

Functional analysis of BBS3 A89V that results in non-syndromic retinal degeneration

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Bardet–Biedl syndrome (BBS) is a syndromic form of retinal degeneration. Recently, homozygosity mapping with a consanguineous family with isolated retinitis pigmentosa identified a missense mutation in BBS3, a known BBS gene. The mutation in BBS3 encodes a single amino acid change at position 89 from alanine to valine. Since this amino acid is conserved in a wide range of vertebrates, we utilized the zebrafish model system to functionally characterize the BBS3 A89V mutation. Knockdown of *bbs3* in zebrafish alters intracellular transport, a phenotype observed with knockdown of all BBS genes in the zebrafish, as well as visual impairment. Here, we find that BBS3 A89V is sufficient to rescue the transport delays induced by the loss of *bbs3*, indicating that this mutation does not affect the function of BBS3 as it relates to syndromic disease. BBS3L A89V, however, was unable to rescue vision impairment, highlighting a role for a specific amino acid within BBS3 that is necessary for visual function, but dispensable in other cell types. These data aid in our understanding of why patients with the BBS3 A89V missense mutation only present with isolated retinitis pigmentosa.

INTRODUCTION

Bardet–Biedl syndrome (BBS, OMIM 209900) is a genetically heterogeneous autosomal recessive disorder characterized by retinitis pigmentosa, obesity, polydactyly, renal abnormalities, hypogenitalism and cognitive impairment (1–4). Moreover, BBS is associated with an increased risk for hypertension, diabetes and heart defects (1,2,5). BBS patients present with early and progressive photoreceptor degeneration and are blind by the third decade of life (2,6–13). To date, 12 BBS (*BBS1–12*) genes are reported to individually cause BBS (14–27). Additionally, hypomorphic mutations in *MKSI* and *CEP290* have been associated with BBS, representing *BBS13* and *BBS14*, respectively (28). The BBS genes belong to multiple protein families and function cannot be defined based on homology; however, recent advances in molecular

pathophysiology and animal models have helped to elucidate why 14 different genes can lead to the same phenotype. Work in mouse, zebrafish, *Caenorhabditis elegans* and *Chlamydomonas* has provided multiple lines of evidence supporting a role for BBS proteins in cilia function and intraflagellar and/or intracellular transport (19,22,23,26,29–36). Although progress has been made in understanding the pathophysiology of BBS, there are major gaps in our understanding of the precise cellular function of the BBS proteins.

BBS3 (ARL6, ADP-ribosylation factor-like), a member of the Ras family of small GTP-binding proteins, was initially identified as a BBS gene through computational genomics and high-density single nucleotide polymorphism (SNP) genotyping (21,22). Several mutations (G2X, T31M, T31R, P108L,

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R122X, G169A and L170W) leading to BBS have been reported throughout BBS3 (21,22,37). Knockdown of *bbs3* using an antisense oligonucleotide [Morpholino (MO)] results in two cardinal features of BBS in the zebrafish: reduced size of the ciliated Kupffer's Vesicle and delays in intracellular melanosome transport (35,38). These prototypical phenotypes are preset with knockdown of all BBS genes in the zebrafish (26,34,35,38). Recently, we identified a second longer eye-specific transcript of BBS3, BBS3L, which is required for retinal organization and function in both the mouse and zebrafish (38). Knockdown of either both *bbs3* transcripts or *bbs3L* alone leads to vision impairment in zebrafish. To determine the functional requirement of each transcript, RNA encoding either human BBS3 or BBS3L was co-injected with the *bbs3* aug MO, which targets both transcripts. We determined that human BBS3 RNA is sufficient to suppress the melanosome transport delays, but not the vision defect. In contrast, BBS3L RNA was sufficient to rescue the vision defect; however, it was unable to suppress the cardinal phenotypes of BBS seen in the zebrafish, supporting a retina specific role for BBS3L (38).

BBS is rare in the general population; however, the study of this disease can offer insight into normal retinal development as well as provide an understanding of the pathophysiology involved in non-syndromic forms of BBS. Homozygosity mapping of a consanguineous Saudi Arabian family has identified a missense mutation (A89V) in BBS3 that leads to non-syndromic retinitis pigmentosa (39,40). The identification of specific mutations in the same gene that results in either syndromic or non-syndromic retinitis pigmentosa will provide insight into tissue-specific functional regions of BBS3 in the retina. Moreover, understanding the functional domains of proteins involved in vision aids in our understanding of not only the disease state, but also normal vision development.

