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A novel H395R mutation in MKKS/BBS6 causes retinitis pigmentosa and polydactyly without other findings of Bardet-Biedl or McKusick-Kaufman syndrome

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Purpose: To identify the causative mutation in two siblings from a consanguineous family in India with retinitis pigmentosa (RP) and polydactyly without other findings of Bardet-Biedl syndrome (BBS). We also performed functional characterization of the mutant protein to explore its role in this limited form of BBS.

Methods: The siblings underwent a thorough ophthalmological examination, including retinal optical coherence tomography (OCT) imaging, and an extensive physical examination with abdominal ultrasonography to characterize the disease phenotype. Next-generation sequencing (NGS) using a panel targeting retinal degeneration genes was performed on genomic DNA samples from the siblings and parents. Upon identification of the causative mutation, functional characterization was accomplished by performing protein–protein interaction studies in human embryonic kidney (HEK-293T) and human adult retinal pigmented epithelium (ARPE-19) cells.

Results: The two siblings showed signs of RP and polydactyly. The patients did not have truncal obesity, renal anomalies, hydrometrocolpos, congenital heart disease, or overt cognitive defects. NGS identified a homozygous c.1184A>G mutation in the *MKKS/BBS6* gene in both patients resulting in a p.H395R substitution in the MKKS/BBS6 protein. This mutant protein decreased the interaction of MKKS/BBS6 with BBS12 but did so to a different extent in the HEK-293T versus ARPE-19 cells. Nonetheless, the effect of the H395R variant on disrupting interactions with BBS12 was not as profound as other reported *MKKS/BBS6* mutations associated with syndromic RP.

Conclusions: We identified a novel H395R substitution in MKKS/BBS6 that results in a unique phenotype of only RP and polydactyly. Our observations reaffirm the notion that mutations in *MKKS/BBS6* cause phenotypic heterogeneity and do not always result in classic MKKS or BBS findings.

Retinitis pigmentosa (RP) is the most common inherited form of retinal degeneration, affecting nearly 1/4,000 individuals [1]. RP is caused by progressive rod photoreceptor degeneration ultimately leading to peripheral vision loss, night blindness, and, sometimes, complete blindness. Mutations in more than 50 genes have been identified to cause nonsyndromic RP, while mutations in a smaller subset of genes have been found to cause syndromic RP (i.e., RP combined with additional symptoms in other tissues/organs). Common forms of syndromic RP include Usher syndrome [2], Refsum disease [3], and Bardet-Biedl syndrome (BBS) [4]. Of these disorders, BBS is one of the most studied.

Genetic mutations that trigger BBS cause defects in primary cilia biogenesis and/or function [5,6]. The primary cilium plays an important role in extracellular signal transduction in several different tissues. Thus, the loss of primary cilia or cilia dysfunction leads to several abnormalities, including truncal obesity, genital anomalies, situs inversus, polydactyly, nephropathy, and learning difficulties (in addition to RP). At the molecular level, BBS is a genetically heterogeneous disease caused by recessive mutations in several proteins involved in regulating primary cilium formation and function. To date, mutations in 16 genes have been implicated in causing BBS [4]. These mutations are primarily found in genes that encode for the BBSome complex (*BBS1-2* [BBS1 - Gene ID: 582, OMIM 209901; *BBS2* - Gene ID: 583, OMIM 606151], *BBS4-5* [*BBS4* – Gene ID: 585, OMIM 600374; *BBS5* – Gene ID: 129880, OMIM 603650], *BBS7-9 [BBS7* – Gene ID: 55212, OMIM 607590; *BBS8* – Gene ID: 123016, OMIM 608132; *BBS9* – Gene ID: 27241, OMIM 607968]) [6], which is responsible for organizing cilogenesis and cilium maintenance, or the BBS chaperonin complex (*MKKS/BBS6* [Gene ID: 8195, OMIM 604896], *BBS10* [Gene ID: 79738,

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OMIM 610148], *BBS12* [Gene ID: 166379, OMIM 610683]) [7]). Mutations in *MKKS/BBS6* have also been identified in patients with McKusick-Kaufman syndrome (MKKS) [8] and Meckel syndrome [9]. These syndromes are generally less severe than BBS but, nonetheless, share key diagnostic features, including polydactyly and hydrometrocolpos (fluid-filled vaginal cavity) [8]. During early childhood, it is difficult to make a clinical diagnosis with any certainty whether a patient presenting with polydactyly and slight obesity has BBS or MKKS [10]. The distinction between BBS and MKKS becomes more clear only as the affected individual ages and develops additional phenotypes indicative of one or the other syndrome.

