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ORIGINAL ARTICLE

Visual impairment and progressive phthisis bulbi caused by recessive pathogenic variant in *MARK3*

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Abstract

Developmental eye defects often severely reduce vision. Despite extensive efforts, for a substantial fraction of these cases the molecular causes are unknown. Recessive eye disorders are frequent in consanguineous populations and such large families with multiple affected individuals provide an opportunity to identify recessive causative genes. We studied a Pakistani consanguineous family with three affected individuals with congenital vision loss and progressive eye degeneration. The family was analyzed by exome sequencing of one affected individual and genotyping of all family members. We have identified a non-synonymous homozygous variant (NM_001128918.2: c.1708C > G: p.Arg570Gly) in the *MARK3* gene as the likely cause of the phenotype. Given that MARK3 is highly conserved in flies (I: 55%; S: 67%) we knocked down the *MARK3* homologue, *par-1*, in the eye during development. This leads to a significant reduction in eye size, a severe loss of photoreceptors and loss of vision based on electroretinogram (ERG) recordings. Expression of the *par-1* p.Arg792Gly mutation (equivalent to the *MARK3* variant found in patients) in developing fly eyes also induces loss of eye tissue and reduces the ERG signals. The data in flies and human indicate that the *MARK3* variant corresponds to a loss of function. We conclude that the identified

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mutation in *MARK3* establishes a new gene-disease link, since it likely causes structural abnormalities during eye development and visual impairment in humans, and that the function of *MARK3/par-1* is evolutionarily conserved in eye development.

Introduction

Developmental eye birth defects are of clinical significance due to severely reduced visual impairment (VI), yet the genetic cause of only a small number of gene variants associated with developmental eye defects has been identified (1). VI causes a significant burden in the life of individuals and has a strong impact on their psychological and socioeconomic experiences. The elucidation of the genomic causality provides a better understanding of the molecular pathophysiology of the various forms of VI, enhances the diagnostic possibilities and provides targets and possible strategies for therapeutic interventions (2,3). Mendelian disorders provide a direct way to identify genes and variants of high phenotypic impact (4). Large consanguineous families with multiple affected individuals are an obvious source to identify pathogenic genetic variants in recessive disorders (5–8). It has been estimated that more than 10% of marriages occur among close relatives worldwide (9); in some countries, this reaches almost 70%, and this is the case in Pakistan (www.consang.net). We argue that a considerable number of additional recessive genes implicated in VI could be identified in these populations (https://sph.uth.edu/retnet/).

Here, we report a Pakistani consanguineous family of three affected individuals with severe VI and progressive eye loss. The family was analyzed by combining whole exome sequencing and homozygosity mapping, which is a powerful and cost-effective methodology to identify candidate genes in undiagnosed families with likely autosomal recessive disorders (10–12). We identified a homozygous, likely pathogenic missense variant in *MARK3* (microtubule-associated protein/microtubule affinity-regulating kinase 3) gene (OMIM* 602678) that is likely to cause VI and the progressive phthisis bulbi in patients after birth. Modeling of the variant in the *Drosophila* homologue of the *MARK3* gene leads to reduced eye development and dysfunction, supporting the pathogenicity of the *MARK3* variant.

Results

Phenotypes

Family F105 with three affected individuals (Fig. 1), is of Pakistani origin from the area of Jamshoro, Sindh, Pakistan. Parents are first cousins once removed. All three probands had poor vision at birth. Individuals V: 2 and V: 3, who are 30- and 18-years old, respectively, developed eye phthisis by adulthood. Individual V: 5, a 15-years old female, has poor vision with low acuity along with hazy cornea (Figs 1 and 2).

The magnetic resonance imaging (MRI) findings revealed that bilaterally the eye globe was deformed and smaller in size in both probands V: 2 and V: 3. In addition, there was a loss of the normal structural differentiation between the anterior and posterior chambers, including aqueous and vitreous humor. The right and left eye globes were measured as 1.7× 1.4 cm and 1.7×1.5 cm, respectively, in V: 2; while in V: 3 they were 2.0×1.8 cm and 2.0×1.7 cm (normal = 2.42 ± 0.96×2.41 ± 0.97 cm) (13) (Fig. 2). B-scan and A-scan ultrasonography of affected individual V: 3 showed that the posterior wall was thick along with vitreous opacity, and the interior–posterior chamber was small with an axial diameter of about 14 mm (normal = 24 mm). No posterior eye wall was detected in patient V: 2 on the B-scan (Fig. 2). The ophthalmologic examination of patient V: 5 showed hypermetropia with corneal plana and ptosis. The best corrected visual acuity was 2/60 and 6/60 in the right and left eye, respectively. Fundoscopic examination revealed a clear fundus with a normal cup and disk ratio and the tonometry indicated normal intraocular pressure (IOP) in both eyes (17 mm/Hg OD, 12 mm/Hg OS).