Here we report the functional characterization of the BBS3 missense mutation (A89V), which occurs in a highly conserved region of BBS3. The function of the BBS3 A89V mutation was evaluated by utilizing gene knockdown of *bbs3* coupled with RNA rescue in the zebrafish. We examined the intracellular transport of melanosomes, a cardinal feature of BBS gene knockdown in the zebrafish, and visual function using a vision startle assay. The A89V mutation can suppress the melanosome transport defects, but not the vision impairment observed with the loss of *bbs3*. Thus, the missense mutation identified in patients with non-syndromic retinal degeneration has uncovered an amino acid in BBS3 that is necessary for vision. The A89V mutation is able to function in melanosome transport, demonstrating that the mutant form of the protein retains the ability to function in tissues typically affected by BBS.

RESULTS

BBS3 conservation and BBS3L mutant expression

Previous homozygosity mapping with a consanguineous family from Saudi Arabia identified a novel missense mutation (A89V) in BBS3 that results in non-syndromic retinitis pigmentosa (39,40). Comparison across vertebrate species with available full-length BBS3 sequences demonstrates that the

A89V mutation occurs in an evolutionarily conserved region of the protein, suggesting that the alanine at position 89 may have an essential function within the protein (Fig. 1A). The location of the mutation upstream of the C-terminus would impact both BBS3 and BBS3L, as this region is identical between the two isoforms (Fig. 1B). Additionally, the missense mutation is located downstream of the phosphate binding loop (P-loop), which is important for binding triphosphates of the GTP nucleotide (41). To test whether BBS3L A89V is stably expressed, we performed western blot analysis on embryos injected with either human *myc*-tagged BBS3L or BBS3L A89V RNA. We found that similar to BBS3L, BBS3L A89V was present through 5 days post-fertilization (dpf) (Fig. 1C). Thus, the BBS3 A89V missense mutation is in an evolutionarily conserved region of the protein and would be found in both BBS3 and BBS3L. Moreover, the mutation does not impact BBSL expression, indicating that the mutation does not inhibit protein expression.

BBS3 A89V functions in melanosome transport

Knockdown of *bbs3* using a MO that targets both transcripts (*bbs3* aug MO) results in intracellular melanosome transport delay, a phenotype related to syndromic disease and shared among all BBS genes (26,34,35,38). In response to light or hormonal stimuli, zebrafish alter their skin pigmentation through intracellular melanosome transport within the melanophores (42–45). To test the rate of cellular trafficking, 6-day-old zebrafish embryos were dark adapted and treated with epinephrine to induce retrograde melanosome transport (Fig. 2A). Retrograde transport results in the movement of the dispersed melanosomes to a perinuclear location (Fig. 2B). Rescue experiments were performed by co-injecting RNA encoding human BBS3 or BBS3 A89V with the *bbs3* aug MO. Wild-type embryos demonstrate rapid melanosome aggregation, averaging 1.45 min, whereas knockdown of *bbs3* leads to a statistically significant delay in transport [analysis of variance (ANOVA) with Tukey, $P < 0.01$] (Fig. 2C and Table 1). We previously demonstrated that human BBS3, but not human BBS3L, can suppress the melanosome transport delays, thus we focused on BBS3 to investigate the role of the A89V mutation in syndromic disease (38). Similar to BBS3 RNA, co-injection of BBS3 A89V RNA with the *bbs3* aug MO can restore transport times to wild-type levels (Fig. 2C and Table 1). These results demonstrate that the BBS3 A89V missense mutation can function to suppress the cardinal BBS phenotype of intracellular melanosome transport. This is consistent with the observation that the human patients harboring the BBS3 A89V mutation do not present with BBS-related phenotypes, such as obesity, polydactyly, renal anomalies or cognitive impairment.

