

RESEARCH ARTICLE

The splicing factor Prp31 is essential for photoreceptor development in *Drosophila*

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ABSTRACT

Retinitis pigmentosa is a leading cause of blindness and a progressive retinal disorder, affecting millions of people worldwide. This disease is characterized by photoreceptor degeneration, eventually leading to complete blindness. Autosomal dominant (adRP) has been associated with mutations in at least four ubiquitously expressed genes encoding pre-mRNA splicing factors—Prp3, Prp8, Prp31 and PAP1. Biological function of adRP-associated splicing factor genes and molecular mechanisms by which mutations in these genes cause cell-type specific photoreceptor degeneration in humans remain to be elucidated. To investigate the *in vivo* function of these adRP-associated splicing factor genes, we examined *Drosophila* in which expression of fly Prp31 homolog was down-regulated. Sequence analyses show that CG6876 is the likely candidate of *Drosophila melanogaster* Prp31 homolog (DmPrp31). Predicted peptide sequence for CG6876 shows 57% similarity to the Homo sapiens Prp31 protein (HsPrp31). Reduction of the endogenous Prp31 by RNAi-mediated knockdown specifically in the eye leads to reduction of eye size or complete absence of eyes with remarkable features of photoreceptor degeneration and recapitulates the bimodal expressivity of human Prp31 mutations in adRP patients. Such transgenic DmPrp31RNAi flies provide a useful tool for identifying genetic modifiers or interacting genes for Prp31. Expression of the human Prp31 in these animals leads to a partial rescue of the eye phenotype. Our results indicate that the *Drosophila* CG6876 is the fly ortholog of mammalian Prp31 gene.

KEYWORDS *Drosophila*, photoreceptor degeneration, retinitis pigmentosa, Prp31

INTRODUCTION

Retinitis pigmentosa (RP) is the most common form of retinal degeneration with an incidence of 1 in 4000. The disease is characterized by progressive degeneration of photoreceptor cells leading to night blindness, abnormal electroretinogram and a loss of peripheral vision with the appearance of bone spicule-like pigment deposits on retina. This is followed by gliosis and atrophy of the retina (Daiger et al., 2007) and eventually complete blindness. Familial RP can occur in autosomal dominant (adRP), autosomal recessive (arRP) and X-linked (xlRP) fashions. Genetic studies have identified a number of RP-associated loci including a number of retina-specific genes and four ubiquitously expressed genes encoding proteins important for pre-mRNA splicing: Prp31, Prp8, Prp3 and PAP-1.

Pre-mRNA splicing is the most upstream step in eukaryotic gene expression that removes intervening sequences (introns) from messenger RNA (mRNA) precursors to produce functionally mature mRNAs. The biochemical reactions of pre-mRNA splicing occur in a complex macromolecular machinery named the spliceosome which consists of five snRNPs (U1, U2, U4, U5, U6 snRNPs) and a host of accessory proteins (Wahl et al., 2009). During spliceosome assembly, the U1 and U2 snRNPs first associate with the pre-mRNA, followed by joining of U4/U6-U5 tri-snRNP and conversion of pre-spliceosome to catalytically active spliceosome in which splicing reactions take place. The tri-snRNP is formed by the association of U4/U6 di-snRNP and the U5snRNP. Prp31 is a U4/U6 di-snRNP specific protein necessary for the formation of the catalytically active spliceosome.

Mutations in human Prp31 gene has been identified in patients affected by autosomal dominant retinitis pigmentosa (RP11) including missense and deletion mutations. The AD5

mutation is caused by an 11bp deletion (Vithana et al., 2001), leading to formation of a truncated protein product of 371 amino acids. Using a minigene-cotransfection method in HEK cells, our previous studies show that the AD5 mutation significantly inhibits splicing of a subset of retina-specific transcripts (Yuan et al., 2005; Mordes et al., 2007).