Genetic analysis

Exome sequencing was performed in one affected individual (V: 3) of the family, and 95.3% of the target exome regions were covered by more than 10 reads. We did not find any potential likely pathogenic variant by considering all of the 261 genes that have been documented to affect vision, including those in the RetNet (https://sph.uth.edu/retnet/) database. Subsequently, we used a 720K SNP-array to genotype all five siblings (V: 1, V: 2, V: 3, V: 4 and V: 5) and both parents (III: 1 and IV: 1) of family F105. By combining both the exome sequence and genotyping data through CATCH (14) analysis, we identified two candidate variants that segregated in the family with a recessive pattern of inheritance; the first was in the *MARK3* gene (NM_001128918.2: c.1708C > G: p.Arg570Gly) on chromosome 14 (OMIM* 602678), and the second in the *PAH* gene (NM_000277.2: c.355C > T: p.Pro119Ser) on chromosome 12 (OMIM *612349). The segregation of both variants was confirmed through Sanger sequencing in all members of the family (Fig. 1 and Supplementary Material, Fig. S1).

The missense variant in *MARK3*, segregated with the disease phenotype in a recessive fashion in the seven members of the family studied, with the LOD score of 1.45 (Fig. 1). The frequency of this variant in the gnomAD database (http://gnomad.broadinstitute.org/) is 0.000052 and the probability of LoF intolerance (pLI) for *MARK3* in the ExAC database (http://exac.broadinstitute.org/) (15) is zero, suggesting that null mutations in one allele of *MARK3* are not uncommon and that a disease associated with the gene would likely be recessive (MARRVEL.org) (16). The *MARK3* variant is predicted to be pathogenic by various prediction algorithms [DANN (17) score: 0.99]. MARK3 Arg570 is very well conserved down to Drosophila (MARRVEL.org) (16) (Supplementary Material, Fig. S2).

The non-synonymous variant PAH p.Pro119Ser has been classified as likely pathogenic in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) because in a compound heterozygote P119S/IVS12 + 1G > A case, the phenotype was a mild phenylketonuria (PKU) with phenylalanine levels of 440 μmol/l (18). Furthermore, the p.Pro119Ser variant is categorized as tetrahydrobiopterin (BH_4) responsive type of PKU (19). The phenotype in the subjects V: 2, V: 3 and V: 5 was not compatible with the PKU; however, we have measured phenylalanine and tyrosine levels from Guthrie cards. Individual V: 2 has phenylalanine 117 μmol/l and tyrosine 36 μmol/l and individual V: 3 has 148 μmol/l phenylalanine and 46 μmol/l tyrosine (normal levels are phenylalanine: 26–98 μmol/l and tyrosine: 35–107 μmol/l). These results confirm that the phenylalanine levels were slightly increased but not in the PKU disease range. We conclude that the homozygosity of the PAH p.Pro119Ser variant does not result in PKU in this family. This 'incidental' finding, which is not related to the eye

