The Tumor Suppressor Gene Retinoblastoma-1 Is Required for Retinotectal Development and Visual Function in Zebrafish

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Abstract

Mutations in the retinoblastoma tumor suppressor gene (rb1) cause both sporadic and familial forms of childhood retinoblastoma. Despite its clinical relevance, the roles of rb1 during normal retinotectal development and function are not well understood. We have identified mutations in the zebrafish space cadet locus that lead to a premature truncation of the rb1 gene, identical to known mutations in sporadic and familial forms of retinoblastoma. In wild-type embryos, axons of early born retinal ganglion cells (RGC) pioneer the retinotectal tract to guide later born RGC axons. In rb1 deficient embryos, these early born RGCs show a delay in cell cycle exit, causing a transient deficit of differentiated RGCs. As a result, later born mutant RGC axons initially fail to exit the retina, resulting in optic nerve hypoplasia. A significant fraction of mutant RGC axons eventually exit the retina, but then frequently project to the incorrect optic tectum. Although rb1 mutants eventually establish basic retinotectal connectivity, behavioral analysis reveals that mutants exhibit deficits in distinct, visually guided behaviors. Thus, our analysis of zebrafish rb1 mutants reveals a previously unknown yet critical role for rb1 during retinotectal tract development and visual function.

Introduction

Biallelic mutations in the retinoblastoma susceptibility gene rb1 are causal for intracocular childhood retinoblastomas. Rb1 is a member of a gene family that consists of three members, p105/Rb1, p107/Rb-like1, and p130/Rb-like2, collectively known as "pocket proteins" [1]. The activity of these proteins is controlled, in part, by cyclin/cyclin-dependant kinase complexes. Upon activation, Rb proteins bind to an array of proteins, including members of the E2F family of transcription factors to execute a range of cellular functions, including cell cycle exit, terminal differentiation, and cortical cell migration [2]. In humans, germline or somatic mutations occur throughout the 180 kb genomic region spanning the rb1 gene, including its promoter region, exons, and intronic essential splice sites, resulting in bilateral or unilateral retinoblastomas within the first 2 years of life [3,4].

Given its clinical relevance, the role of Rb1 during embryonic development and during tumor suppression has been studied intensely, mostly using mouse models [5]. Rb1 is expressed ubiquitously during murine development, postnatally, and continues to be expressed in adults. Embryos harboring non-conditional Rb1 knockout alleles exhibit ectopic proliferation and apoptosis throughout the nervous system and die premortally at embryonic day 14.5 [6,7,8]. Embryos with conditional loss of Rb1 in the retina display ectopic division and considerable apoptosis of retinal transition cells starting at E10 [9,10,11,12]. Retinas in these animals contain reduced numbers of rods, bipolar cells, and RGCs, yielding a retina with a thin outer nuclear layer and a hypoplastic optic nerve. However, the etiology of optic nerve hypoplasia and if/how Rb1 functions in RGC axonal guidance has not been examined. Similarly, electroretinogram recordings from Rb1 deficient mouse retinas have revealed reduced photoreceptor to bipolar to amacrine signal transmission [10], yet the behavioral consequences have not been examined.

Here, we report that zebrafish space cadet mutants carry a rb1 mutation found in cases of sporadic and familial human retinoblastoma [13,14,15,16]. In zebrafish rb1 mutants, RGC precursors show delayed exit from the cell cycle and hence a delay in the generation of early-born, postmitotic RGCs, whose axons are critical for pioneering the retinotectal tract. This delay leads to RGC intrinsic axon guidance defects, aberrant retinotectal connectivity, and deficits in phototactic behaviors. Together, this work describes a novel model for understanding the developmental role of rb1 and reveals a previously unknown role for rb1 in the formation of the retinotectal tract.

Results

space cadet harbors a disease causing mutation in rb1

We previously identified two mutant space cadet alleles, based on abnormal startle response behavior to acoustic or tactile stimuli...
Defects in the cDNAs isolated from rb1 between humans, mice and zebrafish (Figure 1B). Sequencing of cysltr2 interval contains several annotated genes, including binants/2688 meioses, respectively (Figure 1A). This genomic recombinants/2688 meioses, and in the locus (12 recombinants/2688 meioses), respectively (Figure 1A). This genomic recombinants/2688 meioses), which have retained syntenic positional conservation between humans, mice and zebrafish (Figure 1B). Sequencing of rb1 cDNAs isolated from spc4226a larvae revealed the presence of 4 nucleotides inserted between exon 19 and exon 20. Subsequent sequencing of genomic DNA isolated from spc4226a larvae confirmed a single nucleotide change in the splice donor sequence of intron 19 (nt1912 +1 , to A; Figure 1C). This generates a cryptic splice site donor, resulting in the 4 base pair insertion into the rb1 mRNA. This 4 base pair insertion causes a premature stop codon in exon 20, predicted to truncate the protein at amino acid 677, thereby severely truncating the B domain and cyclin domain essential for Rb1 function (Figure 1D) [15]. Interestingly, identical mutations have been reported in human patients with familial and sporadic forms of retinoblastoma [13,14,15,16].