To investigate the *in vivo* function of Prp31 gene, especially its role in the retina, we used *Drosophila*, a powerful model organism for studying mammalian gene function and human pathogenesis. Genomic sequence analyses show that *Drosophila* genes encoding components of the splicing machinery are highly similar to those of human, however, only a limited number of studies have provided direct experimental evidence. Using human Prp31 protein sequence, we identified a transcript CG6876 (Flybase, www.flybase.org) as a possible *Drosophila* homolog of Prp31 protein. The predicted peptide sequence of CG6876 shows 57% similarity in overall protein sequence with the human Prp31 (Fig. 1A). In this study, we report generation and characterization of transgenic flies expressing RNAi against *Drosophila* Prp31. Reducing the endogenous Prp31 levels in the fly eye leads to abnormal eye formation and severe defects characteristic of photoreceptor degeneration at the ultrastructural level. Expression of the human Prp31 protein in such flies partially rescued this eye defect. These results demonstrate that human and *Drosophila* Prp31 genes are functionally equivalent, and that proper expression of *Drosophila* Prp31 gene is essential for the development of photoreceptor cells.

RESULTS

Generation of transgenic flies expressing Prp31 RNAi and human Prp31

We obtained two independent RNAi lines of CG6876 from National Institute of Genetics, Japan. Several lines of transgenic flies were created that express the human wild type or hAD5 mutant PRP31 protein fused at the carboxyl terminus with green fluorescent protein (GFP). The corresponding constructs were prepared by ligating the respective cDNA fragments to the GFP coding sequence in the pGMR vector (Fig. 1B). Transgenic animals expressing hPRP31 protein or the control GFP were created by injecting the construct into *yw* fly embryos. Transgenic flies with red eyes, indicative of the insertion of the transgene, were selected for crossing with appropriate balancer lines to make stock lines. The pGMR promoter allows eye-specific expression of the corresponding transgenes. The expression of the transgenes was detected by GFP expression using fluorescent microscopy, RT-PCR and Western blotting. We obtained at least two different lines with insertions on separate chromosomes for each transgene to exclude non-specific phenotypes as a result of positional effects of transgene insertion.

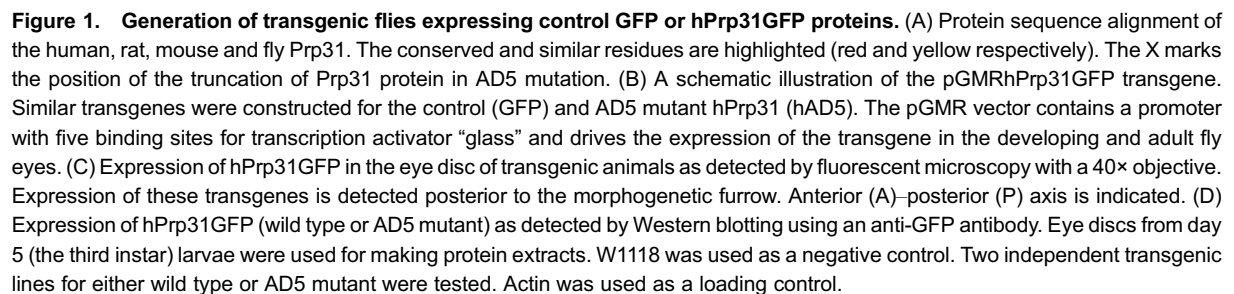
We analyzed transgenic flies expressing human Prp31 in

fly eye discs (Fig. 1C and 1D). Several transgenic lines expressing human Prp31 tagged with GFP showed robust nuclear GFP signals as detected in the cells posterior to the morphogenetic furrow, which is consistent with the predicted expression pattern for genes under the control of the GMR promoter. The expression of the corresponding transgenes was also detected at the protein level by Western blotting with a monoclonal antibody against GFP antibody. Both the wild type and hAD5 Prp31 proteins were detected with their molecular weight as predicted (Fig. 1D). Expression of either wild type or hAD5 mutant Prp31 protein by themselves in the photoreceptors did not cause any obvious detrimental effect in the fly eyes (unpublished results).

Reducing *Drosophila* Prp31 expression causes eye defects

One explanation for the observation that expressing the mutant human Prp31 did not lead to visible effects in flies is the high level of expression of the endogenous *Drosophila* Prp31 gene in the eye (hereafter referred to as DmPrp31). Therefore, we examined flies in which the endogenous Prp31 gene expression was reduced using RNA interference approach. Flies carrying inducible RNAi specifically targeting CG6876 (UAS-DmPrp31RNAi) were crossed to driver flies expressing eyGal4 and the hPrp31 transgenes (wild type, mutant or GFP control). RNA was extracted from heads of these fly lines and subjected to RT-PCR using DmPrp31 specific primers (Fig. 2). The level of DmPrp31 mRNA is significantly reduced in the flies expressing the DmPrp31RNAi as compared to control flies (Fig. 2A, compare lane 2 to 3, lane 4 to 5, and lane 6 to 7).

