts along with its co-repressor, Bcor, to prevent colobomata, and that both require Rnf2 and Hdac activity in order to achieve this (Fig. 10).

Bcl6 function is required in a variety of contexts to suppress apoptosis (23). Most notably is during immune system development when Bcl6 represses p53-dependent apoptosis that would otherwise result from DNA damage induced by immunoglobulin class switching and somatic hypermutation (22). Bcl6 has also been shown to repress the programmed cell death 2 gene (PDCD2) in lymphoma cell lines, and its expression is inverse to that of PDCD2 in lymphocytes and follicular mantle cells in vivo (70), suggestive of a critical role during immune system development and function. Bcl6 knockout mice display elevated levels of apoptosis during spermatogenesis (71), and Bcl6 is required for the survival of differentiating olfactory neurons (24). Indeed, in many cell types that express Bcl6,
terminal differentiation requires that apoptosis be inhibited, and it is thought that Bcl6 may mediate this inhibition (72–76). In various cell lines, sustained expression of Bcl6 confers a pro-survival effect, in some cases leading to oncogenic transformation (77). Importantly, however, sustained expression of Bcl6 is context-dependent as other cell lines and tissue types undergo apoptosis when Bcl6 levels are elevated (23).

During eye development in zebrafish, there are very few detectable apoptotic cells (63,78), indicating that either apoptosis is not critical for eye development or that it is suppressed in order for the eye to properly form. Data presented here support the latter of these hypotheses. Deletion of the cell death regulator FADD correlates with coloboma in human patients, and loss of fadd function in zebrafish leads to elevated cell death and coloboma (56). Cell death in Fadd-deficient embryos was shown to be necrotic in nature, however, fadd expression is regulated by Pax2 and Vax2, supporting a gene regulatory network in which Pax2 and Vax2 act upstream of fadd to mediate cell survival in the developing optic cup. Our data place also Bcl6a in this cascade, downstream of Vax1 and Vax2, and they implicate Bcor, Rnf2 and Hdac as co-regulators required to suppress cell death in the eye. p53 levels were elevated in embryos deficient for each of these factors, and inhibition of p53 expression rescued coloboma in all of them, supporting an underlying mechanism for coloboma prevention wherein Bcl6a and its co-repressors prevent p53 expression and thus, prevent apoptotic cell death in the developing optic cup. Previous studies in zebrafish and mice lacking Vax1 and/or Vax2 function revealed that coloboma stemmed from a failure to restrict retinal cell fates to the optic cup (40,52–55). Our data, along with that of Viringgipurampeer et al. for fadd (56), suggest an additional role for Vax1 and Vax2 during optic cup development in preventing apoptotic and necrotic cell death that would also lead to coloboma. Cell death is not restricted to the ventral optic cup in any of these perturbations, however. Moreover, while bcl6a expression is restricted to the ventral retina, fadd, bcor, rnf2 and hdac1 are expressed throughout the developing retina (68,79). Thus, cell death outside of the choroid fissure in loss-of-function embryos for each of these factors likely reflects additional pro-survival functions, possibly in combination with other DNA-recruitment factors, functioning similarly to Bcl6a, that are expressed in these retinal regions. Additionally, each of these proteins could function to repress the expression of other, cell non-autonomously acting factors that are pro-apoptotic in the retina.

Bcl6 is known to require co-factors in order to repress transcription of target genes, and these include BCOR and HDACs (10,69). Evidence from cultured cells demonstrates that Bcl6 interacts with Bcor through its POZ/ BTB domain and recruits Bcor with other corepressors to Bcl6 target genes like p53 and cyclin D2 (15). Mutations in BCOR are responsible for OFCD syndrome (8), and loss of BCOR function results in coloboma in zebrafish and Xenopus embryos (8,11). Moreover, loss of either Bcl6 or Bcor function in Xenopus embryos also leads to laterality defects (11,25), and our data indicate conserved functions for Bcl6a; Xenopus embryos deficient in Bcl6 function possess coloboma (Fig. 3G and H), and zebrafish deficient in Bcl6a possess laterality defects (Supplementary Material, Fig. S2). Moreover, the synergistic interaction data reported here for Bcl6a and Bcor indicate that Bcl6a and Bcor functionally interact with each other in vivo, and this interaction is required for normal eye development.

