Mutations in the splicing regulator Prp31 lead to retinal degeneration in Drosophila

Sarita Hebbar*, Malte Lehmann*‡, Sarah Behrens§, Catrin Hälsig, Weihua Leng, Michaela Yuan, Sylke Winkler and Elisabeth Knust¶

ABSTRACT

Retinitis pigmentosa (RP) is a clinically heterogeneous disease affecting 1.6 million people worldwide. The second-largest group of genes causing autosomal dominant RP in human encodes regulators of the splicing machinery. Yet, how defects in splicing factor genes are linked to the aetiology of the disease remains largely elusive. To explore possible mechanisms underlying retinal degeneration caused by mutations in regulators of the splicing machinery, we induced mutations in Drosophila Prp31, the orthologue of human PRPF31, mutations in which are associated with RP11. Flies heterozygous mutant for Prp31 are viable and develop normal eyes and retina. However, photoreceptors degenerate under light stress, thus resembling the human disease phenotype. Degeneration is associated with increased accumulation of the visual pigment rhodopsin 1 and increased mRNA levels of twinfilin, a gene associated with rhodopsin trafficking. Reducing rhodopsin levels by raising animals in a carotenoid-free medium not only attenuates rhodopsin accumulation, but also retinal degeneration. Given a similar importance of proper rhodopsin trafficking for photoreceptor homeostasis in human, results obtained in flies presented here will also contribute to further unravel molecular mechanisms underlying the human disease.

This paper has an associated First Person interview with the co-first authors of the article.

KEY WORDS: Spliceosome, Photoreceptor cells, Rhodopsin, scarlet, twinfilin

INTRODUCTION

Retinitis pigmentosa (RP; OMIM 268000) is a clinically heterogeneous group of retinal dystrophies, which affects more than one million people worldwide. It often starts with night blindness in early childhood, continues with the loss of the peripheral visual field (tunnel vision), and progresses to complete blindness in later life due to gradual degeneration of photoreceptor cells (PRCs). RP is a genetically heterogeneous disease and can be inherited as autosomal dominant (adRP), autosomal recessive (arRP) or X-linked (xlRP) disease. So far >90 genes have been identified that are causally related to non-syndromic RP (Ali et al., 2017; Verbakel et al., 2018). Affected genes are functionally diverse. Some of them are expressed specifically in PRCs and encode, among others, transcription factors (e.g. CRX, an orthologue of human CRX like photoreceptor homeobox gene), components of the light-induced signalling cascade, including the visual pigment rhodopsin (Rho/RHÒ in Drosophila/human), or genes controlling vitamin A metabolism (e.g. RLBP1, encoding Retinaldehyde-binding protein). Other genes are associated with a more general control of cellular homeostasis, for example genes involved in trafficking or cell polarity (e.g. CRB1) [reviewed in (Daiger et al., 2014, 2013; Hollingsworth and Gross, 2012; Nemet et al., 2015)]. Interestingly, the second-largest group of genes causing adRP, comprising 7 of 25 genes known, encodes regulators of the splicing machinery. So far, mutations in five pre-mRNA processing factor (PRPF) genes, PRPF3, PRPF4, PRPF6, PRPF8 and PRPF31, have been linked to adRP, namely RP18, RP70, RP60, RP13 and RP11, respectively. Pim1-associated protein (PAP1) and small nuclear ribonucleoprotein-200 (SNRP200), two genes also involved in splicing, have been suggested to be associated with RP9 and RP33, respectively (Maita et al., 2004; Zhao et al., 2009) [reviewed in (Liu and Zack, 2013; Mordes et al., 2006; Poulos et al., 2011; Ruzickova and Stanek, 2016)]. The five PRPF genes encode components regulating the assembly of the U4/U6/U5 tri-snRNP, a major module of the pre-mRNA spliceosome machinery (Nguyen et al., 2015; Patel and Bellini, 2008; Will and Luhrmann, 2011). Several hypotheses have been put forward to explain why mutations in ubiquitously expressed components of the general splicing machinery show a dominant phenotype only in the retina. One hypothesis suggests that PRCs with only half the copy number of a gene encoding a general splicing component cannot cope with the elevated demand of RNA-/protein synthesis required to maintain the exceptionally high metabolic rate of PRCs in comparison to other tissues. Hence, halving their gene dose eventually results in apoptosis. Although this model is currently favoured, other mechanisms, such as impaired splicing of PRC-specific mRNAs or toxic effects caused by accumulation of mutant proteins have been discussed and may contribute to the disease phenotype [discussed in (Mordes et al., 2006; Scotti and Swanson, 2016; Tanackovic et al., 2011)]. More recent data obtained from retinal organoids established from RP11 patients showed that removing one copy of PRPF31 affects the splicing machinery specifically in retinal and retinal pigment epithelial (RPE) cells, but not in patient-derived fibroblasts or iPSC cells (Buskin et al., 2018).