Reducing DmPrp31 expression led to morphological defects in the eye. The fly eyes were smaller in size and abnormal in shape or completely absent (Fig. 3A), with significant phenotypic variations. A small fraction of flies (5%–10%) had morphologically normal eyes, whereas the majority of flies showed various degrees of eye defects. Flies expressing the UAS-DmPrp31RNAi under eyGal4 driver showed eye defects in about 90% of the progeny (Fig. 3A). Microscopic images of flies overexpressing the control GFP or wild type or mutant forms of human Prp31 in the RNAi background revealed their eye morphology. One interesting phenotype was the presence of one normal on one side and one abnormal eye on the opposite side of the same animal (Fig. 3A, panel f) in some of the flies when the RNAi was expressed. This was observed for all fly lines tested, including those overexpressing the only the RNAi transgene as well as the human Prp31 or the control transgenes. These fly lines showed a wide range of eye abnormalities from complete absence of eye, to formation of deformed or small eyes. This recapitulates the bimodal expressivity observed among RP11 patients carrying Prp31 mutations. In these patients, there is no obvious correlation between their Prp31 genotype with



RNAi controls not related to Prp31 examined did not show any eye defects (unpublished data), Prp31RNAi flies showed significant eye defects. The flies expressing the GFP control in the presence of DmPRP31RNAi had the highest number of animals with eye defects followed by flies expressing mutant hAD5. A significantly smaller number of flies expressing the human Prp31 in the DmPrp31-RNAi background showed eye

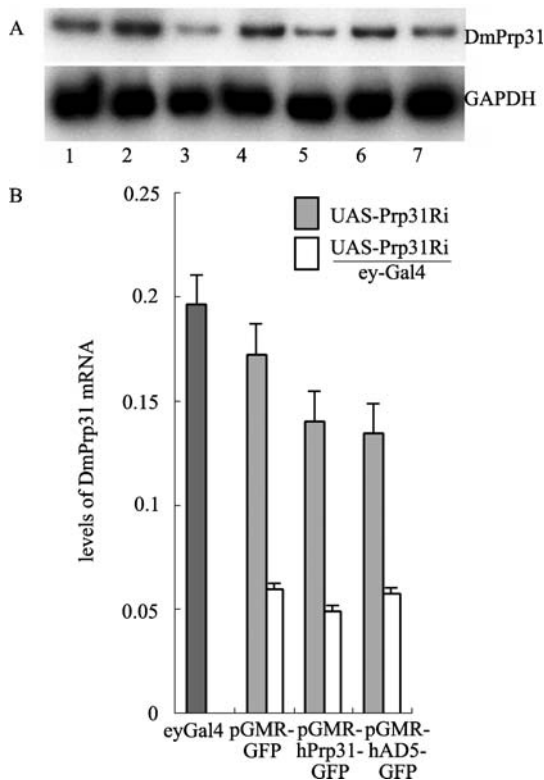


Figure 2. Reduction of DmPrp31 gene expression levels by in RNAi lines expressing Gal4 under the control of *eyeless* (*ey*) promoter. (A) The levels of DmPrp31 in fly heads as detected by RT-PCR. Transgenic flies expressing RNAi against DmPrp31 (CG6876) and the pGMR driven hPrp31 or hAD5 or GFP were crossed to eyGal4 flies. Heads from 5-day old transgenic flies were used for RNA extraction and RT-PCR. DmPrp31RNAi expressing flies show significant reduction in the levels of DmPrp31 transcript as compared to the parental line, eyGal4. Lanes 1–6 contain respectively: 1, eyGal4 parental flies; 2, UAS-DmPrp31RNAi/pGMRGFP in the absence of Gal4; 3, eyGal4/UAS-DmPrp31RNAi/pGMRGFP; 4, UAS-DmPrp31RNAi/pGMR-hPrp31GFP; 5, eyGal4/UAS-DmPrp31RNAi/pGMR-hPrp31GFP; 6, UAS-DmPrp31RNAi/pGMR-hPrp31AD5GFP; 7, eyGal4/UAS-DmPrp31RNAi/pGMRhPrp31AD5GFP. (B) Quantification of the RT-PCR results from three independent experiments showing a significant reduction in DmPrp31 levels in the presence of RNAi. Dark grey bars represent the parental line carrying the RNAi transgene against DmPrp31 and the human transgenes in the absence of the Gal4 driver whereas white bars represent the lines containing the Gal4 drivers, the RNAi construct and the hPrp31 transgenes.

defects as compared to flies expressing either vector control or hAD5 mutant Prp31 (Fig. 3B).