A prominent, defining feature of BBS that makes it unique when compared to similar syndromes such as MKKS or Meckel syndrome is the development of RP. Given that photoreceptors are comprised of highly complex, dense stacks of sensory cilia in the form of outer segments, it is no surprise that photoreceptor degeneration is a hallmark of several ciliopathies [11]. Mutations to critical ciliary-regulating proteins involved in the trafficking of the components of outer segments results in the accumulation of rhodopsin in the inner segment and eventual photoreceptor cell death [12,13], causing peripheral vision loss, tunnel vision, and, potentially, blindness. At the heart of the primary cilia in the photoreceptor cell is the BBS chaperonin complex [7]. This complex consists of three chaperonin-like BBS proteins (MKKS/BBS6, BBS10, and BBS12) along with six chaperonin-containing TCP-1/TCP-1 ring complex (CCT/TRiC) proteins and is required for the proper formation of the BBSome complex [7,14]. In turn, the BBSome is required for protein and vesicle trafficking along the microtubules and primary cilium function [15,16]. Due to the reliance of the BBSome assembly on the proper formation of the BBS chaperonin complex, mice lacking *Bbs6* as well as human patients who have *MKKS/BBS6*, *BBS10*, or *BBS12* mutations phenotypically resemble mice and humans with defects in BBSome genes [17-20]. However, mutations in one particular BBS chaperonin gene, *MKKS/BBS6*, seem to give rise to several phenotypically variable, yet similar, syndromes [8,9,21]. Thus, it is apparent that mutations in genes encoding for BBS proteins do not always cause BBS but instead may cause syndromes that phenotypically overlap with BBS [8,10,22].

Herein, we describe a pair of siblings from a consanguineous Indian family who display RP and polydactyly in the absence of other findings typically found in ciliopathies. We have discovered that both patients share a novel homozygous c.1184A>G missense mutation in the *MKKS/BBS6* gene that results in a H395R substitution in the MKKS/BBS6 ciliary protein. Furthermore, this mutation reduces the interaction between MKKS/BBS6 and another important ciliary protein in the BBS chaperonin complex, BBS12. To our knowledge, this novel missense mutation is the first reported mutation in the *MKKS/BBS6* gene and one of only a handful of known mutations to result in the limited form of BBS that includes RP and polydactyly [23-26].

METHODS

Study subjects: The study was approved by the Institutional Review Board of the Srikiran Institute of Ophthalmology, adhered to the ARVO statement on human subjects, and followed the tenets of the Declaration of Helsinki. Two affected siblings and their unaffected parents were recruited and examined after informed consent was obtained. All subjects underwent detailed ophthalmologic evaluations, including a fundus examination, by a retina fellowship–trained ophthalmologist. Affected siblings underwent retinal optical coherence tomography (OCT) examination with the Topcon 3D OCT-2000 (Topcon, Tokyo, Japan). The siblings underwent a thorough physical examination by a pediatrician followed by ultrasonography of their abdomen.

Mutational analysis: DNA from siblings and parents was used to perform targeted gene capture (retinal degeneration genes) using a custom capture kit (NimbleGen EZ Choice XL, Roche NimbleGen, Madison, WI). The libraries were sequenced to mean >80–100X on-target depth on an Illumina sequencing platform (Illumina, San Diego, CA). The sequences obtained were aligned to the GRCh37/hg19 human reference genome using a Burrows-Wheeler Aligner program [27] and analyzed using the Picard and GATK-Lite toolkit to identify variants in the targeted genes relevant to the clinical condition. Annotation of the variant was performed against the Ensembl release 75 gene model using an internal annotation pipeline. Clinically relevant mutations were annotated using published variants in the literature and a set of variant databases, including ClinVar, OMIM, GWAS, HGMD, and SwissVar. Only non-synonymous and splice-site variants found in the retinal degeneration panel genes were used for clinical interpretation. Silent variations that did not result in any change in amino acids in the coding region were not reported. Sequences of the MKKS/BBS6 protein from different species were obtained from the UniProt website and aligned to human MKKS/BBS6 using ClustalOmega.

Construct generation: pCS2 plasmids encoding for Myc wild-type (WT) BBS12 and FLAG WT MKKS/BBS6 were kind gifts of Dr. Val Sheffield and Dr. Seongjin Seo, University of Iowa [7]. FLAG MKKS/BBS6 mutants encoding for T57A,

L277P, and H395R were generated using site-directed mutagenesis (Q5 Site Directed Mutagenesis Kit, New England Biolabs, Ipswich, MA). All constructs were verified with sequencing.