The observation that all adRP-associated genes involved in splicing are highly conserved from yeast to human allows to use model organisms to unravel the genetic and cell biological functions of these genes in order to obtain mechanistic insight into the origin of the diseases. In the case of RP11, the disease caused by mutations...
in PRPF31, three mouse models have been generated by knock-in and knock-out approaches. Unexpectedly, mice heterozygous for a null allele or a point mutation that recapitulates a mutation in the corresponding human gene did not show any sign of retinal degeneration in 12- and 18-month-old mice, respectively (Bujakowska et al., 2009). Further analyses revealed that the retinal pigment epithelium, rather than the PRCs, is the primary tissue affected in Prp31 heterozygous mice (Farkas et al., 2014; Graziotto et al., 2011; Hamieh and Nandrot, 2019). Other data show that homozygous PRPF31 mice are not viable (Dickinson et al., 2016). Morpholino-induced knockdown of zebrafish Prp31 results in strong defects in PRC morphogenesis and survival (Linder et al., 2011). Defects induced by retina-specific expression of zebrafish Prp31 constructs that encode proteins with the same mutations as those mapped in RP11 patients (called AD5 and SP117) were explained to occur by either haplo-insufficiency or by a dominant-negative effect of the mutant protein (Yin et al., 2011). In Drosophila, no mutations in the orthologue Prp31 have been identified so far. RNAi-mediated knockdown of Prp31 in the Drosophila eye results in abnormal eye development, ranging from smaller eyes to complete absence of the eye, including loss of PRCs and pigment cells (Ray et al., 2010).

In order to get better insights into the mechanisms by which Prp31 prevents retinal degeneration we aimed to establish a meaningful Drosophila model for RP11-associated retinal degeneration. Therefore, we isolated two mutant alleles of Prp31, Prp31P17 and Prp31P18, which carry missense mutations affecting conserved amino acids. Flies heterozygous for either of these mutations are viable and develop normally. Strikingly, when exposed to constant light, these mutant flies undergo retinal degeneration, thus mimicking the disease of RP11 patients. Degeneration of mutant PRCs is associated with accumulation and abnormal distribution of the visual pigment rhodopsin, Rh1, in PRCs. Reduction of dietary vitamin A, a precursor of the chromophore 11-cis-3-hydroxretinal, which binds to opsin to functionalize the rhodopsin, mitigates both aspects of the mutant phenotype, rhodopsin accumulation and retinal degeneration. From this we conclude that Rh1 accumulation and/or misdistribution reflect a degeneration-prone condition in the Prp31 mutant retina.

RESULTS
Two Prp31 alleles were discovered by TILLING
It was recently shown that RNAi-mediated knockdown of Drosophila Prp31 in the eye using eye-specific Gal4-lines [eyeless (ey)-Gal4 or GMR-Gal4] results in abnormal eye development, ranging from smaller eyes to complete absence of the eye, including loss of photoreceptor cells (PRCs) and pigment cells (Ray et al., 2010). Both Gal4-lines are expressed throughout eye development. Therefore, some of the defects observed could be the result of impaired early development of the eye, such as defective cell fate specification, which would only indirectly affect PRC development. Here, we aimed to establish a more meaningful Drosophila model for RP11-associated retinal degeneration, a human disease associated with mutations in the human orthologue PRPF31, which would allow a deeper insight into the role of this splicing factor in the origin and progression of the disease.