To examine the phenotype of the flies expressing DmPrp31RNAi at the subcellular level, TEM analysis was performed on 20-day old flies grown in a 12-h light/dark cycle (Fig. 4). Abnormal eye morphology was detected in flies

expressing RNAi against DmPrp31. Such DmPrp31RNAi flies expressing the GFP control under pGMR driver showed extensive signs of neurodegeneration in the intra-ommatidial space around the rhabdomeres of R1–R6 and R7, with significant reduction in the sizes of the rhabdomeres. Additional ultrastructural findings include loss of pigment cells and the presence of multi-vesicular bodies (MVBs) and autophagic vacuoles, well-defined pathological features of neurodegeneration (Fig. 4D). In DmPrp31RNAi flies that overexpressed the human wild type or hAD5 mutant Prp31, the rhabdomeres appeared normal in comparison to the group overexpressing only GFP (Fig. 4, compare panels B and C to panel A), with no MVBs or vacuoles detected in the retina of these flies. Our results show that down-regulating DmPrp31 in the *Drosophila* eye leads to degeneration of photoreceptors, and that expressing hPrp31 (either wild type or hAD5 mutant) alleviates such photoreceptor degeneration phenotypes. This suggests that DmPrp31 is the *Prp31* homolog in *Drosophila melanogaster*. We propose that CG6876 can be re-named as *Drosophila* Prp31 gene.

In order to ensure that the partial rescue seen in flies expressing hAD5 is not due to the reduction of the Prp31 transgene expression by DmPrp31RNAi, we analyzed the levels of hPrp31 (and hAD5) by RT-PCR (Fig. 5). The results show that the level of expression of the Prp31 transgenes were not affected by expression of DmPrp31RNAi, thus confirming that the RNAi effect was specific.

DISCUSSION

Prp31 was originally discovered as a gene essential for *S. cerevisiae*. Although human Prp31 is a ubiquitously expressed gene, its mutations affect only the photoreceptor neurons. Identification of Prp31 mutations in patients affected by retinitis pigmentosa highlights the importance role of human Prp31 in photoreceptors. The physiological function of mammalian Prp31 gene and molecular mechanisms underlying the photoreceptor-specific defect seen in RP11 patients remain to be elucidated.

So far no animal model has been reported for RP11. Several published studies are based on results obtained from patient lymphoblast cultures or cell lines, which may not fully reflect the neurobiology in living animals. There have been no published studies on biological function of Prp31 in animals or photoreceptor cells *in vivo*. Our results show that down-regulating the endogenous Prp31 in the eye led to photoreceptor cell death in *Drosophila*, demonstrating the requirement of Prp31 gene in development and maintenance of photoreceptors *in vivo*.

Discovery of adRP associated mutations in ubiquitously expressed genes such as those encoding pre-mRNA splicing factors poses a complex problem. Different hypotheses have been put forward to explain cell type-specific effect of such adRP gene mutations. Our previous studies show that that