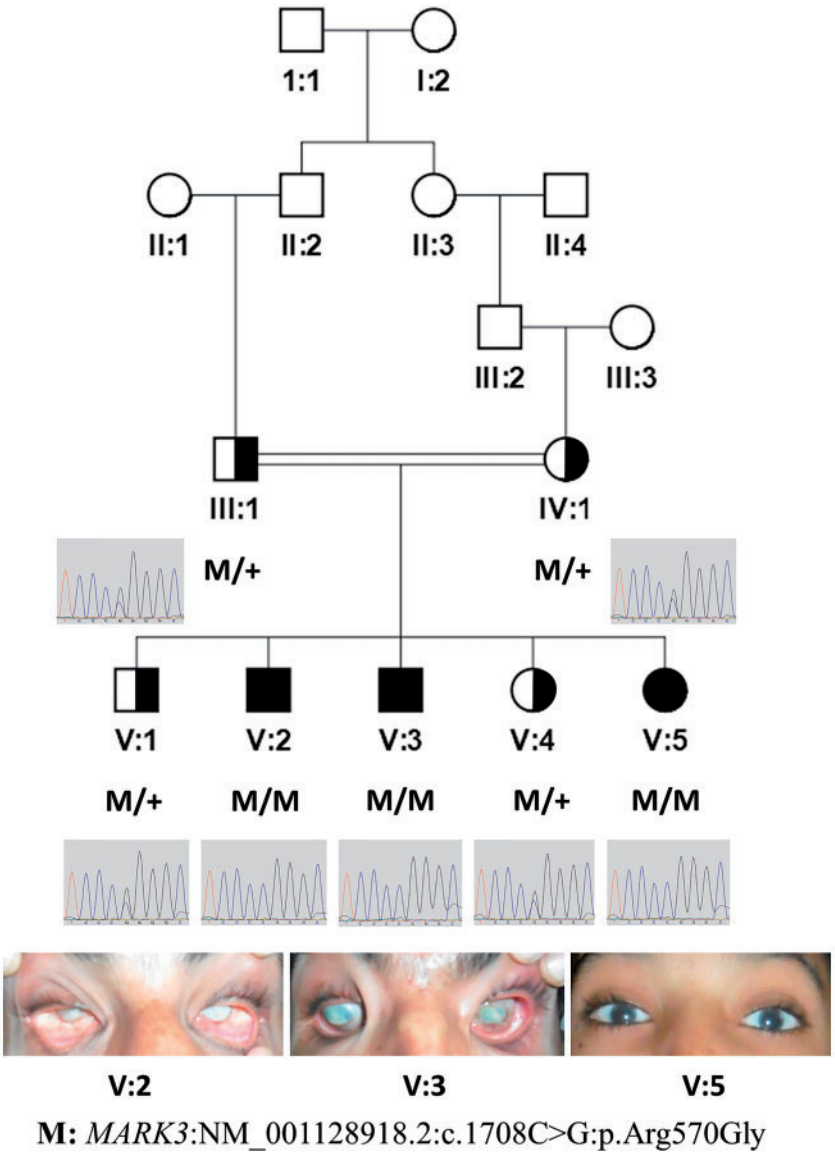

Figure 1. *MARK3* variant segregation in the Pakistani family pedigree F105 and eye phenotype. Genotypes of the variant (M), also shown by chromatograms (reverse complement), in the pedigree diagram of a consanguineous family of three affected and two unaffected individuals. Photographs of eyes of the affected individuals are shown in the lower panel. Half-filled symbols denote a carrier for the pathogenic variant. Filled symbols denote affected individuals.

phenotype, provides additional information in the PKU field related to the interpretation of this variant. It helps populate public databases with genomic variants from consanguineous individuals so that homozygosity for likely pathogenic variants can be evaluated against certain phenotypes.

Drosophila model analysis

The homologue of *MARK3* with the highest DIOPT score (11/12) in Drosophila is *par-1*, indicating that *par-1* is the orthologue of *MARK3* (16). We therefore used two independent RNAis to reduce Par-1 expression in the developing eye by driving the RNAis with the *eyeless GAL4* (*ey-GAL4*) driver (20) which is expressed throughout eye development. As shown in Figure 3, this leads to a significant reduction in eye size (Fig. 3A–C) and a reduced amplitude of the electroretinograms (ERGs) (Fig. 3D–F). Control RNAi experiments do not affect the eye morphology (Supplementary Material, Fig. S3). The most severe phenotype associated with RNAi-2 causes not only a severe reduction in amplitude, arguing that the retinal function is severely impaired, but also a loss of on-off transients (arrows in Fig. 3F, H and I), indicating a loss of vision. We confirmed that the *ey > par-1 RNAi* successfully knocked down the Par-1 protein level in developing eye using a polyclonal Par-1 antibody (Fig. 3J and K). Importantly, the number of developing photoreceptors marked by Elav antibody which labels neurons/photoreceptors, is severely reduced in eye imaginal discs when compared to control discs (Fig. 3N and O). In summary, the data show that Par-1 is required for proper eye development and photoreceptor differentiation and that its loss leads to vision loss.

To investigate the functional effects of the identified *MARK3* variants, we created the equivalent Drosophila *par-1* mutation (p.Arg792Gly) and generated transgenic animals harboring this