BBS3L A89V does not function in vision

Both the mouse and zebrafish model systems have demonstrated that BBS3L is necessary for proper retinal function. Additionally, rescue experiments in the zebrafish have shown that human BBS3L is sufficient to suppress the *bbs3* aug MO-induced vision defect (38). Since patients with the BBS3 A89V mutation present with only retinitis pigmentosa,

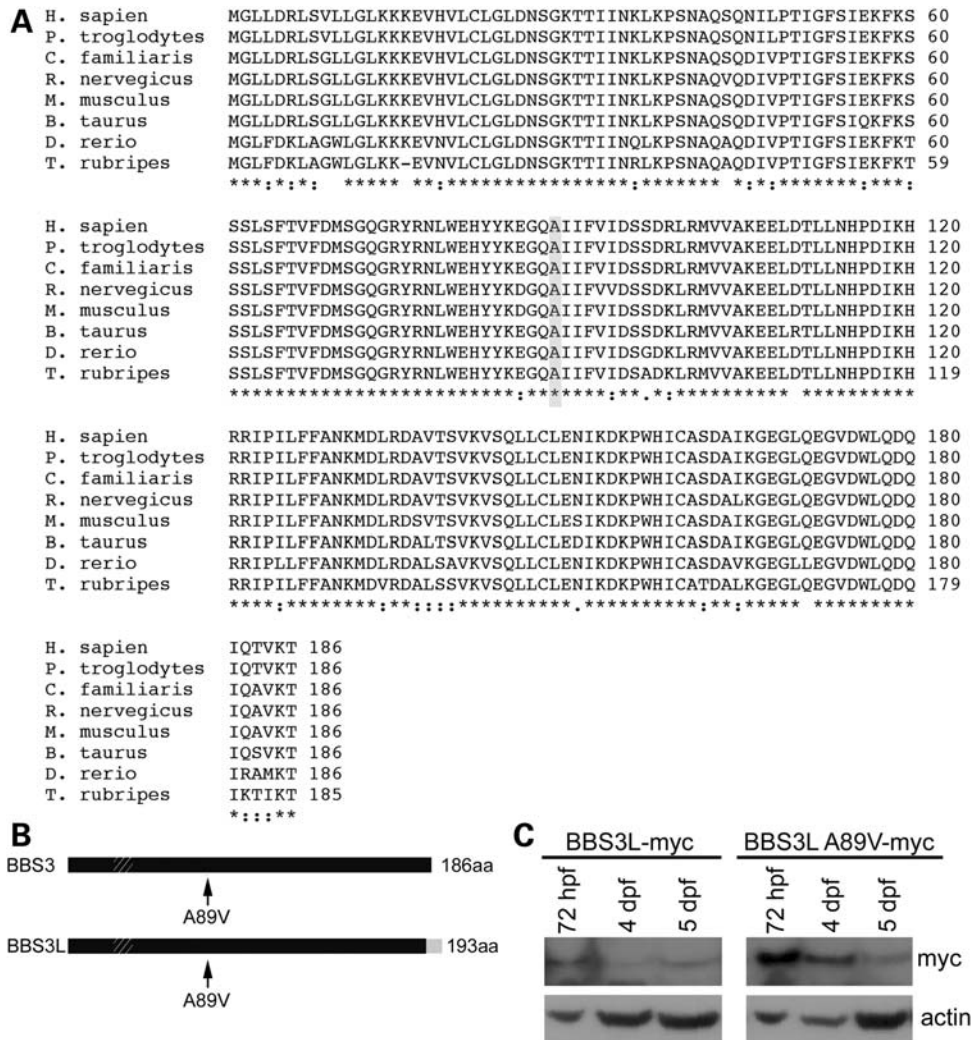


Figure 1. BBS3 conservation and protein expression. (A) Multi-species alignment of BBS3 demonstrating the conservation among vertebrates. Shaded box highlights the location of the A89V mutation. Asterisks (*) indicate identical amino acids, while colons (:) and periods (.) represent conserved amino acids. (B) Schematic depicting the location of the A89V mutation in human BBS3 and BBS3L isoforms. Hatched box depicts the location of the P-loop motif and the grey box on the C-terminus of BBS3L denotes the region of difference between the two isoforms. (C) Western blot analysis of staged zebrafish embryos injected with either human BBS3L or BBS3L A89V *myc*-tagged RNA. Both proteins are present through 5 dpf. Actin served as a control.