Cell culture: Human embryonic kidney (HEK-293T) cells (authenticated with short tandem repeat DNA profiling, University of Arizona Genomics Core, Tucson, AZ) were cultured in DMEM media (Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA) and penicillin/streptomycin/glutamine (Corning). Human adult retinal pigmented epithelium (ARPE-19) cells (also authenticated as described above) were cultured using identical conditions as for the HEK-293T cells but in DMEM/F12 media (Corning). For the HEK-293T cotransfection immunoprecipitation (IP) experiments, 300,000 cells were plated into wells of a six-well plate, allowed to attach overnight, and transfected with 2.5 µg of total DNA (1.25 µg MKKS/BBS6 and 1.25 µg BBS12) and 5 µl of XtremeGENE HP (Roche, Piscataway, NJ). Cotransfection experiments in ARPE-19 cells were performed by reverse transfection [28] using Lipofectamine 3000 (Life Technologies). Briefly, 300,000 cells were placed on top of transfection complexes composed of 1.25 µg MKKS/BBS6 DNA, 1.25 µg BBS12 DNA, 2.5 µl of P3000, and 7.5 µl of Lipofectamine 3000 in a six-well plate. Media were changed 24 h post transfection for the HEK-293T and ARPE-19 cells.

Immunoprecipitation (IP) and western blotting: Forty-eight hours post transfection, HEK-293T and ARPE-19 cells were harvested and resuspended in lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, and 0.5% Triton-X supplemented with Halt protease inhibitor cocktail [Thermo Scientific Pierce, Waltham, MA]). About 75 – 200 µg total cell lysate was IP'd using 5 µl of anti-FLAG M2 magnetic beads (Sigma, St. Louis, MO) for ≥2 h at 4 °C. Before the IP, 10% of the total cell lysate sample was used for the input. M2 beads were washed four times in lysis buffer before elution in 1X Laemmli buffer. Input and IP samples were run under reducing, denaturing conditions on a 4–20% Tris Glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to a nitrocellulose membrane using a semidry G2 blotter (Thermo Scientific Pierce). The membrane was probed with the following primary antibodies: rabbit anti-FLAG (1:800, Sigma), mouse anti-Myc (1:1,500, Thermo Scientific Pierce), and rabbit anti-β actin (1:10,000, LI-COR, Lincoln, NE) followed by an appropriate infrared dye-conjugated secondary antibody (LI-COR). All blots were imaged and quantified on an Odyssey Fc (LI-COR).

RESULTS

Two Indian siblings from a consanguineous family display features of RP and polydactyly and no other common signs of ciliopathies:

Case A—An 18-year-old male subject (study ID SIO101) presented with a chief complaint of poor vision and night blindness since the age of 10. His best spectacle-corrected visual acuity was 20/40 in the right eye (OD) and 20/70 in the left eye (OS). Slit-lamp examination of the anterior segments was remarkable only for trace bilateral subcapsular cataracts. A fundus examination revealed bilateral optic nerve head pallor, retinal vascular attenuation, and RPE degeneration of the macula and the peripheral retina. Fundus images and macular optical coherence tomography (OCT) are shown in Figure 1A,B, respectively. Significant retinal thinning was observed in both maculas (Figure 1C). A thorough physical examination was performed by a pediatrician. The subject's height was 175 cm (50th percentile), and his weight was 47.9 kg (<5th percentile) without any evidence of truncal obesity (BMI 16). Patient was noted to have post-axial polydactyly of the hands and feet (Figure 1D). Cardiac, abdominal, and genitourinary exams were normal. Subject had normal sexual development for his age. Although no overt cognitive deficits were noted, the subject was found to have poor scholastic performance in school. Ultrasonography of his abdomen revealed a small right renal calculus (stone), but no kidney or other organ abnormalities were noted.

Case B—A 13-year-old female subject (study ID SIO102) presented with a chief complaint of poor vision and night blindness. Her best spectacle-corrected visual acuity was 20/300 in OD and 20/40 in OS. A slit-lamp examination of the anterior segments was unremarkable. A fundus examination revealed bilateral optic nerve head pallor, retinal vascular attenuation, and RPE degeneration of the macula and the peripheral retina with mild bone spicule-like formation (Figure 2A,B). Bilateral retinal macular thinning was also observed in this subject (Figure 2C). On systemic evaluation by a pediatrician, the subject was noted to have normal growth and development. Her height was 150 cm (10th percentile), and her weight was 24 kg (10th percentile) without any truncal obesity (BMI 15). She was noted to have post-axial polydactyly of the hands and feet (Figure 2D). Cardiac, abdominal, and genitourinary exams were normal. The subject had normal sexual development for her age and had attained menarche with a regular cycle. Although no overt cognitive deficits were found, she was also noted to have poor scholastic performance in school. Ultrasonography of her abdomen was unremarkable.