Therefore, we set out to isolate specific mutations in Drosophila Prp31 by targeting induced local lesions in genomes (TILLING), following a protocol described recently (Spann et al., 2017). In total, 2,400 genomes of ethyl methanesulfonate (EMS)-mutagenised flies were screened for sequence variants in two different amplicons of Prp31. Four sequence variants were identified, which were predicted to result in potentially deleterious missense mutations. Two of the four lines, named Prp31P17 and Prp31P18 were recovered from the living fly library and crossed for three generations to control, white-eyed (w*) flies to reduce the number of accompanying sequence variations. We outcrossed the mutants with white-eyes flies (w*) rather than with wild-type, red-eyed flies to generate a sensitised background for light-dependent degeneration experiments, since the presence of the pigment granules surrounding each ommatidium contributes towards lower sensitivity to light (Stark and Carlson, 1984). Prp31P16 flies were viable as homozygotes and in trans over any of three deficiencies, which remove, among others, the Prp31 locus (Fig. 1A). In contrast, no homozygous Prp31P17 flies were obtained. However, Prp31P17 was viable in trans over Prp31P18 and over Df(3L)ED217. This suggests that the lethality was due to a second site mutation, which was not removed during outcrossing. We noticed that outcrossing Prp31P17 and Prp31P18 did not remove scarlet (st), one of the markers of the original, mutagenised chromosome (ru st e ca) mapping close to Prp31. Therefore, the correct genotypes of the two mutant lines are w*, Prp31P17, st1 and w%; Prp31P18, st'. For simplicity, we will refer to them as Prp31P17 and Prp31P18 throughout the text.

The molecular lesions in the two Prp31 alleles were mapped in the protein coding region. Drosophila PRP31 is a protein of 501 amino acids, which contains a NOSIC domain (named after the central domain of Nop56/SIK1-like protein), a Nucleolar protein (Nop) domain required for RNA binding, a PRP31_C-specific domain and a domain of Nop56/SIK1-like protein, a Nucleolar protein (Nop) domain of Nop56/SIK1-like protein, a Nucleolar protein (Nop). The molecular lesions in the two Prp31 alleles are less severe and more variable than that observed in RP11 patients (called AD5 and SP117) (Chartier et al., 2012; Johnson et al., 2002; Spann et al., 2017). In the two crb alleles crb1422 and crb1349 only 5 to 11% of all ommatidia displayed seven rhabdomeres upon exposure to constant light, respectively (Fig. 2E).

Flies hetero- or hemizygous for Prp31 undergo light-dependent retinal degeneration
Hom- and heterozygous Prp31P18 and heterozygous Prp31P17 animals raised and kept under regular light–dark cycles (12 h light; 12 h dark) have eyes of normal size. Histological sections revealed normal numbers of PRCs per ommatidium (distinguished by the number of rhodromeres) and a normal stereotypic arrangement of PRCs (Fig. 1C–F and Fig. S2A). This indicates that the development of the retina was not affected by these mutations. However, PRCs of Prp31P17/e, Prp31P18/e and Prp31P17/Prp31P18 flies showed clear signs of retinal degeneration when exposed to constant light for several days, manifested by a partial or complete loss of rhodomeric integrity (Fig. 2C, D and Fig. S2B). Quantification of the number of surviving rhodromeres in Prp31 mutant retinas revealed only about 48% of ommatidia with the full complement of seven PRCs (Fig. 2E), while w* mutant control flies exhibited 82% of all ommatidia displaying the full complement of rhodromeres (Fig. 2A, E). The degree of degeneration observed in Prp31 alleles is less severe and more variable than that observed in the well-established RP12 disease model induced by mutations in the gene crumbs (crb) (Chartier et al., 2012; Johnson et al., 2002; Spann et al., 2017). In the two crb alleles crb1422 and crb1349 only 5 to 11% of all ommatidia displayed seven rhodromeres upon exposure to constant light, respectively (Fig. 2E).