HDACs are known to be involved in silencing transcription through the epigenetic modification of histones, maintaining a transcriptionally inactive chromatin state (80), or through direct regulation of the activity of transcription activators by controlling their cytosolic/nuclear localization, affinities with co-factors or abilities to bind DNA (81). It has also been reported that acetylation of BCL6 by p300 inactivates its function as a transcriptional repressor and prevents it from recruiting HDACs to a repression complex (69). Zebrafish hdac1 mutants possess severe coloboma and defects in cell cycle exit and differentiation of proliferative retinoblasts (29,30). In the developing mouse retina, HDAC activity is involved in regulating expression of the pro-apoptotic factors like Apaf-1 and Caspase3 in order to prevent apoptosis (31,32). Our interaction data between Bcl6a, Bcor and Hdac1 support a model in which these factors functionally interact with each other during eye development to repress p53 expression to prevent apoptosis and coloboma.

Although Bcl6 function, target genes and corepressors have been studied in various contexts, the molecular mechanisms by which Bcl6 acts to suppress transcription remain to be elucidated. Bcl6 recruits the BCOR repression complex to target loci, and this complex also includes FBXL10 and RNF2 (15). RNF2 possesses E3 ubiquitin ligase activity for H2A, which facilitates the maintenance of repressed chromatin and thus, Bcl6-mediated recruitment of an RNF2-containing BCOR complex to target gene promoters could mediate transcriptional repression. Our data support a model in which Rnf2 functionally interacts with Bcor and is required to prevent coloboma. FBXL10, another BCOR complex member, demethylates H3K36 and could represent an additional biochemical mechanism through which Bcl6-mediated regulation leads to transcriptional repression. Interestingly, Fbxl10-deficient mice possess retinal coloboma and exencephaly correlating with increased cell death of neural progenitor cells (82). It will be of interest
to determine the molecular mechanisms through which Bcl6a acts in order to repress p53 and prevent apoptosis and coloboma during optic cup formation, and to determine whether Bcl6a’s functions are also mediated through the Bcor complex member, Fbxl10 in mediating this repression.

MATERIALS AND METHODS

Zebrafish maintenance and strains

Zebrafish (Danio rerio) were maintained at 28.5°C on a 14 h light/10 h dark cycle. Embryos were obtained from the natural spawning of wild-type (AB) fish setup in pairwise crosses. Embryos were collected and raised at 28.5°C after Westerfield (1995) and were staged according to Kimmel et al. (1995). hda- c1 hi1618Tg outcrosses were provided by the Zebrafish International Resource Center at the University of Oregon and were propagated by repeated outcrosses to AB fish. All animals were treated in accordance with provisions established at the University of Texas at Austin governing animal use and care. hda- c1 hi1618Tg mutants were genotyped by PCR (forward primer: 5′-CTG ACC ATC TGA ACT TCT CTA TTC-3′) (reverse primer: 5′-CAC GCC TAC AGT GAT GGA ACC-3′).

Histology

Histology was performed as described in Nuckels and Gross (2007). Briefly, embryos were collected and fixed overnight at 4°C in a solution of 1% (w/v) paraformaldehyde (PFA), 2.5% glutaraldehyde and 3% sucrose in phosphate buffered saline (PBS). They were washed three times for 5 min (min) in PBS, re-fixed for 90 min at 4°C in a 2% OsO4 solution, washed three times for 5 min in PBS at room temperature (RT) and dehydrated through a graded ethanol series (50, 70, 80, 90, 2× 100%). Embryos were further dehydrated two times 10 min in propylene oxide and infiltrated 1–2 h in a 50% propylene oxide/50% Epon/Araldite mixture (Polysciences, Inc.). Embryos were then incubated overnight at RT in 100% Epon/Araldite resin with caps open to allow for propylene oxide evaporation and resin infiltration, embedded and baked at 60°C for 2–3 days. Sections 1–2.5 µm were cut, mounted on glass slides and stained in a 1% methylene blue/1% borax solution. Sections were mounted in DPX (Electron Microscopy Sciences) and photographed with a Leica DMRB microscope mounted with a DFC480 digital camera.