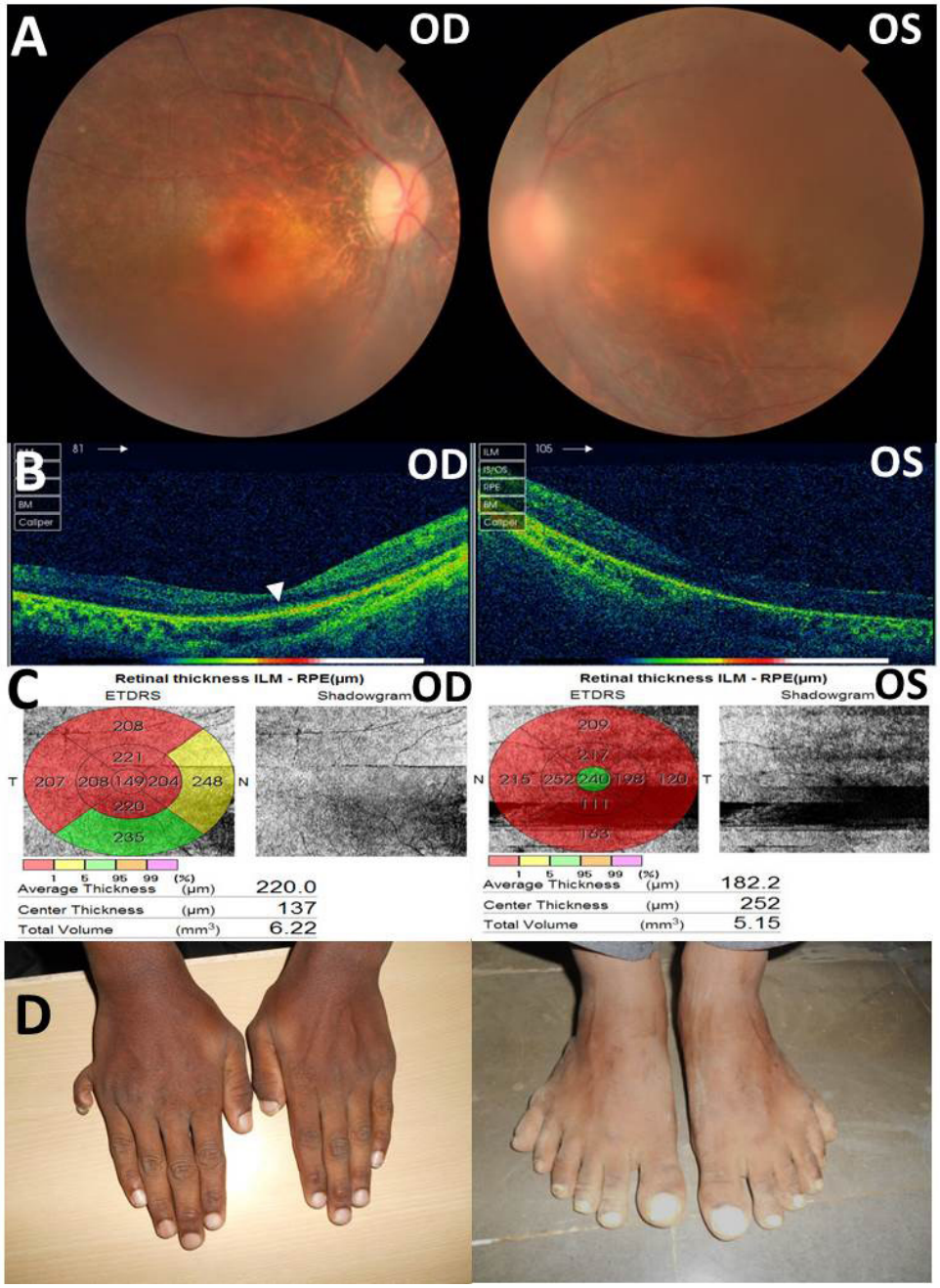

Figure 1. Findings of retinitis pigmentosa and polydactyly in the male subject. **A**: Fundus photos demonstrate marked vascular attenuation in both eyes, diffuse RPE atrophic changes with some foveal preservation, and prominent luteal pigment visualization. **B**: Loss of the outer retinal layer on optical coherence tomography (OCT) imaging (arrowhead). **C**: The OCT images show significant retinal thinning in both maculas. **D**: Post-axial polydactyly of the hands and feet.