To further confirm that the degeneration phenotype observed in Prp31P18 and Prp31P17 heterozygous flies is due to mutations in
Prp31, we used additional strategies to reduce/inactivate Prp31 function. First, we knocked down Prp31 by overexpressing Prp31 RNAi, mediated by Rh1-Gal4, which drives expression late in retinal development, from 70% pupal development into adulthood (Kumar and Ready, 1995). Thereby, we can rule out any early effects on PRC specification or morphogenesis induced by loss of Prp31. To make
the data comparable to those obtained with Prp31 alleles (which are in a \textit{w} background), we reduced the red-coloured screening pigments encoded by the \textit{w}+-gene on the transgenes by expression of another transgene, GMR-\textit{wIR}, which expresses \textit{white} RNAi under the control of the GMR-promoter (Lee and Carthew, 2003). RNAi-mediated knockdown of Prp31 in PRCs (and concomitant ubiquitous
knockdown of \( w \) resulted in clear signs of degeneration upon light exposure, such as loss of rhabdomeres and accumulation of intensely stained structures reminiscent to apoptotic bodies (Fig. 3B). In fact, while 71% of control ommatidia revealed 7 identifiable rhabdomeres and no major morphological defects (Fig. 3A,C), the number of ommatidia with a full complement of rhabdomeres decreased to 48% upon induction of \( Prp31 \) RNAi (Fig. 3C).

As a second alternative strategy to confirm the role of \( Prp31 \) in retinal degeneration, we analysed the phenotype of three deficiency lines that remove the \( Prp31 \) locus (see Fig. 1A). For a proper comparison with the data obtained for the \( Prp31 \) alleles (which are in a \( w \) background), we removed the red pigments of the deficiency lines (caused by the presence of a \( w^+ \)-minigene) by studying their phenotype in a \( cn bw \) background, an alternative way to remove all screening pigments. \( Df(3L)Exel6262/+, \ Df(3L)ED217/+, \) and \( Df(3L)ED218/+ \) flies exhibited retinal degeneration similar as \( Prp31^{P17} \) or \( Prp31^{P98} \) heterozygous flies (Fig. 4), with only about 20% of their ommatidia showing seven rhabdomeres. These
deficiency lines also had no obvious effects on retinal development (Fig. S2D–F). Degeneration was also observed in Prp31<sup>P13</sup>/Df (3L)217 and Prp31<sup>P17</sup>/Df (3L)217 flies (Fig. S2G,H).

We noticed that retinal degeneration in <sup>w<sup>*</sup>; <sup>st</sup>1/+ flies was enhanced compared to that of <sup>w<sup>*</sup></sup> flies (Fig. 2A,B and E). To rule out that retinal degeneration observed in Prp31 mutant flies is...
influenced by the presence of a mutation in st mapping close by (Fig. 1A), we overexpressed st in the retina of Prp31P18 flies (simultaneously knocking-down w gene activity provided by the transgenes). Expression of st did not modify the degree of retinal degeneration of Prp31 mutants (Fig. 5, Table S2).

Taken together, these data support the conclusion that reducing the function of the Prp31 locus causes light-induced retinal degeneration.

**Prp31 mutant photoreceptor cells show increased rhodopsin accumulation**

A common cause of retinal degeneration, both in flies and in mammals, is abnormal localisation/levels of the visual pigment rhodopsin (Rh1) (Hollingsworth and Gross, 2012; Xiong and Benell, 2013). Therefore, we asked if the degeneration observed in Prp31 mutant retinas is associated with altered Rh1 localisation/levels. *Drosophila* Rh1, encoded by *ninaE*, is the most abundant rhodopsin expressed in the outer PRCs R1-R6 (Harris et al., 1976; Ostroy et al., 1974). In control flies raised under regular light conditions (12 h light, 12 h dark), Rh1 is concentrated in the rhabdomeres. As reported previously, Rh1 either fills the entire rhabdomere, forms a crescent-shaped pattern, or is restricted to the base or the lateral edges of the rhabdomere (Chen et al., 2017; Chinchore et al., 2009; Mitra et al., 2011; Orem et al., 2006; Wang et al., 2014; Xiong et al., 2012). Differences in localisation have been attributed to inconsistencies in antibody penetration due to either the dense packing of microvilli in the rhabdomeres (Xiong et al., 2012) or to a light-induced staining artefact (Schopf et al., 2019). Rh1 staining is observed in rhabdomeres (red arrowheads in Fig. 6A–E) and outlines the rhabdomeric structure along its length (Fig. 6A–C). Rh1 could also be detected in cytoplasmic punctae (blue arrowheads in Fig. 6A–E). This intracellular pool of Rh1 presumably represents internalised Rh1 following light exposure (Satoh and Ready, 2005), since these flies were raised under 12 h light/12 h darkness. Strikingly, PRCs of adult flies heterozygous for Prp31 exhibited increased accumulation of Rh1 in the rhabdomeres in comparison to genetic controls (Fig. 6C,C′). Increased Rh1 immunostaining was observed in mutants independent of light conditions applied (Fig. S3). Prp31 homozygotes exhibited a similar phenotype of enhanced Rh1 immunostaining intensity (Fig. 6E as compared to D). Further, all three deficiencies that remove the Prp31 locus exhibited increased Rh1 staining when heterozygous (Fig. S4B–D) in comparison to the genetic controls (Fig. S4A). Finally, RNAi-mediated knockdown of Prp31 also resulted in increased accumulation of Rh1 in rhabdomeres (Fig. S4F) as compared to genetic control (Fig. S4E). Increased intensity of Rh1 immunostaining is due to increased levels of Rh1 as revealed by western blots of protein extracts isolated from adult heads of Prp31 hetero- and homozygotes. On average, Rh1 levels were significantly increased by over 300% in heads from Prp31 P18 heterozygous and by 140% in Prp31P18 homozygous flies as compared to heads of genetic controls (Fig. 6F,G). The variability in the magnitude of increased Rh1 levels (see biological replicates in Fig. 6G) parallels the variability in the degenerative phenotype in the Prp31 mutants.