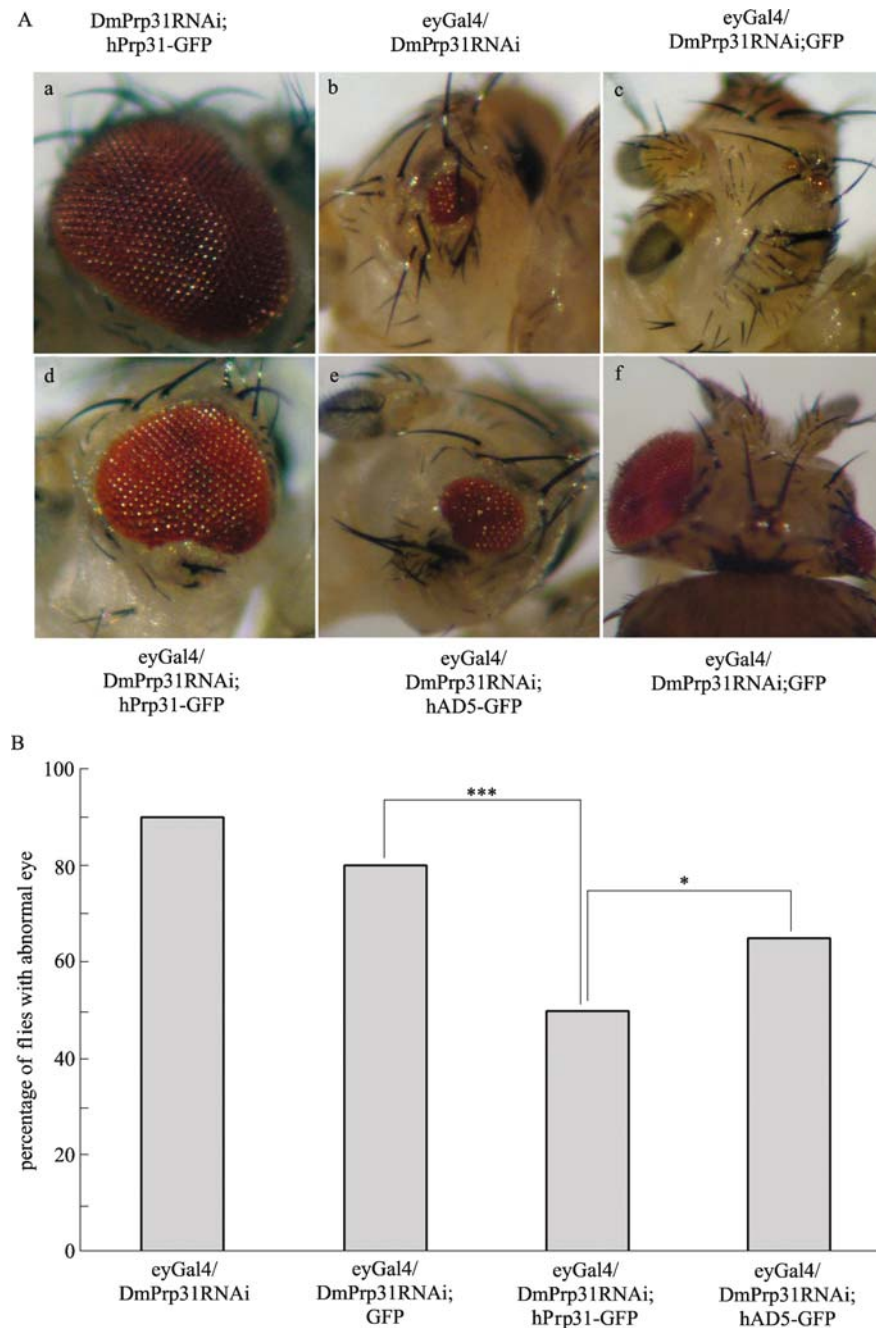


Figure 3. Knockdown of *Drosophila* Prp31 using eyGal4 driver leads to eye defects. (A) Panels a–f show the various levels of eye phenotypes in flies expressing the DmPrp31RNAi in the presence of the eyGal4 driver. a, the parental line without the eyGal4 driver; b, DmPrp31RNAi with eyGal4 driver; c and f, DmPrp31RNAi in the presence of pGMR-GFP; d and e, DmPrp31RNAi in the presence of wild type hPrp31 or hAD5 mutant Prp31 transgenes. (B) Quantification of the eye defects in different fly lines. A total of 500 flies from each group were analyzed.

overexpression of hAD5 mutant form of human Prp31 gene in cultured cells leads to neuronal death and reduced splicing efficiency of a subset of retinal genes (Yuan et al., 2005; Mordes et al., 2007). In our current study, simply expressing hAD5 Prp31 mutant in *Drosophila* eye did not lead to detectable effect on photoreceptor cells. This observation

may be interpreted in a number of ways. First, hAD Prp31 expression in fly photoreceptors from the transgene may not be at a sufficiently high level to exert its effect in the fly photoreceptors in the presence of endogenous DmPrp31 gene expression. Second, *Drosophila* photoreceptor cells may have physiological and metabolic requirements that are