NGS identifies a homozygous mutation in MKKS/BBS6 in both patients: An NGS panel targeting retinal degeneration genes revealed a shared, homozygous missense mutation in exon 5 of the *MKKS/BBS6* gene in the siblings (Figure 3A), which results in the substitution of histidine by arginine (p.H395R) in a conserved amino acid position (Figure 3B). This rare variant is absent in the 1000 Genomes and Exome Aggregation Consortium (ExAC) databases and is predicted by the Polymorphism Phenotyping v2 software (PolyPhen-2) to be 'possibly damaging' (score of 0.818). This missense mutation was confirmed with Sanger sequencing (Figure 3A).

The H395R mutation differentially disrupts interactions between MKKS/BBS6 and BBS12 according to the cell type used: Previously, Seo et al. found that several BBS-associated mutations in MKKS/BBS6 reduced the ability of the protein to bind to another chaperonin-like BBS protein, BBS12 [7]. Importantly, these authors, as well as others, have found that tagged forms of MKKS/BBS6 interact with endogenous

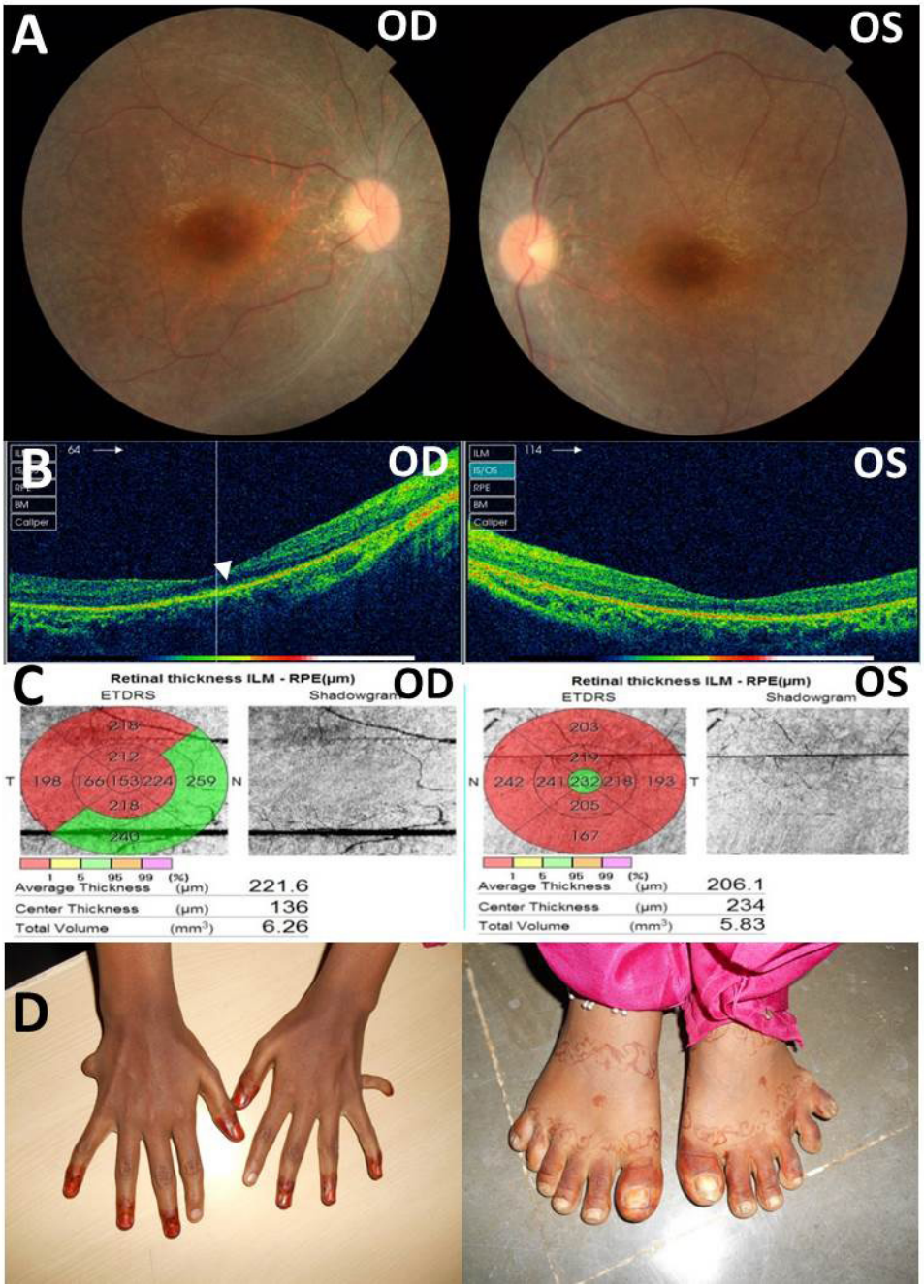

Figure 2. Findings of retinitis pigmentosa and polydactyly in the female subject. **A**: Fundus photos demonstrating marked vascular attenuation in both eyes and diffuse RPE atrophic changes. **B**: Optical coherence tomography (OCT) imaging shows loss of the outer retinal layer (arrowhead). **C**: The OCT images show significant bilateral retinal macular thinning. **D**: Post-axial polydactyly of the hands and feet.

BBSome proteins, achieve correct centrosomal subcellular localization, and are functional in transfected cells [7,29-32]. We therefore decided to test whether the newly identified H395R mutation in MKKS/BBS6 also disrupted the MKKS/BBS6-BBS12 interaction. The H395R mutation reduced the ability of FLAG MKKS/BBS6 to bind to Myc WT BBS12 in HEK-293T cells, on average, by 43% (57±17% of FLAG WT MKKS/BBS6 levels, Figure 4A, B). As a positive control, we included two additional MKKS/BBS6 mutants (T57A and L277P [21]) that had previously been found to disrupt the MKKS/BBS6-BBS12 