**Impaired Prp31 function does not affect splicing or abundance of opsิน mRNA, but results in increased twinfilin mRNA**

To better understand the underlying cause of increased Rh1 in rhabdomeres, we aimed to find out whether *ninaE/opsin1* mRNA levels were altered in these mutants. Using Real time qRT-PCR and primers targeting each of the exons (Table 1A) and the exon-intron junctions (Table 1B), no significant change in *opsin1* mRNA levels was detected in heads of heterozygous and homozygous Prp31P18 flies. This implies that abundance and splicing of *opsin1* mRNA is unaffected in these mutants. We next investigated whether trafficking of ops1n/rodopsin along its biosynthetic route is altered. Carotenoids are precursors of the chromophore 11-cis-3-hydroxylretinal, which binds to ops1n to generate the functional visual pigment rhodopsin in flies (von Lintig et al., 2010). Reduction of the chromophore halts endoplasmic reticulum (ER) to Golgi transport and maturation of rhodopsin, resulting in the accumulation of an intermediate form in the perinuclear ER (Colley et al., 1991; Ozaki et al., 1993). Upon supplementation of retinal and induction of its isomerization by blue light, mature Rh1 is now trafficked to the rhabdomere (Satoh et al., 1997). An assay, called blue-light induced chromophore supply (BLICS) (Iwanami et al., 2016; Ozaki et al., 1993), allows to follow Rh1 trafficking along its biosynthetic route. Using the BLICS assay, no qualitative difference was observed in Rh1 reaching the rhabdomere in control and Prp31 P18 heterozygote flies (Fig. S5). There was no substantial increase in Rh1 reaching the rhabdomere upon its release from the ER. This suggests that the amount of Rh1 produced and trafficked to the rhabdomere (at least via Rab11) was not substantially altered in the Prp31 mutant.

Finally, we evaluated mRNA levels of three genes (Table 2) that have been recently implicated in Rh1 trafficking (Laffajian and Tepass, 2019). Of these, mRNA levels of only twinfilin (*twf*), which encodes an actin monomer-binding protein, is increased in Prp31 mutants (heterozygous and homozygous alleles) as compared to those of the respective genetic background. Taken together, impaired Prp31 function is associated with increased rhodopsin protein levels and increased twinfilin mRNA, but does not affect the amount or splicing of *opsin1* mRNA.

**Light-dependent photoreceptor degeneration in Prp31 mutants is suppressed upon elimination of Rh1 accumulation**

There is evidence suggesting that increased levels of rhodopsin and/or mis-localised rhodopsin contributes to retinal degeneration, both in mammals and in flies (Hollingsworth and Gross, 2012). In *crb* mutant retinas, for example, the degree of light-dependent retinal degeneration could be strongly reduced in animals raised on carotenoid-depleted food (Johnson et al., 2002). To determine whether rhodopsin accumulation makes the retina of Prp31 mutant flies prone to light-induced degeneration, we experimentally reduced rhodopsin levels by raising animals in carotenoid-free diet from embryonic stages onward. In fact, in retinas of flies raised under this condition, Rh1 levels are reduced and rhabdomeric localisation of Rh1 is abolished. Instead, Rh1 can now be found perinuclear, both in control and in Prp31 +/+ retinas (Fig. 7B–C). In contrast, in the retina of control flies raised on normal food, Rh1 is detected on the lateral edges of the rhabdomeres (Fig. 7A,A′, white arrowheads).