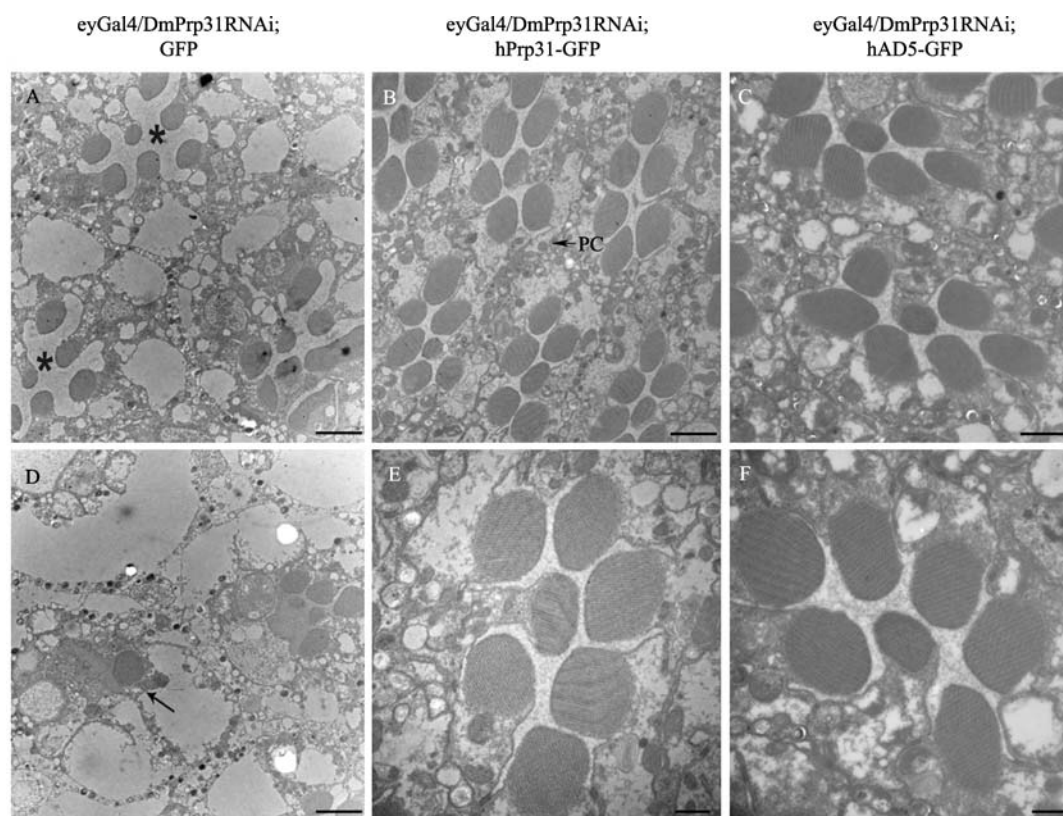


Figure 4. The expression of hPrp31 rescues eye defects induced by down-regulating DmPrp31 expression. Ommatidia of 20-day old flies were examined by TEM. Expression of DmPrp31-specific RNAi photoreceptor defects including an increase in the space between the rhabdomeres (panels A and C, marked by asterisk in A), the lack of pigment cells (PC) and the appearance of autophagic vacuoles (AV) (the arrow in panel D). Lower panel images are four times magnification of the respective upper panels. The different fly strains are indicated on the top panels. The scale bars in panels A, B and C is 2 μ m and that in panels D, E and F represents 500 nm. The expression of wild type or AD5 mutant hPrp31 leads to partial rescue of the eye phenotype (panels B, C, E and F).

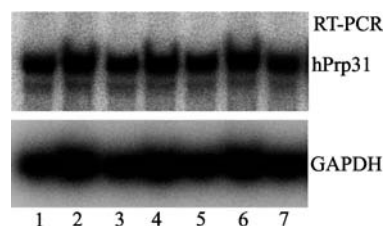


Figure 5. DmPrp31 RNAi is specific and does not affect levels of hPrp31 expression. Levels of hPrp31 were analyzed by RT-PCR in the different lines of flies. GAPDH expression levels were used as loading control. The different lanes are as follows 1: eyGal4 parental line, 2: UAS-DmPrp31RNAi/pGMRGFP in the absence of Gal4, 3: eyGal4/UAS-DmPrp31RNAi/pGMRGFP, 4: UAS-DmPrp31RNAi/pGMR-hPrp31GFP, 5: eyGal4/UAS-DmPrp31RNAi/pGMR-hPrp31GFP, 6: UAS-DmPrp31RNAi/pGMR-hAD5GFP, 7: eyGal4/UAS-DmPrp31RNAi/pGMRhAD5GFP.

different from those in human photoreceptor cells, making the photoreceptors in flies and in human show different phenotypes when hAD5 mutant Prp31 is expressed. Third, it remains possible that hAD5 mutant protein interacts with different set of interaction partners in photoreceptors in flies and in human. These issues require further studies to be clarified in the future.