we sought to functionally test the role of this mutation in vision by co-injecting *BBS3L* and *BBS3L A89V* RNA in *bbs3* knockdown embryos. Visual function was evaluated by using a natural escape response that is elicited when zebrafish embryos are exposed to rapid changes in light intensity (38,46). In the vision startle assay, an embryos response to five short blocks in bright light are monitored and recorded. Visually responsive embryos change swimming directions in response to short blocks of bright light (Fig. 3A). Wild-type embryos respond on average 3.77 times (Fig. 3B). Cone-rod homeobox (*crx*) gene knockdown was used as a control for vision impairment as *crx* is necessary for photoreceptor formation in the zebrafish (47,48). *crx* knockdown embryos respond an average of 2.39 times, indicative of vision impairment (Fig. 3B) (ANOVA with Tukey, $P < 0.01$). *bbs3* aug MO-injected embryos show a statistically significant reduction in the number of responses when compared with controls (Fig. 3B) (ANOVA with Tukey, $P < 0.01$) (38). Co-injection

of *BBS3L* RNA with the *bbs3* aug MO restored visual responsiveness back to wild-type levels, indicating that *BBS3L* RNA was sufficient to rescue vision (Fig. 3B). Conversely, *BBS3L A89V* RNA was not able to restore visual function back to wild-type levels as embryos responded on average 1.60 times (Fig. 3B). Western blot analysis confirms expression of BBS3L A89V through 5 dpf, when the vision assay is performed (Fig. 1C). The inability of BBS3L A89V RNA to restore vision provides strong functional support that this missense mutation leads to non-syndromic retinitis pigmentosa.

DISCUSSION

The present study utilizes the zebrafish to examine the function of BBS3 A89V in intracellular transport, a phenotype associated with the knockdown of *BBS* genes, and vision. This mutation was identified in a consanguineous Saudi