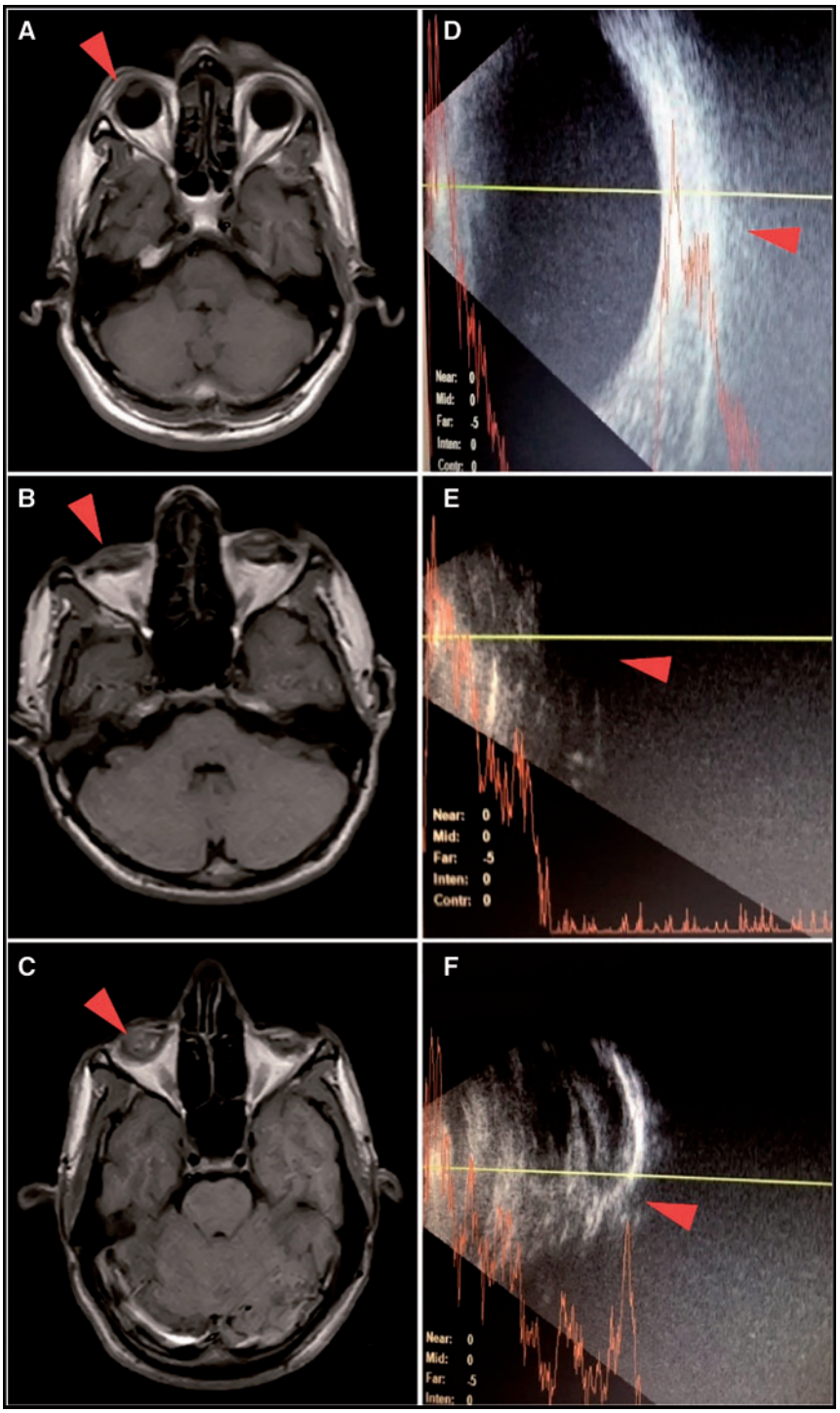

Figure 2. MRI and B-scans of two affected individuals (V: 2 and V: 3). **B** and **C**: T1 weighted MRI images of patients V: 2 and V: 3, respectively, arrows indicate the shrunken eye globes; **A** is the representative MRI image with normal eye globes. **E** and **F** are the B-scan images of patients V: 2 and V: 3, respectively, showing abnormal eye globe; **D** is a representative B-scan of a normal eye.

variant cDNA (*UAS-par-1* p.Arg792Gly) as well as the wild-type Drosophila cDNA (*UAS-par-1 WT*). We again used *ey-GAL4* to express these cDNAs in the developing eye (21). As a negative control, we expressed the *lacZ* gene with *ey-GAL4* and observed a normal eye morphology and ERG (Fig. 4B and F). Expression of the wild-type Par-1 protein in the developing eye causes a mild defect in eye morphology and a subtle reduction in ERG amplitude (Fig. 4C and G). In contrast, expression of the Par-1 p.Arg792Gly variant induces severely reduced eyes that are very rough and causes a near complete loss of amplitude of the ERG (Fig. 4D, H and I). In summary, these data suggest that Par-1 p.Arg792Gly acts as a dominant negative mutation in flies when overexpressed.