The zebrafish rb1 gene is 67% similar (52% identical-based on amino acid sequence) to the mouse and human rb1. The Rb1 protein consists of an A and B domain forming Rb1’s binding “pocket”, and a cyclin binding domain (Figure 1D), and shows 81% amino acid similarity (66% identical) between zebrafish and mammalian rb1 in these critical domains. Thus, sequence homology, syntenic conservation, and cDNA sequence analysis provide compelling evidence that space cadet phenotypes are caused by an rb1 gene mutation known to cause retinoblastoma. Sequence analysis of the second mutant space cadet allele did not reveal any changes in the coding sequence or in any of the splice donor and acceptor sites, suggesting that this allele is caused by a regulatory mutation in the rb1 locus. Importantly, analyses of spc4226a and spc4226a mutants revealed no significant differences with regards to the strength of the phenotypes examined below (Table 1). From here on, we will refer to the space cadet allele as rb1 and describe anatomical and behavioral defects in the rb14226a allele.

rb1 mutant embryos show delayed RGC axon outgrowth and reduced tectal innervation

During zebrafish embryogenesis, the earliest born RGCs begin extending axons at 32 hpf, cross the ventral midline of the diencephalon to form the optic chiasm at 36 hpf, and project dorsally to the contralateral optic tectum by 48 h to form a retinotectal pathway critical for mediating visually guided behaviors by 120 hpf [19]. We previously reported that 120 hpf stage rb1 mutants display wild type like retinal lamination and visual circuitry. Before an organism can execute necessary behavioral responses to environmental stimuli, the underlying neural circuits that regulate these behaviors must be precisely wired during embryonic development. A properly wired neural circuit is the product of a sophisticated collaboration of multiple genetic pathways that orchestrate cell type specification, the extension and growth of the cell processes that connect each circuit component, and the refinement of these connections. In an unbiased genetic screen designed to identify the genes required for proper circuit formation in developing zebrafish embryos, we identified a human disease causing mutation in the retinoblastoma-1 (rb1) gene that disrupts the formation of the zebrafish visual circuit. rb1 canonically functions to regulate the cell cycle, and when mutated the loss of rb1-mediated cell cycle control elicits childhood ocular tumor formation. Genetic models of rb1 have been developed to study the developmental role of rb1 in the retina; however, ectopic cell proliferation and death within the retina have largely precluded the ability to evaluate the formation and integrity of neural circuits connecting the retina with the brain. In this study, through genetic and cellular analysis of a zebrafish rb1 mutant, we reveal a novel role for rb1 in regulating the establishment and functionality of the visual circuitry.

[17,18]. Using recombination mapping, we mapped the space cadet allele to a 1.1 cM interval on chromosome 21 between single nucleotide polymorphic markers in the myo5b locus (20 recombinants/2688 meioses), and in the ncam1 locus (12 recombinants/2688 meioses), respectively (Figure 1A). This genomic interval contains several annotated genes, including rb1, lpar6, and cysltr2, which have retained syntenic positional conservation between humans, mice and zebrafish (Figure 1B). Sequencing of rb1 cDNAs isolated from spc4226a larvae revealed the presence of 4 nucleotides inserted between exon 19 and exon 20. Subsequent sequencing of genomic DNA isolated from spc4226a larvae confirmed a single nucleotide change in the splice donor sequence of intron 19 (nt1912 +1 , to A; Figure 1C). This generates a cryptic splice site donor, resulting in the 4 base pair insertion into the rb1 mRNA. This 4 base pair insertion causes a premature stop codon in exon 20, predicted to truncate the protein at amino acid 677, thereby severely truncating the B domain and cyclin domain essential for Rb1 function (Figure 1D) [15]. Interestingly, identical mutations have been reported in human patients with familial and sporadic forms of retinoblastoma [13,14,15,16].

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Figure 1. spc4226a mutation induces a premature stop codon and truncation of Rb1. (A) Recombination mapping places the spc4226a mutant locus within a 1.1 cM interval on chromosome 21 that shows syntenic conservation (B) of rb1, lpar6, and cysltr2 transcripts with human chromosome 13, q14.2 and mouse chromosome 14, d3. (C) rb1 exons depicted as alternating gray/white blocks. spc4226a mutation at splice donor site of intron 19 (nt1912+1) causes frameshift and premature stop codon in exon 20. (D) Estimated Rb1 protein product with truncated B- and cyclin-domains. doi:10.1371/journal.pgen.1003106.g001
expression of terminal RGC cell differentiation markers, but exhibit various RGC axonal pathfinding defects [18]. To determine the temporal onset and spatial site of RGC pathfinding errors in rb1 mutant embryos, we used the ath5:GFP transgenic line expressed in RGCs to examine the development of the retinotectal trajectory [20]. Importantly, between 28–96 hpf we did not detect a difference in the intensity of GFP fluorescence in the retinas of rb1 mutant compared to wild type retinas (Figure 2B and data not shown). At 36 hpf wild type RGCs have exited the retina and pioneered across the ventral midline to form an optic chiasm (n = 40, Figure 2A). In contrast, only 13% (n = 30) of rb1 mutant retinas had RGC axons that exited the retina (Figure 2B, 2C), suggesting that the loss of rb1 function causes a delay in the initial outgrowth of RGC axons from the retina. At 48 hpf the optic nerve in rb1 mutants was significantly thinner, with a mean diameter of 3.04 μm (n = 18), compared to the thicker optic nerves in wild type siblings (15.76 μm, n = 22; Figure 2D–2F). At 72 and 96 hpf, when wild type RGC axons have reached and innervated the optic tectum, rb1 mutant tecta show a significant reduction in RGC axonal tectal innervation (Figure 2G–2L; see Methods for quantification details). Together, these results reveal that rb1 mutants exhibit a delay in RGC axonal outgrowth, leading to a delay in optic nerve development, and reduced innervation of the optic tectum.