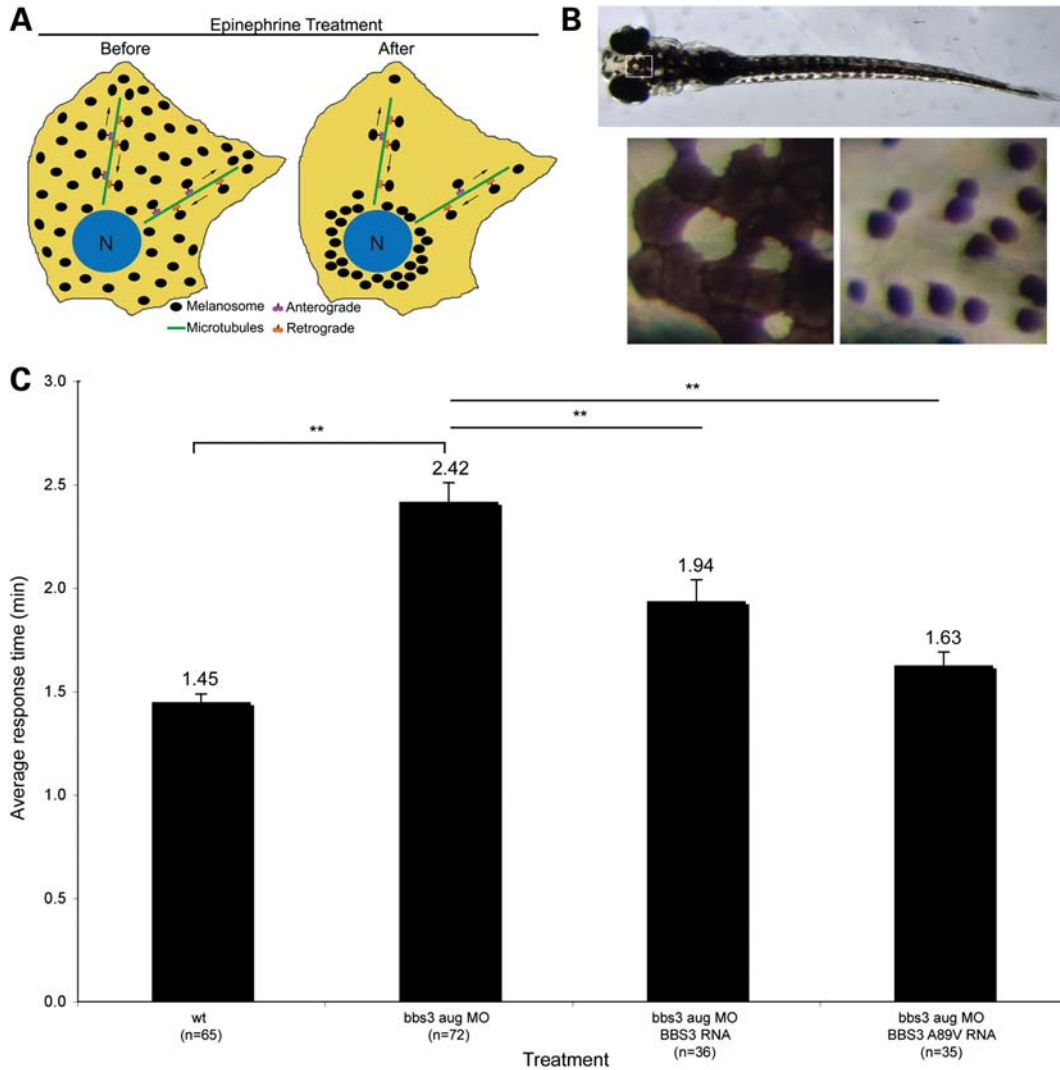


Figure 2. BBS3 A89V functions in melanosome transport. (A) Schematic illustrating the retrograde movement of melanosomes within the melanocyte from the periphery before epinephrine treatment to the perinuclear region after epinephrine treatment. (B) Dorsal view of a dark-adapted wild-type 6-day-old zebrafish embryo. Boxed head region is magnified below the full embryo. The left-hand image shows melanocytes prior to epinephrine treatment, while the right-hand panel depicts the same melanocytes at the endpoint of retrograde transport. (C) The graph summarizes the average epinephrine-induced response times in minutes for each experimental group. Both human *BBS3* and *BBS3 A89V* RNAs are able to suppress the transport time seen with knockdown of *bbs3*. Sample size (n) is denoted on the X-axis. ** $P < 0.01$, ANOVA with Tukey. Data presented as the mean \pm SEM.

Table 1. Melanosome transport (MT) times and vision assay responses

Treatment	MT (min)	n	Vision (# responses)	n
wt	1.45 ^a	65	3.77	64
bbs3 aug MO	2.42 ^b	72	1.91 ^b	75
bbs3 aug MO + hBBS3 RNA	1.94 ^{a,b}	36	1.00 ^b	15
bbs3 aug MO + hBBS3 A89V RNA	1.63 ^a	35	1.05 ^b	42
bbs3 aug MO + hBBS3L RNA	NA	NA	3.46	26
bbs3 aug MO + hBBS3L A89V RNA	NA	NA	1.60 ^b	25

^aANOVA and Tukey test, $P < 0.01$ when compared with *bbs3* aug MO.

^bANOVA and Tukey test, $P < 0.01$ when compared with wt.

Arabian family that presented with only retinitis pigmentosa (39,40). The polymorphism phenotyping program, PolyPhen, predicts that the BBS3 A89V missense mutation is a benign non-synonymous SNP; however, the alanine at position 89 is highly conserved in vertebrates, suggesting that this amino acid has an essential function. Using RNA rescue experiments, we demonstrate that unlike *BBS3L* RNA, the *BBS3L A89V* RNA does not rescue the vision defect observed with the loss of *bbs3*. Although the A89V mutation is not functional in vision, *BBS3 A89V* RNA is able to suppress the cardinal zebrafish BBS phenotype of melanosome transport. Taken together, these data demonstrate that the BBS3 A89V mutation identified in patients with non-syndromic retinal degeneration is critical and specific for the vision defect.

This study highlights the importance of functionally evaluating mutations that fall within different splice variants of a single gene for disease or tissue-specific relevance. It is estimated that