interaction [7]. We confirmed that these mutants disrupt the MKKS/BBS6-BBS12 interaction, but they do so to a greater extent than the H395R mutation. T57A, a clearly disrupting mutation, nearly abolished the ability of FLAG MKKS/BBS6 to bind to Myc WT BBS12 in HEK-293T cells (5.7±4.2% of FLAG WT MKKS/BBS6 levels), whereas the L277P interacted with Myc WT BBS12 at 43±12% of FLAG WT MKKS/BBS6 levels (Figure 4A,B).

Due to the lack of syndromic RP in H395R homozygous individuals, we speculated that the H395R mutation might affect the MKKS/BBS6-BBS12 interaction differently in different cell types (i.e., that the H395R mutation would be disruptive in certain instances but less so in other instances). Therefore, we tested how the H395R mutation affected MKKS/BBS6 interactions with BBS12 in an RPE-derived cell line, ARPE-19 [33]. We found that the H395R mutation only slightly reduced the ability to interact with Myc WT BBS12, on average, by 15% (85±10% of WT MKKS/BBS6 levels, Figure 4C,D). The other two mutations, T53A and L277P, showed a similar reduced ability to interact with BBS12 in ARPE-19 cells (0.74±0.23% and 41±13% of WT MKKS/BBS6 levels in ARPE-19 cells, respectively) when compared with their effects in HEK-293T cells. In the comparison of the two cell lines, the effects of the MKKS/BBS6 mutations on disrupting the interaction with BBS12 were only significantly different for H395R (Figure 4C,D).

DISCUSSION

We report a mutation in the *MKKS/BBS6* gene that causes only RP and polydactyly without other common BBS/MKKS findings, such as obesity, hydrometrocolpos, heart disease, or nephropathy. Given the poor scholastic performance of both siblings, we cannot rule out a subtle learning disability as part of the H395R mutation phenotype. Several independent groups have found that patients with the M390R mutant in BBS1 appear to have a less severe form of BBS that may include only retinal dysfunction and polydactyly [24,25,34]. Another study found that mutations in *C8orf37,* which encodes for a separate ciliary protein, cause a similar RP/