While our study was in progress, an independent study was published showing that the Prp31 homolog is essential in mouse in that down-regulating Prp31 using an actin driver led to embryonic lethality (Bujakowska et al., 2009). It has not escaped our attention that DmPrp31-RNAi flies show incomplete penetrance in their eye phenotypes. Different individual flies with the same genotype show different levels of eye phenotypes, including eye sizes and shapes or the presence of tumor-like growth in the eyes. Sometimes even two eyes of the same fly showed different levels of defects. Approximately 5%–15% of these flies have normal eyes, whereas the majority of the flies showed some form of eye

defect. This phenomenon recapitulates incomplete penetrance or bimodal expressivity observed in RP11 patients, that is, significant variations in clinical manifestations of subjects with the same Prp31 genotype and from the same family (Evans et al., 1995). The molecular mechanisms underlying this incomplete penetrance of Prp31 mutations in patients remain to be investigated. Possible explanations include differential expression of the wild type allele among different carriers or asymptomatic patients (Vithana et al., 2003) or the presence of a modifier gene (Rio Frio et al., 2008).

The *Drosophila* eye has served as a powerful system for screening for genes important for development (Pepple et al., 2007; Wolff et al., 2007). The observations that DmPrp31RNAi flies show dramatic eye defects and that the expression of human Prp31 partially rescues the eye defects indicate that DmPrp31 is required for the formation or maintenance of photoreceptors and that human and fly Prp31 genes are functionally equivalent *in vivo*.

These results together with the previously published bioinformatics data (Mount and Salz, 2000) clearly indicate that CG6876 is the *Drosophila* homolog of human Prp31. The observation of partial phenotypic rescue by the expression of wild type hPrp31 suggests that hPrp31 expression by the pGMR driver is not sufficiently high. Because pGMR is a constitutive driver and not an inducible system we were unable to further increase hPrp31 expression. Contrary to our prediction, expression of the hAD5 mutant also led to partial rescue, albeit to a lesser extent, of the eye defects. Ultrastructural analyses showed that the eye abnormalities were accompanied by striking morphological features of photoreceptor degeneration, including reduced size of rhabdomeres, severe reduction in the intra-ommatidial space, the appearance of MVB and autophagic vacuoles as well as the loss of pigment cells. It is likely such severe photoreceptor degeneration leads to deficits in retinal function.

The fact that down-regulating DmPrp31 gene expression leading to photoreceptor degeneration supports a haplo-insufficiency model that has been suggested from studies using non-photoreceptor cell lines derived from RP11 patients (Deery et al., 2002; Wilkie et al., 2006; Rio Frio et al., 2008; Wilkie et al., 2008). This is different from the gain-of-function toxicity model suggested by our previous studies using the mammalian cell culture system. It should be pointed out that it remains possible that photoreceptors from different species may have different properties and sensitivities to the presence of mutant Prp31 gene products. Differences in processing of pre-mRNA transcripts have been observed in three separate studies on the rodopsin transcript (Deery et al., 2002; Yuan et al., 2005; Wilkie et al., 2008). The first study reported no effects on the splicing of bovine rodopsin transcripts in the presence of the Prp31 mutant protein in an *in vivo* splicing assay whereas the latter studies demonstrated dominant effects of Prp31 mutations in affecting splicing efficiency of human rodopsin transcript. These observations suggest that pre-mRNA transcripts from

different species may be differentially sensitive to mutations in splicing factors.

The incomplete penetrance and bimodal expressivity associated with RP11 mutations suggest that modifier genes may influence the disease severity. *Drosophila* models of various neurodegeneration diseases have been successfully used to identify enhancer and suppressor genes (Shulman and Feany, 2003; Lessing and Bonini, 2008). To our knowledge, our DmPrp31RNAi transgenic flies represent the first animal model for photoreceptor degeneration associated with human Prp31 mutation. Our *Drosophila* model reported in this study recapitulates the essential features of the RP11 including photoreceptor degeneration with bimodal expressivity and incomplete penetrance. Such model will be useful in future identification of modifier genes for adRP associated with Prp31 mutations.