Morpholinos and mRNA injections

bcl6a-SPMO (5′-GCA AGC CCT CAA TTG TAC CTG C-3′), bcl6a-ATGMO (5′-TTG CGG CTG CAC AAG CCA TTT TTT C-3′), bcl6a-MM (5′-GGA AGC ACC CTT GAA TTC TAC GTG C-3′), hecor-MO (5′-AGC TCT ACC GGA AAG AAA ACA C-3′) (8), xbccl6-MO (5′-TTG AGT TTG AGA TGC CAT AGT GCC C-3′), p53-MO (5′-GCC CCA TTT TCG TGC AAG AAT TG3′-3′) (83), vax1-MO (5′-GTC TTG ACT GTA GCG GAC TTC CAT A-3′) (40), vax2-MO (5′-ATG TGT CCA AGT TTT CTT TCT GAG C-3′) (40), ryn2-MO (5′-ACA CCA CCG TTT TTA TCT CCA TGT T-3′) (68) and conMO (5′-CCT TTT ACC TCA GGT ACA ATT TAT A-3′) were purchased from Gene Tools (Philomath, OR, USA). MOs were resuspended in water and injections were performed at the one-cell stage into wild-type Oregon AB embryos.

Full-length zebrafish bcl6a was sub-cloned from clone 4145622 (Open Biosystems) for mRNA synthesis (details available upon request). Capped mRNA synthesized using mMESSAGE mMACHINE (Ambion).

Riboprobe, in situ hybridization and immunohistochemistry

Hybridizations were performed essentially as described by Jowett and Lettice using digoxigenin-labeled antisense RNA probes (84). p53 was cloned from 24 hpf complementary DNA (cDNA), ligated into pGEM-T and used for probe synthesis (cloning details available upon request). Probe synthesis constructs for bcl6a were purchased from Open Biosystems.

Immunohistochemistry with pH3 antiserum (Millipore) was performed as described by Uribe and Gross (2007). Imaging was performed on a Zeiss LSM5 Pascal laser scanning confocal microscope. Five to eight optical sections (1 µm in thickness) were collected and projected using Zeiss confocal software.

Trichostatin A (TSA) treatment

TSA stock solution (Sigma, 1 mg/ml in DMSO) was diluted in fish water for use. Embryos were incubated in TSA-containing fish water from 24 to 72 hpf and washed three times in fish water before imaging.

Preparation of Xenopus embryos and morpholino injection

Xenopus laevis embryos were de-jellied using a 3% cysteine solution in 1/3 × MMR (Marc’s Modified Ringers). xbcl6-MO was injected into one of dorsal cells at the four-cell stage and embryos were grown until stage 41 when they were fixed with 1× MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde, pH 7.4), and imaged on a Leica MZ16F microscope mounted with a DFC480 digital camera.

Quantitative real-time RT–PCR (qRT–PCR)