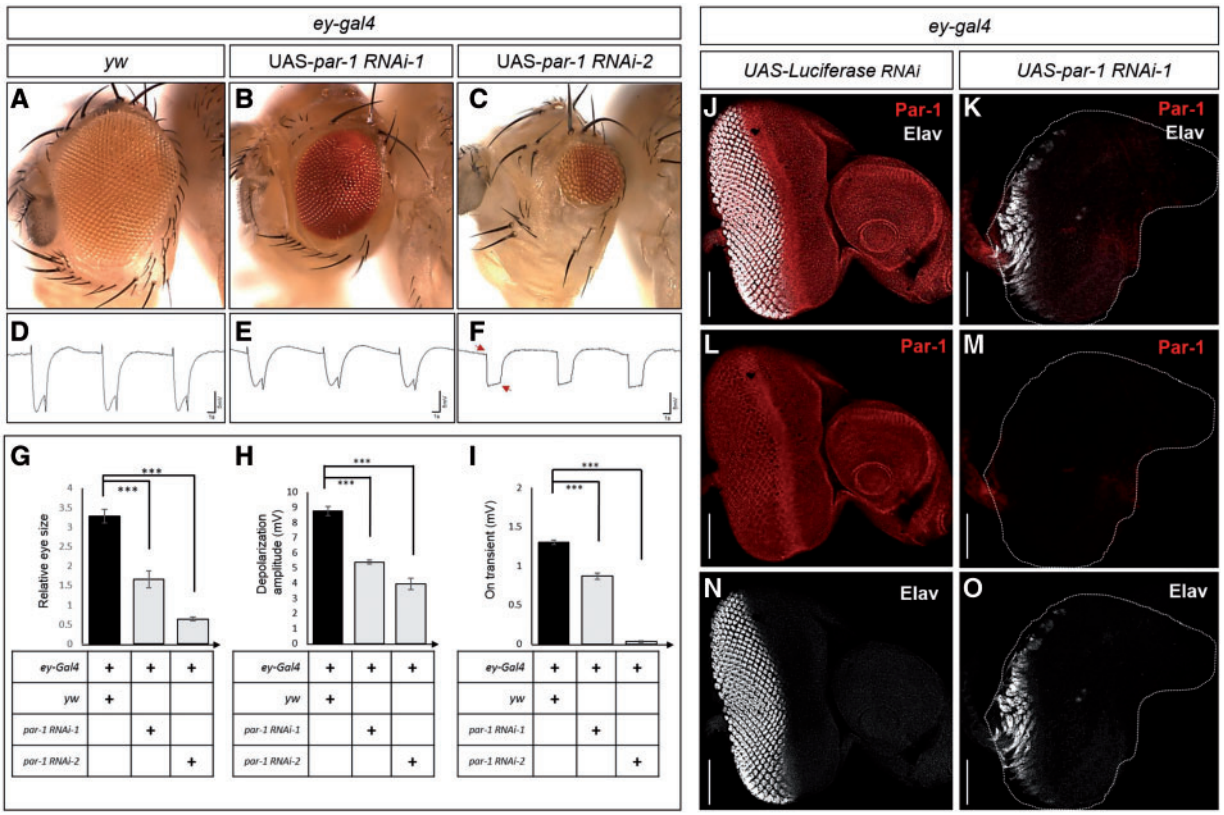

Figure 3. Drosophila Par-1 is required for proper eye development and photoreceptor differentiation. (**A–C**) Adult heads with eyes in which various transgenes were expressed: ey-Gal4 in a *y w* background (A), *ey-Gal4* driving *UAS-par-1 RNAi-1* (B), or *UAS-par-1 RNAi-2* (C). Expression of either *par-1 RNAi-1* (BL#35342) or *par-1 RNAi-2* (BL#32410) by *ey-Gal4* causes small eyes (B and C). (**D–F**) ERGs of flies shown above. Arrows indicate the loss of on- and off-transients of ERGs. Quantification of eye sizes (**G**) (n=10; error bars represent ±SEM, ***P<0.001, t-test) (**H** and **I**). Quantification of the ERG amplitude (H) and on-transients (I) of ERG traces in (D–F) (n=13; error bars represent ±SEM, ***P<0.001, t-test). (**J–O**) Expression of *par-1 RNAi-1* driven by *ey-Gal4* significantly reduces the levels of Par-1 in eye discs. (J and K) Eye imaginal disc is stained for Par-1 (red) and the pan-neural marker Elav (white). Par-1 is ubiquitously expressed in the developing eye disc (L). The expression of Par-1 is severely reduced in eye disc of *ey > UAS-par-1 RNAi-1* (M). (N and O) Knockdown of Par-1 results in a dramatic reduction in photoreceptor numbers. Elav staining in wild-type control discs (N). Knockdown of Par-1 severely reduces the number the photoreceptors in developing eye disc. Scale bars, 50 μm (J–O).