MATERIALS AND METHODS

Generation of transgenic flies carrying GFP tagged wild type and mutant human Prp31 constructs

The mammalian expression plasmids carrying either wild-type or mutant PRPF31 were constructed by inserting the corresponding cDNA fragments into the pGMR vector, a kind gift from Dr. Ross Cagan. PCR primers used for cloning are as follows: forward primer, 5'-GGATCCATGTCTCTGGCAGATGAGC-3'; reverse primer, 5'-ACCACAACCTTCCTGGCTGGAT-3'. For cloning mutant fragment following reverse primer was used 5'-GGAGATCTGGCCTGCTTCCG-3'. The human Prp31 open reading frame or fragments containing N-terminal 371 residues (AD5 mutant) fused with GFP were inserted at the *Bgl*III site downstream of the promoter in the pGMR vector that carries the w⁺(white marker). The corresponding cDNA inserts as well as the junction sequences were confirmed by using PRISM Ready reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Oligonucleotides were purchased from IDT Integrated DNA Technologies (Coralville, IA). These constructs were injected into 5–30min old W1118 fly embryos together with the $\Delta 2-3$ helper plasmid. Progeny was screened by the red eye color, and appropriate stocks made after crossing into balancer lines. Primers used for single fly PCR are as follows: forward primer, 5'-GAGTATATCAGCAAGCAAGCC-3' and reverse primer, 5'-GGTTCTCATTGTTCTTGCACTTGTC-3'.

Fly stocks and crosses

Flies were maintained on standard cornmeal agar medium at 25°C under 12-h light/dark cycle with 50% humidity. GMR-Gal4 and eyGal4 stocks were kind gifts of Dr. Richard Carthew (Northwestern University). Balancer stocks were obtained from Bloomington Stock Center, Bloomington, Indiana. UAS-DmPrp31RNAi lines were obtained from National Institute of Genetics, Japan.

RNA Extraction and RT-PCR

Age-appropriate fly heads were isolated (~30 mg) for RNA extraction using Trizol reagent (Invitrogen, Catalog No. 155-96-011) following a

slightly modified version of the Invitrogen manual. Briefly, frozen fly heads were homogenized in Trizol and centrifuged to remove debris. Supernatant was transferred to a fresh tube and incubated with chloroform. RNA was precipitated using isopropanol and washed twice with 70% ethanol. RNA concentration was quantified using spectrophotometer and equal amounts of RNA (100 ng) used for cDNA synthesis with oligodT primer. After 2 rounds of RT-PCR amplification cDNA was used for PCR using Prp31 specific primers in the presence of [α - ^{32} P]-dCTP. The levels of GAPDH (as a control for the RNA levels) or corresponding genes were detected using gene-specific primers. The levels of PCR products were quantified using a Phosphorimager (Fuji).

Electron microscopy

Dissected heads from wild type and mutants were prepared for TEM using standard protocol. Briefly, heads were fixed in 2% glutaraldehyde at 4°C for at least 48 h. Samples were then washed in 2–10 min. changes of phosphate buffered sucrose (PBS), transferred to 1% OsO₄ in phosphate buffer for 2 h. Fly heads were rinsed with distilled water, dehydrated through a series of graded alcohol (50%–100%), then treated propylene oxide for 30 min., immersed in propylene oxide/spur-mixture (1:1) for 30 min, and then embedded in pure resin overnight in 60°C to for resin to polymerize. Ultra-thin sections (50–60 nm) of fly heads were cut on a Leica Ultracut UCT 54 Ultramicrotome and collected on Synaptek 2mm×1 mm gold slot notch grids (EMS). Grids were stained on a drop of uranyl acetate for 25 min with a drop of lead citrate added and incubated for additional 5 min. The sections were examined on a Phillips CM12 TEM, equipped with a 2-mega pixel CCD camera. Digital electron microscopy (EM) images were taken of cross sections of the entire head.

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ABBREVIATIONS

adRP, autosomal dominant RP; arRP, autosomal recessive RP; AV, autophagic vacuole; DmPrp31, *Drosophila melanogaster* Prp31; GFP, green fluorescent protein; HsPrp31, Homo sapiens Prp31; MVB, multi-vesicular body; PC, pigment cell; RP, retinitis pigmentosa; xIRP, X-linked RP

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