After 7 days of constant light exposure, the overall appearance of the retinae of both the genetic control (w*) and Prp31 +/+ mutants appeared more damaged as revealed by fewer surviving rhabdomeres and more lacunae (compare the retina of w* in Figs 2 and 7). Note that rhabdomeres are smaller under this dietary condition, a result which is consistent with previous reports (Johnson et al., 2002; Sapp et al., 1991; Satoh et al., 1998). More importantly, the percentage of ommatidia with seven rhabdomeres was the same in heterozygous Prp31 P18/+ and control (w*) retinas under carotenoid depletion (Fig. 7D,E and G). A similar result was obtained in *crb* mutant retinas.
prepared from flies raised under the same conditions (Fig. 7F,G) (Johnson et al., 2002), supporting the conclusion that carotenoid depletion prevents retinal degeneration.

To conclude, these results suggest that Rh1 accumulation in Prp31 mutant flies makes the retina more susceptible to light-induced degeneration.
DISCUSSION

Here we present a fly model for RP11, an autosomal-dominant human disease caused by mutations in the splicing regulator PRPF31, which leads to blindness in affected patients. Our results reveal that mutations in the Drosophila orthologue Prp31 induce RPR degeneration under light stress, thus mimicking features of RP11-associated symptoms. Similar to those in human, mutations in Drosophila Prp31 are haplo-insufficient and lead to retinal
degeneration when heterozygous. This is in stark contrast to mice heterozygous for Prpf31, which did not show any signs of PRC degeneration (Bujakowska et al., 2009), but rather late-onset defects in the retinal pigment epithelium (Farkas et al., 2014; Graziotto et al., 2011).

By using three different genetic approaches we provide convincing evidence that the knockdown of Prp31 is the cause of the retinal degeneration observed. (1) The two Prp31 alleles induced by TILLING (Prp31P17 and Prp31P18) carry missense mutations in conserved amino acids of the coding region, which are predicted to be damaging. (2) Flies heterozygous for any of three deletions, which completely remove the Prp31 locus, exhibit comparable phenotypes as flies heterozygous for Prp31 point mutations. (3) RNAi-mediated knockdown of Prp31 results in light-induced retinal degeneration. The results obtained suggest that the two missense mutations mapped in Prp31P17 and Prp31P18 are strong hypomorphic alleles. First, the two Drosophila alleles characterised here are hemizygous (Prp31/deficiency) and homozygous (in the case of Prp31P18) viable and fertile. Second, mutations in the two established Prp31 fly lines are missense mutations, one located N-terminal to the NOSIC domain in Prp31P17 (G90R) and the other in the Nop domain in Prp31P18 (P277L) (see Fig. 1A), which most likely result in a reduced function of the respective proteins (Fig. S1B). Whether protein levels are also decreased cannot be answered due to the lack of specific antibodies. The mutated amino acid residue in Drosophila Prp31P18 (P277L) is predicted to be damaging. Whether the P277L mutation could similarly weaken, but not abolish, the function of the respective proteins (Fig. S1B). Whether protein levels are also decreased cannot be answered due to the lack of specific antibodies. The mutated amino acid residue in Drosophila Prp31P18 (P277L) is predicted to be damaging. We noticed that the retinal phenotype observed upon reduction of Prp31 is more variable than that observed upon loss of crb (see, for example, Fig. 2E) (Johnson et al., 2002; Spannl et al., 2017). This could be due to the fact that all Prp31 conditions analysed represent hypomorphic conditions, possibly retaining some residual protein function(s). However, the expressivity of the mutant phenotype is not increased in Prp31/deficiency flies (carrying only one mutant copy) in comparison to that of Prp31/+ flies, which carry one mutant and one wild-type allele. Interestingly, human RP11 patients heterozygous for mutations in Prp31 show an unusually high degree of phenotypic non-penetrance and can even be asymptomatic. Various causes have been uncovered to explain this feature (Wheway et al., 2020). These include a highly variable expression level of the remaining wild-type Prp31 allele, possibly due to changes in the expression levels of trans-acting regulators (Rio Frío et al., 2008) (reviewed in Rose and Bhattacharya, 2016). In addition, mutant PRP31 proteins can form cytoplasmic aggregates in RPE cells, thus reducing the amount of protein entering the nucleus (Valdes-Sanchez et al., 2019), or can impair overall transcription or splicing, as described in Prp31 zebrafish models (Linder et al., 2011; Yin et al., 2011). Finally, mutations in unlinked genes have been suggested to modify the disease severity of patients (Venturini et al., 2012).