Total RNA was extracted from embryos at 48 hpf using TRIzol reagent (Invitrogen). The total RNA was reverse-transcribed using an iScript™ cDNA Synthesis kit (BIO-RAD). Relative gene expression levels were quantified using Power SYBR Green dye (Applied Biosystems) and an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.3 software). All samples were analyzed in triplicate and relative transcript abundance was normalized to expression of β-actin using the 2-ΔΔCT method (85). Primers used to assay gene expression were designed across exon boundaries of the genes. Primers were as follows: p53 forward 5′-CCC ATC TCT ACA ATC ACT CTG G-3′, reverse 5′-TCT CCT CAG TTT TCG TCT TGC C-3′; β-actin forward 5′- CCA AAG CCA ACA GAG AGA TGA C-3′, reverse 5′- TAC AGA GAG AGC ACA GCC TGG ATG -3′. qRT–PCR data were analysed by Student’s t-test for statistical significance.
Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a protocol modified by Wardle et al. (86). For exogenous Vax2 expression, embryos were injected with 170 pg of myc-tagged zebrafish vax2 mRNA (subcloning details available upon request). Approximately 200 injected embryos were dechorionated and then cross-linked with 1.8% formaldehyde in fish water and incubated for 20 min at RT. Formaldehyde was deactivated by adding 2.5 M glycine (125 mM final concentration). Cross-linked embryos were homogenized in ChIP dilution buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl, and protease inhibitors) with a 30G syringe and then sonicated using bioruptor (Diagenode) with conditions of: high power, 30 s on and 1 min off for 30 min. The lysate, containing an average size of 300–500 bp DNA fragments, was precleared with protein A agarose beads at 4°C for 3 h to minimize nonspecific binding. Precleared chromatin was incubated with an anti-Myc tag antibody (ab9106, Abcam) or an anti-fibronectin antibody (sc-9068, Santa Cruz Biotechnology) (as a negative control) at 4°C overnight. Protein–DNA complexes were pulled down using prewashed protein A agarose beads at 4°C for 6 h. Immunoprecipitated complexes were washed twice for 5 min with a low salt wash buffer (1% Triton X-100, 1 mM EDTA, 50 mM HEPES pH 7.5, 0.1% sodium deoxycholate, 150 mM NaCl), once with a high-salt wash buffer (1% Triton X-100, 1 mM EDTA, 50 mM HEPES pH 7.5, 0.1% sodium deoxycholate, 500 mM NaCl), once with a LiCl wash buffer (250 mM LiCl, 0.5% NP-40, 1 mM EDTA, 0.5% sodium deoxycholate, 10 mM Tris-Cl pH 8.1), and twice with TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA). SDS elution buffer was applied to the washed sample to recover protein–DNA complexes at 4°C overnight. Protein–DNA complexes were resuspended in 20 μl of Tris-Cl pH 7.5, 1 mM EDTA). SDS elution buffer. RNA and Proteins were removed by treating with RNase A and Proteinase K followed by phenol–chloroform extraction and ethanol precipitation. The DNA pellet from ethanol precipitation was resuspended in 20 μl of TE buffer. Three separate ChIP experiments were performed with three separate batches of injected embryos (i.e. three biological replicates). Enriched DNA was utilized for qRT–PCRs. Primers were as follows: Target forward 5’-CGT GTT TTG TTG TAA GCT TGC ATT TCT TCG G-3’, reverse 5’-GTG CCA CTA TTT TTT TCA ATC GTT ACG GC-3’; negative control forward 5’-CCC TCA CGT TTA AAC TCG ATG ACA G-3’, reverse 5’-CAA AGA CCG TTC TCA AGC AGC AAA CAG-3’. Relative abundance was normalized to expression of β-actin and compared between ChIPs using anti-Myc and ant-Fibronectin antibodies. qRT–PCR data were analyzed by Student’s t-test for statistical significance.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis

Apoptotic cells were examined by TUNEL. Embryos were fixed overnight in 4% paraformaldehyde at 4°C, washed three times with PBS and dehydrated in 100% methanol at −20°C. Rehydration of the embryos through a degraded methanol/PBS series (50, 30, 0%) for 5 min each at RT was followed by incubation in 100% acetone at −20°C (12 min) and three washes in PBS containing 0.1% Tween-20 (PBST) for 5 min each. The embryos were permeabilized with 10 μg/ml protease K for 25 min at RT, followed by 4% parafomaldehyde and three rinses in PBST. The embryos were assayed by TUNEL using the In Situ Cell Death Detection kit, AP (Roche), and the images were examined by light microscopy on a Leica MZ16F microscope mounted with a DFC480 digital camera.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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