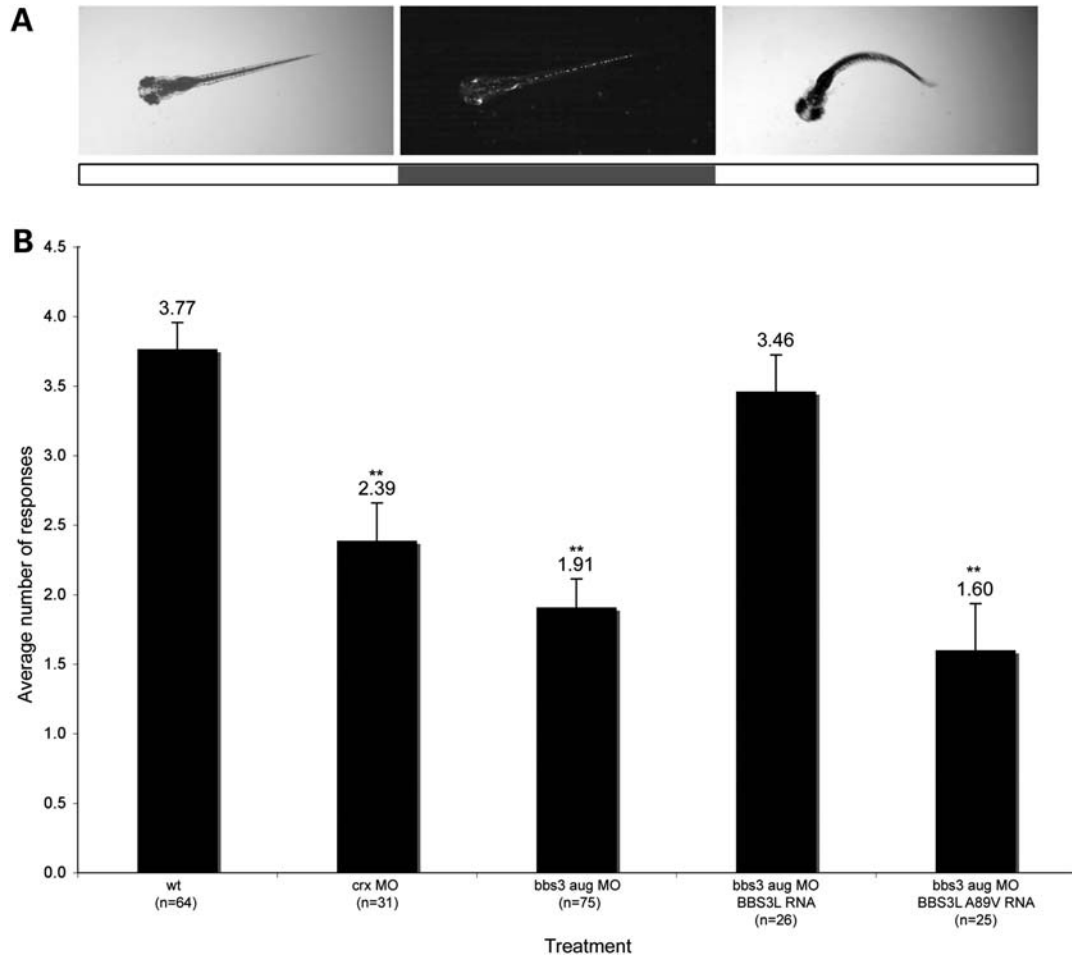


Figure 3. The BBS3L A89V mutation is not functional in vision. (A) Still images of a 5-day-old zebrafish embryo illustrating the characteristic escape response elicited by a sudden change in light intensity. The white boxes below the stills indicate lights on, while the grey box indicates lights off. For visualization of the embryo in the dark, image contrast manipulation was performed in Adobe Photoshop. (B) Graphical representation of the vision startle response data. *crx* gene knockdown was used as a control for visual impairment. Human *BBS3L* can rescue the vision defect, whereas human *BBS3L A89V* is not able to rescue the vision defect in *bbs3* knockdown embryos. The sample size (n) is noted on the X-axis. ** $P < 0.01$, ANOVA with Tukey. Data presented as the mean \pm SEM.

alternative splicing affects 94% of multi-exon human genes (49,50). Multiple splice isoforms with potentially diverse functions can be generated from a single gene, thus contributing to phenotypic complexity in disease. Recently, a retina specific splice variant was identified for another BBS gene, *BBS8* (51). A splice-site mutation was identified in *BBS8* that resulted in the skipping of a retina specific exon (51). This mutation results in affected individuals presenting with non-syndromic retinitis pigmentosa, similar to what was seen with patients harboring the BBS3 A89V missense mutation (51). While the BBS3 A89V mutation does not fall within in a splice site, it is located such that it impacts both the canonical BBS3 isoform as well as the eye-specific BBS3L isoform. Thus, mutations targeting a gene with multiple isoforms can have different affects on disease presentation.

Our finding that the BBS3 A89V missense mutation is specific for vision demonstrates that this region is important for proper function of the protein in the eye. While no known domains are predicated to map to this region of BBS3, it is possible that the A89V mutation abrogates the interaction of BBS3 with a retina specific modifier and/or

retina specific binding partner. Mutations in another BBS gene, *CEP290*, also result in phenotypic variation. This gene has been implicated in numerous diseases, in particular BBS and isolated blindness (52). While this gene has been implicated in several syndromic diseases, a mouse model for *Cep290*, the *rd16* mouse in which a few exons of the gene are missing, only manifests early onset retinal degeneration (53). Moreover, additional splice isoforms are predicted for *Cep290* and western blot analysis supports the notion that there are retina specific isoforms (53). These observations for BBS8, *Cep290* and now BBS3 indicate that mutations need to be evaluated in the context of both isoform and tissue specificity. Moreover, this study highlights the importance of functionally testing human disease mutations to further elucidate their underlying pathophysiology in disease.