Delayed retinotectal development is caused by a near complete loss of rb1 function in RGCs

To determine whether the identified mutation in rb1p1226a is causative of the delay in retinotectal development, we injected wild type rb1 mRNA into one-cell stage rb1p1226a mutants and examined optic nerve diameter at 48 hpf. Microinjection of wild type rb1 mRNA restored optic nerve diameter in rb1 deficient mutants in a dose dependent manner, demonstrating that mutations in zebrafish rb1 cause RGC outgrowth defects (Figure 3A–3C, 3E). To determine if and to which extent the mutant rb1p1226a allele has retained biological activity, we examined the ability of rb1p1226a mRNA to rescue retinotectal development in rb1p1226a mutants (Figure 3D–3E). Injection of rb1p1226a mRNA failed to significantly increase optic nerve diameter in rb1p1226a mutants, suggesting that the rb1p1226a protein product has very limited, if any, functionality. However, we cannot exclude the possibility that the mutant phenotype is ameliorated by maternal rb1 mRNA and/or protein deposition.

Finally, we asked whether Rbl functions within RGCs for their axons to exit from the retina and enter the retinotectal path. Because zebrafish rb1 is expressed ubiquitously throughout development (Figure 4A–4B), we generated chimeric embryos by transplanting cells at the blastula stage between rb1 mutant and wild type embryos, and then examined their ability to exit from the retina (Figure 4C). A significant fraction of axons from genotypically mutant rb1 RGCs transplanted into wild type hosts failed to exit from the retina (19% of retinas showed failure of transplanted rb1 mutant RGC axons to exit, n = 31, Figure 4E–4F), consistent with the low but significant frequency of rb1 mutant retinas in which we observed a complete failure of RGCs to exit from the eye (11%; see below). Conversely, 100% of rb1 mutant retinas showed exit of axons from transplanted wild type RGCs (n = 69, Figure 4D). Thus, during zebrafish development rb1 acts RGC autonomously for axons to exit the retina and to form the optic nerve.

Delayed cell cycle exit of rb1 mutant RGC precursors leads to reduction of “early born” RGCs

Given the RGC intrinsic defects observed in rb1 mutants, we next wanted to determine the primary defect leading to the delay of RGC axons to exit from the retina. Rbl can functionally to regulate cell cycle checkpoints, promoting cell cycle exit and differentiation of progenitors and suppressing cell cycle re-entry of differentiated cells [2]. In the retina, rb1 has been shown to promote the exit of retinal progenitor cells from the cell cycle into the various postmitotic cell types that populate the retinal lamina [9,10,11,12]. To examine rb1 deficient retinas for cell cycle defects, we labeled wild type and rb1 mutant retinas for M-phase positive nuclei with an anti-phosphohistone-H3 antibody (anti-pH3) during the initial phase of RGC birth and axon outgrowth, between 28 and 36 hpf. During this time window, premitotic ath5 positive retinal progenitors divide, with one daughter becoming a postmitotic RGC and the other maintaining its progenitor potency to give rise to other retinal cell types that become postmitotic at later stages of development [21]. Although the total number of M-phase positive increased with time between 28 and 36 hpf in rb1 mutant and wild type retinas, we observed fewer M-phase positive nuclei in rb1 deficient retinas, compared to wild type retinas, at each time point examined (Figure 5).

One possibility is that the reduction of M-phase retinal precursors in rb1 deficient retinas is due to increased cell death. Indeed, compared to wild type retinas, rb1 deficient retinas showed a slight, but significant increase of TUNEL positive nuclei between 28 and 36 hpf (Figure S1). Importantly though, comparing the increased number of TUNEL positive nuclei to the decreased number of pH3 positive nuclei in rb1 mutants at 28, 32, and 36 hpf revealed that apoptosis accounts for only 18–26% of the observed reduction in M-phase positive retinal precursors in rb1 mutant retinas at each time point examined. This suggests that in rb1 mutant retinas cell death contributes only partially to the deficiency of M-phase positive nuclei (Figure S1G). Thus, the reduction in M-phase retinal precursors in rb1 mutant retinas suggests a prolonged terminal cell cycle for the retinal precursors, which need to exit their final cycle to become the earliest population of postmitotic RGCs.