Discussion

We propose here that the biallelic non-synonymous p.Arg570Gly variant in the *MARK3* gene likely causes phthisis bulbi and VI in the affected individuals of the Pakistani consanguineous family F105. MARK3 is involved in the phosphorylation of various microtubule-associated proteins MAP2 (OMIM *157130), MAP4 (OMIM *157132) and tau/MAPT (OMIM *157140) (22). Loss of MAPT protein results in defects in photoreceptor development in Drosophila (23), and a small eye phenotype was observed in transgenic rats bearing a human MAPT gene (24). Also, MARK3 interacts with Mitf (25), which is known to cause the COMMAD syndrome with severe microphthalmia (26). MARK3 together with DLG5 function as negative regulators of the Hippo pathway: the MARK3/DLG5 complex inhibits the kinase activity of MST1/2 and LATS1 by inhibiting the MST1/2 mediated phosphorylation of LATS1 (22). The *MARK3* knockout mouse presented with resistance to weight gain under high fat diet and exhibited defects in gluconeogenesis (27). However, these mice were not studied for phenotypes related to vision. This colony of mice does no longer exists and was not available for further studies.

We studied the *par-1* (ortholog of *MARK3*) gene in Drosophila. We found that the down-regulation of *par-1* by RNAi specifically in the eye leads to a very severe loss of photoreceptors in the developing fly eye and causes a near or complete loss of visual function based on ERG (Fig. 3). Moreover, expression of Par-1 p.Arg792Gly, the equivalent of human variant p. Arg570Gly, results in a small, non-functional eye which closely resembles the *ey > par-1 RNAi* phenotype. These data strongly suggest that the MARK3 p.Arg570Gly variant is pathogenic.

MARK3/par-1 is a Ser/Thr kinase initially identified as an essential polarity gene for a proper asymmetric division in *Caenorhabditis elegans* embryos (28). Prior studies in invertebrates and vertebrates have shown that the MARK3/par-1 homologs are necessary for cell polarity and that loss-of-function mutations are associated with a range of developmental defects (29–32). In Drosophila, Par-1 is necessary for anterior–posterior axis formation, and *par-1* loss-of-function embryos exhibit severe patterning defects (33). Moreover, loss of Par-1 in various imaginal discs leads to a mild reduction in tissue size, induces some apoptotic cell death during development and affects Hippo signaling (34).

In *Xenopus*, early dorsoanterior development of the embryo is disrupted by the loss of *par-1*. This causes a loss of Wnt signaling (32). Interestingly, in the same study, knocking down of *par-1* expression resulted in a lack of eye development as well as a decrease in body axis length. Wnt signaling is involved in eye development and retinal angiogenesis (35–37). Other genes involved in Wnt signaling have been implicated in eye diseases in humans. For instance, individuals with pathogenic mutations in *FZD4*, another core component of the Wnt pathway, disrupts