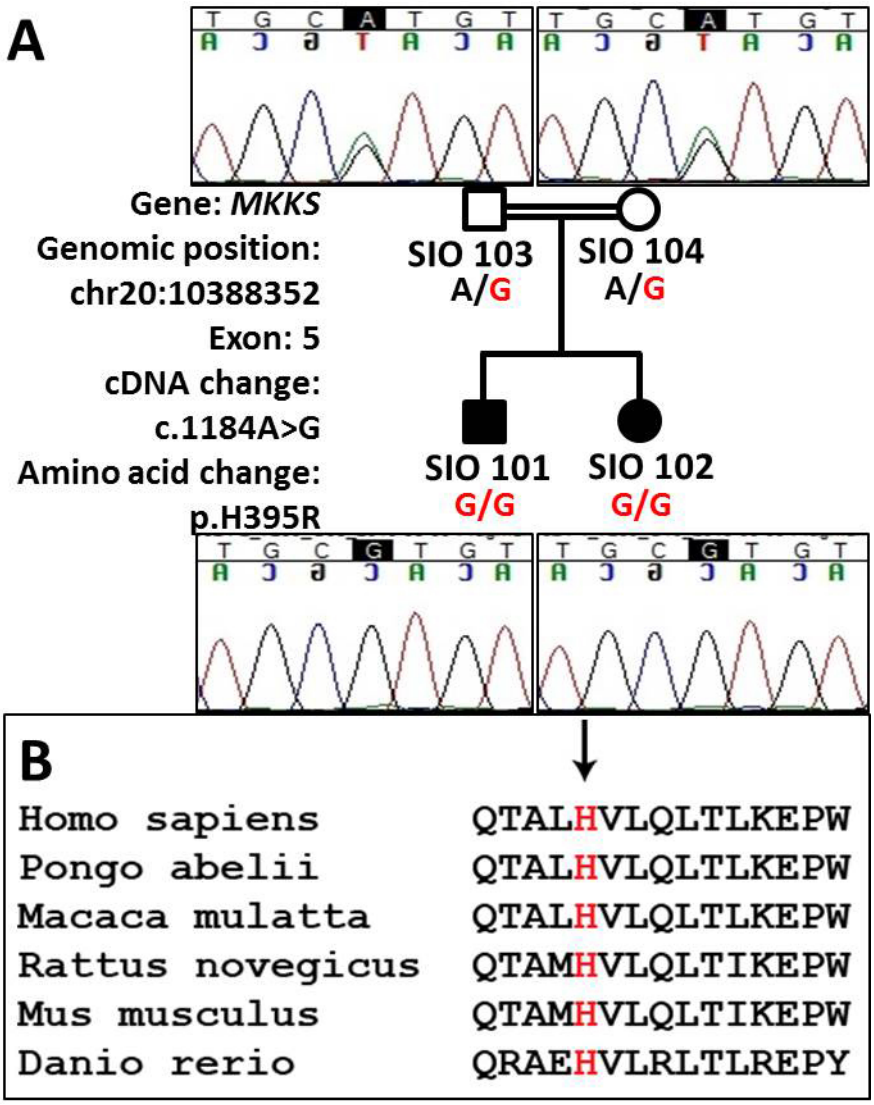

Figure 3. A novel H395R mutation in MKKS/BBS6. **A**: Consanguineous pedigree with homozygous missense mutation in exon 5 of the *MKKS/BBS6* gene found in both siblings. Sanger sequencing confirmed heterozygosity of the variant in parents. **B**: Conservation of exon 5 amino acids of MKKS/BBS6 across species. The arrow indicates the location of the H395R mutation.

polydactyly phenotype in the absence of additional findings associated with primary ciliary defects [23]. These reports, combined with our observations regarding the H395R mutation, reaffirm the general observations that BBS is a genetically heterogeneous disorder with mutations resulting in a wide spectrum of phenotypic clinical features [8,22].

We found that the H395R mutation reduced the ability of MKKS/BBS6 to bind to another component of the BBS chaperonin complex, BBS12, in HEK-293T cells and to a lesser extent in ARPE-19 cells. Although these data suggest that this novel mutation likely disrupts the proper formation of the BBS chaperonin complex and is disease-causing, it is still unknown whether the extent to which mutant MKKS/BBS6 binds to BBS12 impacts disease severity. For example, it is not clear whether patients with a T57A MKKS/BBS6 mutation that markedly disrupts MKKS/BBS6 interactions [7] develop a wider spectrum of BBS-related findings or present with greater disease severity or an earlier age than patients with less disruptive L227P [7] or H395R mutations. We cannot rule out the possible role of secondary disease modifying the genes. However, we speculate that the H395R mutation is a hypomorphic mutation that is differentially tolerated depending on the cell type in which the mutation is expressed. Ultimately, we propose that the H395R mutation disrupts ciliary function and/or biogenesis to result in a less severe BBS phenotype that includes RP and polydactyly.