Table 1. Comparison of the retinal phenotypes observed at 36 hpf in the two rb1 mutant alleles.

| Genotype              | Retinas with complete RGC axon exit defects | Mean M-phase nuclei ±/– SEM per retina (n = number of retinas examined) |
|-----------------------|--------------------------------------------|--------------------------------------------------|
| rb1p1226a/+           | 0/20 (0%)                                  | 85.45+/– 3.22 (20)                                |
| rb1p1226a/+rb1p1226a  | 26/30 (86%)                                 | 42.2+/– 4.7 (15)                                  |
| rb1p1226a/ty85d       | *13/18 (72%)                                | 34+/– 2.9 (13)                                   |

*rb1p1226a/ty85d embryos were generated from a cross between spc+p85d heterozygotes and rb1p1226a heterozygotes, and then genotyped for the presence of the rb1p1226a allele. Of the genotyped rb1p1226a heterozygotes, only half are expected to carry the spc+p85d allele, thus reducing the total number of mutant retinas by half, and numbers for rb1p1226a/ty85d were adjusted accordingly.

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To determine if loss of rb1 function indeed causes an initial delay in the presence of postmitotic, differentiated RGCs, we examined expression of isl2b-gfp, one of the earliest transgenic markers indicative for postmitotic RGCs [22]. We found that in wild type retinas postmitotic, differentiating RGCs marked by isl2b-gfp expression emerged first at 32 hpf, increased significantly in their abundance by 36 hpf, and by 48 hpf isl2b-gfp positive RGCs were densely packed throughout the ganglion cell layer (Figure 6A, 6C, 6E, n = 25, 16, and 29, respectively). In contrast, isl2b-gfp positive RGCs were present in only 13% of rb1 deficient retinas at 32 hpf (n = 23). Because of the cytoplasmic localization of the GFP signal and the density at which RGCs normally populate the ganglion cell layer, it is difficult to determine the total number of isl2b-gfp positive RGCs. Nonetheless, semi-quantitative analysis revealed that by 36 hpf, isl2b-gfp positive RGCs were present in only 13% of rb1 deficient retinas (n = 23). Because of the cytoplasmic localization of the GFP signal and the density at which RGCs normally populate the ganglion cell layer, it is difficult to determine the total number of isl2b-gfp positive RGCs. Nonetheless, semi-quantitative analysis revealed that by 36 hpf, isl2b-gfp positive RGCs were present in only 13% of rb1 deficient retinas (n = 23). However, their distribution with the retina was more similar to that of younger wild type retinas at 32 hpf (Figure 6B, 6D). By 48 hpf, all rb1 mutant retinas harbored isl2b-gfp neurons (n = 32), although differentiation still appeared to lag in 81% (n = 32) of the rb1 mutant retinas compared to the more densely packed ganglion cell layer in wild type retinas (n = 29, Figure 6E-6F). Despite the reduced number of RGCs present at 48 hpf, rb1 mutant isl2b-gfp positive RGCs express DM-GRASP, a late marker of RGC differentiation, demonstrating that mutant RGCs were fully differentiated once becoming postmitotic (Figure S2). Importantly, the number of ath5-gfp positive RGC precursors was unaffected in rb1 mutants (Figure S1 and data not shown). Thus, the rb1 deficiency causes a delay in the transition of RGC precursors to postmitotic RGCs, but not in the specification of RGC precursors.

Aside from the reduced population of early born RGCs, rb1 mutant retinas appear grossly normal, and at 120 hpf, show proper lamination by each retinal cell type [18]. Although we did not determine whether birth dating of other retinal cell types is affected in rb1 mutant retinas, netrin-positive exit glial cells and Muller glia cells are present in appropriate numbers and location, indistinguishable from wild-type retinas (Figure S2). Taken together, these results suggest that a delay in cell cycle exit by rb1 deficient RGC precursors leads to a transient reduction in the early born postmitotic RGCs without consequence to the gross morphology and overall cellular landscape of the rb1 mutant retina.
rb1-deficient RGC axons exhibit intraretinal and midline pathfinding errors

The early born RGCs are located within the central retina and pioneer the retinotectal tract to the contralateral optic tectum [22]. In the absence of the early pioneering RGC axons, the axons of later born, more peripherally located RGCs fail to exit the eye and project aberrantly within the retina [22]. Given the reduced number of these early born, central RGCs in rb1 mutants, we sought to determine whether peripheral RGC axon trajectories were affected. For this, we labeled small groups of RGCs in the anterior peripheral retina with DiO (green) and in the posterior peripheral retina with DiI (red, Figure 7A–7F). In 120 hpf larvae, all labeled axons from anterior and posterior RGCs fasciculated shortly after sprouting from their soma and extended as an axon bundle, forming a path directly toward the retinal exit point (n = 83, Figure 7A–7B, 7D–7E). In contrast, 91% (n = 65) of rb1 deficient retinas harbored a significant subset of axons that had extended aberrantly throughout the retina and failed to exit (Figure 7C, 7F). These results demonstrate that the delayed differentiation of the early born RGCs in rb1 mutants impairs the ability of later born RGC axons to exit the retina.