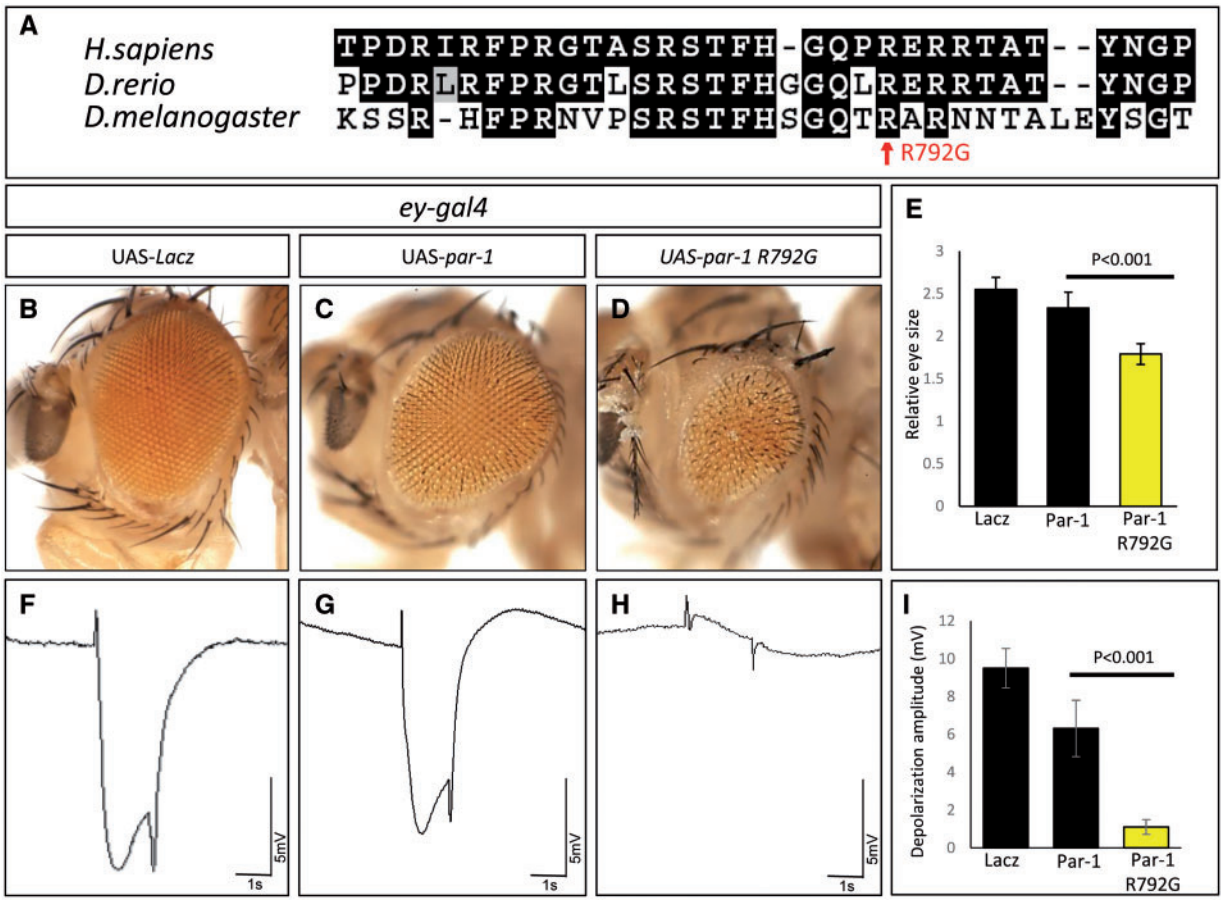

Figure 4. Par-1 p.Arg792Gly, the Drosophila mutation homologous to the human MARK3 p.Arg570Gly variant, affects eye development and function. (**A**) Multiple-sequence alignment of the protein domain of MARK3 that contains the p.Arg570Gly shows that this domain is evolutionarily conserved from flies to human. (**B–D**) Adult fly heads expressing *UAS-lacZ* (B), *UAS-par-1 WT* (C), or *UAS-par-1 p.Arg792Gly* (D), when driven by ey-Gal4. Expression of Par-1 WT causes a mild defect in eye morphology (C), whereas expression of the p.Arg792Gly protein causes severely reduced eyes that are rough (D). Quantification of the eye sizes (**E**) (n=10; error bars represent ±SEM, P<0.001, t-test). (**F–H**) ERGs of flies carrying *ey-Gal4* with *UAS-lacZ* (F), *UAS-par-1 WT* (G), or *UAS-par-1 p.Arg792Gly* (H). Quantification of the ERGs shows that expression of the Par-1 p.Arg792Gly mutation causes a near complete loss of ERG amplitude (**I**) (n=10; error bars represent ±SEM, P<0.001, t-test).

retinal angiogenesis (38,39). Furthermore, individuals with mutations in the FZD4 binding partner, *NDP*, are affected by Norrie disease (40). These mutations cause blindness as well as phenotypes that are very similar to those observed in the patients with the MARK3 p.Arg570Gly variant. Hence, *MARK3/par-1* is evolutionarily critical for early eye development and may affect Wnt signaling.

The study of the incidental finding in the *PAH* gene emphasizes the notion that the pathogenicity of variants for recessive disorders should be evaluated in the context of the other allele. In the case of the family F105 presented here, the PAH p.Pro119Ser variant in homozygosity cause a mild increase of the phenylalanine level but not in the PKU range. On the other hand, the same PAH p.Pro119Ser variant in combination with a 'null' splicing error causes mild PKU that requires treatment (18). The further development of databases of recessive variants would be essential for the interpretation of their phenotypic relationship and the genetic counseling regarding the at-risk carrier status.

We did not find other cases of mutations in *MARK3* by sharing our findings in Genematcher (41). Additional families with *MARK3* pathogenic variants and similar eye phenotype will be necessary to establish causality. Patients with Norrie-like phenotype but with an autosomal mode of inheritance should be evaluated for *MARK3* pathogenic variants if possible. We conclude that the *MARK3* homozygous missense variant is an excellent candidate to cause the progressive phthisis of the eyes and VI at birth observed in our patients.