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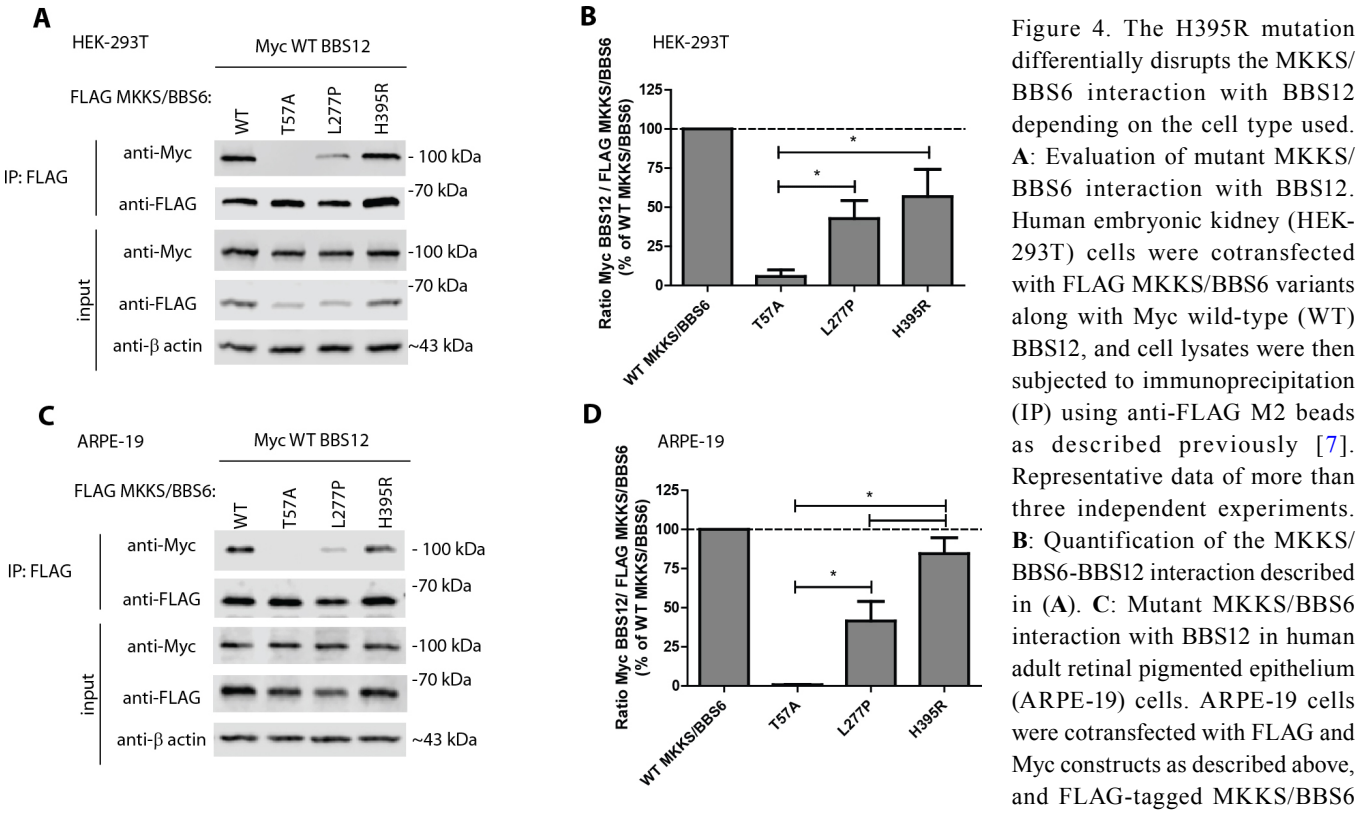

Figure 4. The H395R mutation differentially disrupts the MKKS/BBS6 interaction with BBS12 depending on the cell type used. **A**: Evaluation of mutant MKKS/BBS6 interaction with BBS12. Human embryonic kidney (HEK-293T) cells were cotransfected with FLAG MKKS/BBS6 variants along with Myc wild-type (WT) BBS12, and cell lysates were then subjected to immunoprecipitation (IP) using anti-FLAG M2 beads as described previously [7]. Representative data of more than three independent experiments. **B**: Quantification of the MKKS/BBS6-BBS12 interaction described in (**A**). **C**: Mutant MKKS/BBS6 interaction with BBS12 in human adult retinal pigmented epithelium (ARPE-19) cells. ARPE-19 cells were cotransfected with FLAG and Myc constructs as described above, and FLAG-tagged MKKS/BBS6 was immunoprecipitated. Representative data of three independent experiments. **D**: Quantification of the MKKS/BBS6-BBS12 interaction described in (**C**). The band intensities of the IP'd Myc WT BBS12 and FLAG MKKS/BBS6 proteins were quantified using LI-COR software. The ratio of Myc WT BBS12 to FLAG MKKS/BBS6 was calculated and displayed as a percentage of the Myc WT BBS12/FLAG WT MKKS/BBS6 ratio. n≥3; average ±standard error of the mean (SEM); *=p<0.05, *t* test. Note: The only variant that had a significantly different ability to bind to WT BBS12 in HEK-293T versus ARPE-19 cells was H395R (p<0.05, *t* test).

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