The delayed cell cycle exit and differentiation of pioneering RGCs lacking rb1 may also affect axon navigation by later born RGC axons at key choice points: the ventral midline of the diencephalon and/or the optic tectum. To examine these possibilities, we filled the RGC layer of the left and right eyes of wild type and rb1 mutant larvae with either DiI or DiO, respectively (Figure 7G). In wild type siblings, 99% of dye filled optic nerves projected to their appropriate contralateral tectum (n = 946, Figure 7H). In contrast, rb1 mutant optic nerves displayed a variety of phenotypes. The majority of rb1 deficient optic nerves were significantly thinner than their wild type counterparts (37%, n = 663, Figure 7I–7J, 7L), consistent with what we observed in with ath5:gfp (Figure 2). In a significant portion of rb1 deficient optic nerves, 17%, RGCs projected to both the contralateral but also to the ipsilateral tectum, indicative of midline pathfinding defects (n = 663, Figure 7K–7L). Focal DiI/DiO labeling of RGC axons arising from the anterior and posterior retina revealed that retinotopic mapping, a function of retinal cell body location [23], remains intact in rb1 mutants despite the aberrant pathfinding en route to the optic tectum (Figure S3). Finally, in 11% of rb1 mutant retinas, there was a complete failure of RGCs to exit from the eye, even at 120 hpf (Figure 7J). Taken together, these results suggest that rb1 deficient

![Figure 3. rb1 mRNA overexpression rescues optic nerve hypoplasia in rb1<sup>te226</sup> mutants.](image1)

![Figure 4. rb1 is required in RGC axons to regulate retinal exit.](image2)
RGC axons make intraretinal and midline pathfinding errors, leading to reduced and incorrect tectal innervation.

rb1 mutants display deficits in visually guided behaviors

By 120 hpf, zebrafish larvae perform an array of sensorimotor behaviors, including responses to visual stimulation. For example, changes in visual field illumination, such as the sudden absence of light or a shift from uniform to focal illumination, elicit specific, stereotyped turning behaviors [24,25]. We first examined the ability of rb1 mutant larvae to perform positive phototaxis, defined as navigating toward a target light source that is presented after extinguishing the pre-adapted uniform light field [25]. Positive phototactic navigation is characterized by larvae first turning towards the target light source and then swimming forward towards the target. As previously reported, when presented with a target light source wild type larvae facing away from the light target show significant initiation of turns, which are preferentially biased towards the light target (Figure 8A–8B). Once facing the target, wild type larvae initiate forward scoot swims (Figure 8C). In contrast, turn initiation in rb1 mutant larvae facing away from the light target was dramatically reduced (Figure 8A). On the few occasions when they initiated a turn, turning direction was unbiased with respect to the light target (Figure 8B). Moreover, rb1 mutants facing the light target did not show an increase in forward scoot swim initiation above baseline (Figure 8C). To further determine whether rb1 mutants respond to more extreme changes in illumination, we examined their ability to perform an O-bend response to a visual dark flash stimulus, a sudden extinction of light [24]. Again, compared to their wild type siblings, rb1 mutants displayed a minimal O-bend response to dark flash stimulation (Figure 8D). Despite their impaired visual responses, rb1 mutants showed no difference in the spontaneous initiation of turning or swimming behaviors compared to wild type siblings (Figure 8D). Importantly, the kinematic parameters of spontaneously occurring turning and swimming movements were indistinguishable between rb1 mutants and their wild type siblings, demonstrating that the neural circuits required for initiation and execution of turning behaviors are largely intact in rb1 mutants. Together, these results demonstrate that rb1 mutants exhibit visual deficits.

Discussion

Children with biallelic germline or sporadic inactivation of rb1 are likely to form ocular tumors during early childhood. Initially, the retinas of affected individual show an otherwise grossly normal morphology. In contrast, even conditional rb1 knockout mouse
models exhibit ectopic proliferation and cell death leading to significant morphological defects throughout affected retinas. We find that inactivation of the zebrafish \( rb1 \) gene through a \( rb1 \) causing mutation results in mutant retinas that display very limited signs of cell death, with differentiated retinal cell types that are properly laminated, similarly to childhood retinas lacking \( rb1 \).

Thus, the fairly 'normal' retinal landscape of zebrafish \( rb1^{te226a} \) mutants provided us with a unique opportunity to investigate if and how \( rb1 \) is required to establish the retinotectal projection. Our analysis reveals a RGC autonomous requirement for \( rb1 \) in regulating RGC axon pathfinding within the retina and at presumptive choice points en route to the optic tectum. Moreover, we demonstrate that zebrafish \( rb1^{te226a} \) mutants exhibit deficits in visually guided behaviors, suggesting that the retinotectal path defects in \( rb1 \) mutants may be sufficient to impair vision. Together, this work reveals a novel role for \( rb1 \) in the establishment of RGC axon projections during development and establishes a unique model for understanding the developmental and tumor suppressor roles of the \( rb1 \) gene.