MATERIALS AND METHODS

Ethics statement

The University Animal Care and Use Committee at the University of Iowa approved all animal work in this study.

Animal care

Adult zebrafish were maintained under standard conditions and embryos collected from natural spawnings (54). Embryos were staged using previously described criteria (55).

Conservation

Using the Ensemble genome (release 59), orthologs to BBS3 were identified by performing BLAST algorithms with the human BBS3 sequence (ENST00000335979). Sequences were aligned using ClustalW (56).

Morpholino injections

Antisense MOs were air pressure injected into one- to four-cell-staged embryos at a concentration of 12 ng for *bbs3* and 1 ng for *crx*.

MO sequences:

*bbs3*_{aug} (35,38): 5'-AGCTTGTCAAAAAGCCCCATTTGCT-3';

crx (48): 5'-ATGTAGGACATCATTCTTGGGACGG-3'.

DNA constructs and RNA synthesis

The A89V mutation was generated by introducing the appropriate nucleotide change into C-terminally *myc*-tagged human *BBS3* or *BBS3L* constructs using the Quick Change II site-directed mutagenesis kit (Stratagene).

Primers:

BBS3 A89V-F: 5'-GGAACACTATTATAAAGAAGGCCAAG-3';

BBS3 A89V-R: 5'-CACTACTATCAATGACAAAATAATAA-3'.

RNA was synthesized using the mMessage mMachine transcription kit (Ambion) and injected into 1–2 cell embryos at a concentration of 8 pg.

Melanosome transport assay

The melanosome transport assay was performed as previously described (26,34,35). Briefly, epinephrine (500 μl/ml, Sigma E437) treatment was applied to dark-adapted 6-day-old zebrafish embryos. The movement of the melanosomes from the periphery to the perinuclear region was timed and recorded. A stereoscope equipped with a Zeiss Axiocam camera was used to image live embryos.

Vision startle response

Zebrafish embryos change swimming directions in response to rapid changes in light intensity. Prior to performing the vision startle assay, 5 dpf embryos are light adapted for 1 h. Embryos were exposed to five trials of rapid changes in light intensity spaced at 30 s intervals and the response, a distinct C-bend, scored (38,57). *crx* gene knockdown was used as a control for vision impairment. To ensure motility, embryos were probed with a blunt needle on their flank, invoking the same response observed in the vision assay.

Statistical analysis

Statistics for both the melanosome transport and vision startle assay were calculated using the one-way ANOVA paired with the Tukey honestly significant difference test.

Western blot

Embryos were injected with C-terminal *myc*-tagged human *BBS3L* (8pg) and *BBS3L A89V* (8pg) RNA at the 1–2 cell stage. Embryos ($n = 15$) were collected at the following time points: 72 hpf, 4 dpf and 5 dpf. Collected embryos were homogenized in lysis buffer [20 mM Tris; 100 mM NaCl; 1 mM EDTA (ethylenediaminetetraacetic acid); 0.5% Triton X-100; 0.5% SDS (sodium dodecyl sulfate)] with protease inhibitor [0.1 mM PMSF (phenylmethanesulfonyl fluoride, Roche); 10 μg/ml Leupeptin (Roche)] and the supernatant collected. Whole cell lysates were run and transferred using the X Cell SureLock Mini-Cell System under reduced conditions (Invitrogen). Samples were mixed with NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent (Invitrogen), heated at 70°C for 10 min and run on a pre-cast 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen). Protein was transferred to a PVDF membrane (polyvinylidene fluoride, Amersham). The membrane was blocked in 5% milk (Carnation) for 1 h and probed with either mouse monoclonal anti-Myc (1:2000, 9B11, Cell Signaling) or rabbit polyclonal anti-actin (1:2000, Sigma) antibody overnight at 4°. Horseradish peroxidase-conjugated species-specific secondary antibodies (1:10,000, Jackson ImmunoResearch) were used for detection of primary antibodies.

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Conflict of Interest statement. None declared.

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