Materials and Methods

Patients and study consent

The family was enrolled at the Molecular Biology & Genetics Department, Medical Research Center, Liaquat University of Medical & Health Sciences, Jamshoro, Pakistan, and was studied at the Department of Genetic Medicine and Development, University of Geneva, Geneva, Switzerland. This study was approved by the Bioethics Committee of the University Hospitals of Geneva (Protocol number: CER 11–036), and by the ethical committee of Liaquat University of Medical & Health Sciences, Jamshoro, Pakistan. Informed consent was obtained from the members of this family.

Clinical investigations

The detailed clinical investigations were performed on all affected individuals to characterize the variability of the phenotype.

MRI of orbit was performed on patients V: 2 and V: 3 of Figure 1, and T1, T2 weighted images were obtained without contrast in axial, coronal and sagittal planes. Bilateral B and A scans were also performed on patients V: 2 and V: 3. The Patient V: 5 was subjected to fundoscopy, tonometry and visual acuity assessments.

Laboratory and computational analysis

Exome sequencing was performed using the SureSelect Human All Exon kit v5 (Agilent Technologies, Santa Clara, CA, USA) on an Illumina HiSeq4000. The data analysis was done by using a customized pipeline, based on Burrows–Wheeler aligner tool (BWA) (42), SAMtools (42), PICARD (http://broadinstitute.github.io/picard/) and GATK (43). The alignment was performed by using the reference human genome (GRCh37/hg19) (44). Genotyping of samples from all available family members was done using the 720 K SNP array (HumanOmniExpress Bead Chip by Illumina Inc®, San Diego, CA, USA). PLINK (45) was used to perform homozygosity mapping by taking into account a window of 50 consecutive homozygous SNPs, allowing a maximum of one mismatch in a homozygous region. Runs of homozygosity (ROH) were defined as homozygous regions separated by the first contiguous heterozygous SNPs. The family was analyzed by using CATCH (14), an in-house program which combines the information of family pedigree, ROH, and data of exome sequence. This program identifies variants in the ROHs of patients but not in normal individuals of a family. Subsequently, the variants were filtered as described in previous studies (11,12). All the candidate variants were further verified by Sanger sequencing.

Cloning and transgenesis

Site-directed mutagenesis was performed for full-length cDNA of *par-1* in the pENTR223 Gateway donor vector (RE47050 from Drosophila Genomics Resource Center). To generate a *par-1* p.Arg792Gly (R792G) [equivalent to human MARK3 p.Arg570Gly (R570G)], we performed site-directed mutagenesis using the following primers; FW: 5′-CTTTCACTCTGGTCAAACCGGAGCACGAAACAACACAGCG-3′ RV: 5′-CGCTGTGTTGTTTCGTGCTCCGGTTTGACCAGAGTGAAAG-3′. The fully sequenced pENTR223 *par-1* WT and pENTR223 *par-1* R792G clones were subcloned into pGW-attB-HA (46) vector to generate pGW-attB-HA-*par-1* WT and pGWattB-HA-*par-1* R792G. These constructs were injected into *y, w,ΦC31; VK33* embryos (47) and transgenic flies were selected by the w^+ marker on pGW-attB-HA vector.

ERG recording of fly eye

ERG recordings were performed as described by Verstreken *et al.* (2003) (48). In brief, flies were glued to a glass slide. A recording electrode filled with 3 M NaCl was placed on the eye, and another reference electrode on the fly head. During the recording, a 1 s pulse of light stimulation was given, and the ERG traces of five flies for each genotype were recorded and analyzed using WinWCP V5.3.3 software.

Drosophila genetics

The following stocks were obtained from the Bloomington Drosophila Stock Center in Indiana University (BDSC). *w[*]; P{w[+mC]=UAS-lacZ.B}Bg4–2-4b* (RRID: BDSC_1777), *ey-gal4* (on II), y^1 *sc** v^1*; P{TRiP.HMS00405}attP2* (RRID: BDSC_35342), y^1 *sc** v^1*; P{TRiP.GL00253}attP2* (RRID: BDSC_32410). All flies were maintained at 25°C. For measuring eye size, we used ImageJ.

Immunohistochemistry

Eye discs were dissected from third instar larvae, fixed in periodate-lysine-paraformaldehyde (PLP) fixative for 15 min at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies were as follows: α-Elav (1: 200, 7E8A10, DSHB, Iowa City, IA) (49), α-Par-1 (1: 200) (50). Secondary antibodies conjugated with Cy3, Cy5 or Alexa 488 were from Jackson ImmunoResearch and were incubated for imaging in Vectashield (Vector Laboratories) with 4′, 6-diamidino-2-phenylindole. Fluorescent images were acquired using a Zeiss LSM880 confocal microscope.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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