Zebrafish \( rb1^{te226a} \) mutants harbor a human retinoblastoma causing \( rb1 \) gene mutation. The mutant protein is truncated in the B-domain and lacks the cyclin-binding domain, reducing Rbl’s capacity to form a ‘pocket’, and reducing its capacity for phosphorylation by cyclin dependent kinases [2,26]. Consistent with the notion that the \( rb1^{te226a} \) mutant allele is largely non-functional, mRNA over-expression in \( rb1 \) mutants does not ameliorate the \( rb1 \) mutant phenotype. Despite the absence of biological activity of the truncated \( rb1^{te226a} \) protein, mutant zebrafish show a significantly milder retinal phenotype compared to conditional or even germline \( rb1 \) mouse knockouts [6,7,8,9,10,11,12]. One possible explanation is the strong maternal contribution of \( rb1 \) in zebrafish (Figure 4A), which may suppress phenotypic expressivity at early stages of development. Consistent with this idea, formation of the initial scaffold of axon tracts during the first day of development appears unaffected in \( rb1^{te226a} \) mutants, yet visual and hindbrain pathways that develop after the first day of development show defects [18].
In humans the \( rb1 \) nt1960+1 mutation, which is identical to the zebrafish \( rb1^{P226a} \) mutation, causes ocular tumors [13,14,15,16], raising the possibility that zebrafish \( rb1 \) mutants might also develop tumors as juveniles. However, \( rb1 \) mutants fail to inflate a functional swim bladder, and die \( \sim \) 7 days post fertilization, precluding the analysis of ocular tumors in juveniles. Although wild type \( rb1 \) mRNA injection rescues the early RGC and retinotectal defects through 48 hpf, these transiently rescued \( rb1^{P226a} \) mutants do not survive beyond 7 days of development, indicating that \( rb1 \) plays a critical role after the injected mRNA has been degraded. Establishing stable, inducible \( rb1 \) transgenic lines to rescue developmental deficits will therefore be required to monitor juvenile and adult zebrafish for retinal tumors.

In \( rb1^{P226a} \) mutants, a significant subset of RGC axons fail to exit the retina, and many of the exiting axons then project incorrectly to the ipsilateral tectum, revealing a previously unrecognized requirement for \( rb1 \) function for the development of neural circuitry underlying behavior. The deficits in starrle behavior are due to defects in a small subset of hindbrain neurons, the spiral fiber neurons [18], and giving the results presented here, it is tempting to speculate that \( rb1 \) plays a similar role for the transition of these neurons from precursors to postmitotic neurons. Unfortunately, markers that follow the development of spiral fiber neurons are not available, precluding such analysis. Therefore, we focused on the well-characterized development of RGCs. Although we demonstrate a defect in the early development of these cells and their axonal connectivity, we cannot exclude the possibility that zebrafish \( rb1 \) mutants exhibit defects in the development and/or function of other retinal cell types, and that these defects contribute to the deficits in visual behaviors we observe. Future analysis of transgenic lines expressing the wild type \( Bb1 \) gene in individual retinal cell types will reveal which cell type(s) and connections are causative of the visual deficit.

In summary, we report a zebrafish mutant carrying a human disease causing \( rb1 \) mutation, which reveals novel roles of \( rb1 \) in regulating RGC axon pathfinding and visually guided motor behavior. Furthermore, these mutants provide a non-murine vertebrate model of \( rb1 \) and offer new potential for identifying the elusive retinoblastoma cell of origin and further insight into the developmental role of \( rb1 \).

**Materials and Methods**

**Ethics statement**

All experiments were conducted according to an Animal Protocol fully approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) on January 27, 2011, protocol number 803446. Veterinary care is under the supervision of the University Laboratory Animal Resources (ULAR) of the University of Pennsylvania.

**Animals and fish maintenance**

The zebrafish (\( Danio rerio \)) strain used in this study was the \( spe^{P226a} \) allele (now referred to as \( rb1^{P226a} \)) of space cadet [17,18], maintained on a mixed TLF and Tubingen background. The \( rb1^{P226a} \) allele was also crossed into the \( ath5:gfp \) and \( isl2b:gfp \) transgenic backgrounds for RGC analysis [20,22], \( rb1^{P226a} \) adults were always crossed with \( ath5^{+/-} \) or \( rb1^{P226a} \) adults to ensure that \( rb1^{P226a} \) embryos analyzed for GFP-expressing RGCs were hemizygous for GFP. Throughout the manuscript, \( rb1^{+/-} \), "\( rb1 \) deficient", and \( rb1 \) mutant refers to \( rb1^{P226a} \) homozygotes. The other space cadet allele \( spe^{E80} \) [17] was only used where mentioned. Embryos were collected in the morning, maintained on a 14/10 hour light/dark cycle at 28°C, and staged as described previously [28]. Larvae were raised in 6 cm plastic Petri dishes at a density of 20–30 per 7 mL in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\), 0.33 mM MgSO\(_4\)) with medium changes at 48 hpf (hours.
post fertilization) and 96 hpf. Behavioral experiments were conducted on 120 hpf larvae.