Not only in flies, but also in human, mutations in PRPF31 affect only the retina, despite the importance of this splicing regulator in all cells. Recently published data show that impaired PRPF31 function can affect the splicing of target genes in a cell-type specific manner. Strikingly, retinal cells isolated from RP11 patient-derived retinal organoids exhibit mis-splicing of genes that encode components of the splicing machinery itself. This was not observed in fibroblasts or iPSC cells derived from the same patients (Buskin et al., 2018). These authors obtained similar results from

### Table 1. Summary of Real time qRT-PCR data

<table>
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<th>A</th>
<th>Fold-change (mean±s.e.m.) for amplicon in opsin 1 mRNA corresponding to exon #</th>
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<td>1</td>
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<tr>
<td>w*;;Prp31P18/+ versus w*;;st1/+</td>
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<tr>
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<table>
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<th>B</th>
<th>Fold-change (mean±s.e.m.) for amplicon in opsin 1 mRNA corresponding to exon-exon junction</th>
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<td>2-3</td>
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<tr>
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<td>1.15±0.2 (2)</td>
</tr>
<tr>
<td>w*;;Prp31P18/+ Prp31P18 versus w*;;st1/+ st1</td>
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Number within parenthesis indicates the number of biological replicates. Each biological replicate consists of 10-15 pooled heads. *indicates significant increase as determined by t-test at significance level P<0.05.

### Table 2. Summary of Real time qRT-PCR data

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<th>CdGAPPr</th>
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<td>w*;;Prp31P18/+ versus w*;;st1/+</td>
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<td>2.42±0.16* (4)</td>
<td>1.25±0.09 (3)</td>
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<td>w*;;Prp31P18/+ Prp31P18 versus w*;;st1/+ st1</td>
<td>0.85±0.1 (4)</td>
<td>2.85±0.09* (4)</td>
<td>0.97±0.05 (3)</td>
</tr>
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</table>

Number within parenthesis indicates the number of biological replicates. Each biological replicate consists of 10-15 pooled heads. *indicates significant increase as determined by t-test at significance level P<0.05.
Fig. 7. See next page for legend.
expression of a mutant Prpf31 gene reduced rhodopsin expression, as a result of impaired splicing of the rhodopsin pre-mRNA (Yuan et al., 2005). Similarly, siRNA-mediated knockdown of PRPF31 function in human organotypic retinal flat-mount cultures (HORFC) reduced mRNAs encoding genes involved in phototransduction and photoreceptor structure, including rhodopsin (Azizzadeh Pormehr et al., 2018). Interestingly, the Prpf31 mutants described here show increased mRNA levels of an evolutionary conserved actin monomer binding protein called twinfilin (twf), which inhibits actin polymerisation. Knockdown of twf results in excessive cytoplasmic Rh1 staining, suggesting defects in its trafficking (Laflaifian and Tepass, 2019). In Prpf31 mutants, an increase in rhodomeric Rh1 was observed as well as increased twf mRNA. From this correlation we hypothesise that upregulation of twf mRNA in Prpf31 might be in part responsible for at least the rhodomeric Rh1 accumulation. Rh1 also accumulates in the cytoplasm of Prpf31 mutant PRCs. Our data exclude the role of Rab11-mediated targeting of Rh1 in this accumulation. Now, it remains to be determined if the deregulation of other trafficking routes or the upregulation of twf contributes to the increased Rh1 in the cytoplasm. In the future, it may be interesting to explore the link between increased Rh1 levels as observed in Drosophila Prpf31 mutants, increased mRNA levels of twinfilin and impaired Rh1 trafficking. Additionally, a detailed transcriptome analysis should elucidate possible defects in transcription and/or splicing of target genes, thus also allowing a better understanding of the aetiology of the human disease.