Recombination mapping and molecular cloning of rb1

A three generation mapping cross between \( rb^{p^{229a}} \) heterozygous and WIK fish was generated, and pools of 25 \( F_2 \) mutant and \( F_2 \) sibling 5 dpf larvae were collected in the \( F_2 \) generation and used for bulk segregant mapping (see Table 2 for simple sequence length and single nucleotide polymorphic markers). Mutant larvae were identified by performing successive, unilateral C-bends to acoustic or tactile stimulation [17,18]. To identify the mutation, cDNA was prepared following total mRNA extraction from 5 dpf larvae as previously described [29]. \( rb1 \) cDNA was amplified with the PCR Primers (\( rb1:1 \)–6, Table 2) designed against overlapping regions of the \( rb1 \) reference sequence (Ensembl) with the following RT-PCR conditions: 94°C for 3 min and then 40 cycles of 94°C for 45 sec, 57°C for 1 min, and 70°C for 1 min. Products were gel purified and cloned into the pCR2.1-TOPO-TA vector for sequencing. After detecting a frameshift and 4 nucleotide addition to the end of exon 19 in \( rb^{p^{229a}} \) cDNA clones, gDNA was isolated from 5 dpf larvae, and intron 19 was amplified with the \( rb1 \) primers, using identical PCR conditions to those described above.

For \( rb1 \) RNA injection, cDNA was prepared from genotyped homozygous wild type or \( rb^{p^{229a}} \) mutant 5 dpf larvae (dcAPS protocol, see below) and amplified with the \( rb1:FL \) primers (similar conditions as above, except extension time increased to 3 min), which includes the coding region of \( rb1 \), and cloned into the pCS2+ vector. Wild type \( rb1 \) and \( rb^{p^{229a}} \) mRNA was prepared using the mMessage mMach kit (Ambion, NY) and injected at the 1-cell stage at doses ranging from 1–100 picograms. Embryos injected with 20 or greater picograms of \( rb1 \) mRNA showed gross morphological abnormalities and necrosis, whereas embryos injected with 10 picograms or less appeared morphologically normal.

PCR genotyping \( rb1^{te226a} \)

To genotype \( rb^{te226a} \) embryos, we developed a dCAPS assay [30] using the dCAPS program (http://helix.wustl.edu/dcaps/dcaps.html) to design appropriate primers (Table 2). After gDNA isolation, PCR was performed as described above. The PCR product is then digested with Ssp1 (New England Biolabs, Ipswich, MA), cleaving the \( rb1^{te226a} \) allele and producing a 120 bp fragment that can be distinguished from the 150 bp wild type allele on a 3% agarose gel containing 1.5% Metaphor agarose (Lonza, Rockland, ME). All genotyping, except for BrdU labeled embryos, was performed following immunolabeling experiments.

Immunohistochemistry and in situ hybridization

For immunostaining, embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, permeabilized with 1 mg/mL collagenase, and blocked for 1 hour with 5% normal goat serum in 0.1 M phosphate buffer. Embryos were then incubated in the primary antibodies anti-GFP (1:200 mouse JLI, Clontech, Mountain View, CA or 1:500 rabbit, Invitrogen, Carlsbad, CA), anti-phosphohistone H3 (Millipore, Charlottesville, VA), anti-BrdU (Roche, Branchburg, NJ), and/or 1:50 A2-J-22 polyclonal antiserum (recognizes carbonic anhydrase II, kindly provided by Dr. P. Linser) overnight at 4°C in blocking solution, washed out, and then detected by the addition of AlexaFluor488 or AlexaFluor594 conjugated secondary antibodies (1:500, Invitrogen, Carlsbad, CA). TUNEL assay was performed as previously described [31] using Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). After staining, samples were mounted in DAPI containing Vectashield (Vector Labs, Burlingame, CA). Images were acquired with a Zeiss 710 confocal laser scanning microscope (LSM 710) using ZEN2010 software.

For \textit{in situ} hybridization, digoxigenin-UTP labeled antisense riboprobes for \( rb1 \) were synthesized and hydrolyzed from the full length \( rb1 \) cDNA construct [32]. Whole-mount \textit{in situ} hybridization was performed as described previously [33]. Images were acquired with a Zeiss Axioskop compound microscope. For RT-PCR based expression analysis, the \( rb1:FL \) and \( B\)-actin primers (Table 2) were run against cDNA prepared from total mRNA extracted from 25 embryos/larva at each stage.

| Primer | Forward | Reverse |
|--------|---------|---------|
| \textit{cDNA amplification} | \textit{rb1-1} (180) | CCGACTCAACACCACTAACC | CTAACCCGGAATGTAGGG |
| | \textit{rb1-2} (181) | ACAATTGTGTTAGTCTCC | TAAAGAGCTCCCACTAGGG |
| | \textit{rb1-3} (211) | GCAGAATTTCATACATGTCG | GGTCCAGCATGGAACAGC |
| | \textit{rb1-4} (183) | GCAGCTCTACTCATGGGAC | CTTGGTGACCACTGAGGC |
| | \textit{rb1-5} (184) | TCAGACACTCCATCATGCC | GGTATGTTCACGTCACTCC |
| gDNA amplification | \textit{rb1-6} (208) | AACAGACAGATGGTTCCTC | CATGTGACGTAACTTCGC |
| | \textit{rb1-FL} (Rb1-4) | AACACATCAACAACACTCTG | CCCAGTAATGCTTAACACC |
| | B-actin | TACAGCTTACCACCAACAGC | AAGAAGCGTGAGAAGAGAGC |

Table 2. PCR primers for recombination mapping, molecular cloning, and genotyping of \( rb1^{te226a} \).
Lipophilic dye labeling of retinal ganglion cells

120 hpf larvae were anesthetized (0.01% Tricaine) and fixed in 4% paraformaldehyde at 4°C overnight. Larvae were removed from fix, washed briefly in phosphate buffered saline (PBS), and mounted dorsal side up for whole retinal injection or laterally for discreet RGC labeling on glass microscope slides in a bed of 1.5% agarose. To label all RGCs, the vitreal space of each eye was filled with either of the fluorescent lipophilic dyes Dil (red) or DiO (green) (Molecular Probes, Eugene, OR) dissolved in 1% chloroform, using a WPI PV-920 picopump injector fitted with a glass micropipette. For discreet labeling, a small region of the exposed eye was labeled with pulses of Dil/DiO dissolved in 0.5% dimethylformamide. Injected larvae were kept moist with PBS and incubated overnight at room temperature in a humidity chamber in darkness following transplantation, and were allowed to develop undisturbed until epiboly completed. Embryos were then transplanted into recipients.

Cell transplantation

For transplant direction space cadet host, wild type transgenic Tg(ath5:gfp) and rb1⁵²⁶a heterozygous fish were used to generate wild type GFP expressing donor embryos and non-GFP expressing rb1⁵²⁶a mutant embryos, respectively. For transplant direction space cadet donor into wild type host, rb1⁵²⁶a, Tg(ath5:gfp) double heterozygotes and either TU or TLF strain wild type mating pairs were used to generate rb1⁵²⁶a GFP expressing donor embryos and non-GFP expressing wild type embryos, respectively. Once the appropriate donor-host embryos were collected, embryos were immediately placed in E3 medium and kept at room temperature. Donor embryos were pressure injected into the yolk sac at the 1–2 cell stage with the lineage marker tracer tetramethylrhodamine dextran, 3 Kd, 5% w/v (Molecular Probes, Eugene, OR) dissolved in 0.2 M KCl and filter sterilized. Donor and host embryos were then incubated at 28.5°C in E3 medium in darkness to grow synchronously to the 1000 cell stage. Embryos were then transferred into room temperature complete E2 medium (E2) to retard growth, and dechorionated using Pronase (1:50 in E2 of 30 mg/ml stock, Roche) in glass 60 mm Petri dishes at a density of 12 fish per dish. For all behavioral experiments, N = 48 rb1⁵²⁶a and 48 wild type sibling larvae. For phototaxis experiments, video recordings were triggered every 500 msec, with each recording covering a 400 msec time window, for a total duration of 4 sec of recorded behavior. Each group of 12 larvae were subjected to 3 rounds of phototaxis testing, with 3 min between trials. Orientation of larvae to target light was determined at the beginning of each 400 ms recording as previously described [25], such that the behavior of each larva was tested multiple times and in different orientations with respect to the target light. Therefore, the N for Figure 8A and 8C ranged from 75 to 547 for wild type siblings and 13–33 for rb1⁵²⁶a larvae. For dark flash response experiments, N = 4 groups of 12 larvae. Spontaneous behavior was analyzed on individually housed larvae on a 4 x 4 grid array.

Supporting Information

**Figure S1** rb1⁵²⁶a retinas show increased apoptosis. Retinas removed from wild type (A, C, E) or rb1⁵²⁶a;ath5:gfp embryos (B, D, F) at 28 (A–B), 32 (C–D), or 36 hpf (E–F). Retinas labeled anti-GFP (green), TUNEL (red), and counterstained with DAPI (blue). Lateral view of maximum intensity projection of confocal z-stacks. White dashed circle outlines retina. Anterior to the left, dorsal to the top of each panel. (G) Mean number of TUNEL positive nuclei per retina. Error bars denote SEM. *p<0.01; one-way ANOVA. N retinas shown at base of bar graphs. Scale bar = 50 μm.

(TIF)
project to the rostral tectum in both wild type and \( rb^{1226a} \) larvae. Arrowheads mark misprojecting axons. Scale bar = 50 µm.

(TIF)

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Author Contributions

Conceived and designed the experiments: M Gyda, M Wolman, K Lorent, M Granato. Performed the experiments: M Gyda, M Wolman, K Lorent. Analyzed the data: M Gyda, M Wolman, K Lorent. Contributed reagents/materials/analysis tools: M Gyda, M Wolman, K Lorent. Wrote the paper: M Gyda, M Wolman, M Granato.

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