MATERIALS AND METHODS

Fly strains and genotypes

All phenotypic analyses were performed in age-matched males unless otherwise specified. Genotypes are summarised in Table S1. Flies were maintained at 25°C on standard yeast-cornmeal-agar food unless otherwise stated. To rule out differences in light sensitivity in the light-degeneration paradigm, we used white-eyed flies, bearing mutations in the white gene, both as general controls and in the respective mutant background. The white allele (w*) used here was tested by PCR and shown to carry a deletion that includes the transcription and translation start site of the white gene (data not provided). Loss of scarlet (st) function was rescued by Gal4-mediated expression of a scarlet transgene (Cunningham et al., 2018) in all cells of the retina using GMR-Gal4 (Hay et al., 1994). The RNAi line (ID: 35131) for the Prpf31 gene was obtained from the Vienna Drosophila Resource Centre (VDCR, www.vdrc.at) (Dietzl et al., 2007). RNAi was induced using scarlet-Gal4 (Lee and Carthew, 2003) in combination with Dicer-2 expression and concomitant expression of white RNAi under the control of the GMR-promoter (GMR-w*) (Lee and Carthew, 2003) allowing assay of degeneration in a non-pigmented background. Df(3L)E6C262 with deleted segment 71B3;71C1 (Parks et al., 2004), Df(3L)ED217 with deleted segment 70F4;71E1 and Df(3L)ED218 with deleted segment 71B1–71E1 (Ryder et al., 2007) were obtained from the Bloomington Stock Centre. Since the deficiency lines carry a mini-white transgene due the way they were generated (Ryder et al., 2007), cn bw was recombined into these lines to obtain white-eyed flies and all phenotypes were compared with cn bw.

Isolation of Prpf31 alleles by TILLING

To isolate point mutations in the Prpf31 locus (FlyBase ID: FBgn0036487) a library of 2,400 fly lines with isogenised third chromosomes, which potentially carry point mutations caused by EMS treatment, was screened (Winkler et al., 2005). Our approach targeted exon 1–3 of the Prpf31 locus containing two thirds (67%) of the coding sequence, which includes several predicted functional domains (the NOSIC (IPRO012976), the Nop (IPRO002687) and PRPF31 functional domains (Parks et al., 2004), Df(3L)ED217 with deleted segment 71B3;71C1 (Parks et al., 2004), Df(3L)ED217 with deleted segment 71B1–71E1 (Ryder et al., 2007) were obtained from the Bloomington Stock Centre. Since the deficiency lines carry a mini-white transgene due the way they were generated (Ryder et al., 2007), cn bw was recombined into these lines to obtain white-eyed flies and all phenotypes were compared with cn bw.

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PCR-reactions were performed in 10 µl volume and with an annealing temperature of 57°C, in 384-well format, making use of automated liquid handling tools. PCR fragments were sequenced by Sanger sequencing. Oligonucleotides designed for amplicon re-sequencing in a large-scale format (Winkler et al., 2011) and checked for the phenotypes associated with homozygous null mutations. Conditions for technical advice on western blotting procedures.

**Western blotting**

Five fly heads from each genotype were homogenised in 10 µl of 1× SDS-PAGE sample buffer (200 nM Tris-HCl pH 6.8, 20% Glycerol, 8% SDS, 0.04% Bromophenol blue, 400 mM DTT). After dilution with RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8), lysates were heated at 37°C for 30 min. Lysates equivalent to 2.5 heads were loaded and run on a 15% acrylamide gel, and proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA) for 1 h incubation at room temperature. The fluorescent signal from the dry membrane was measured using LI-COR Odyssey Sa Infrared Imaging System 9260-11P (LI-COR Biotechnology). The intensity of the bands was analysed using the Image Studio Ver 4.0 software. The reported value in Fig. 6 was obtained following normalisation of the intensity values for Rh1 with the corresponding Tubulin intensity values and the number of heads loaded onto the gel.

**Real Time qRT-PCR analyses**

RNA extraction, cDNA generation, qPCR analyses were performed as described in Hebbar et al. (2020) (doi: https://doi.org/10.1083/jcb.201911100). Primers are listed in Table S3.

**Figure panel preparation**

All figure panels were assembled using Adobe Photoshop CS5.1 or Adobe Illustrator CS3 (Adobe Systems, USA). Statistical analyses and graphs were generated using GraphPad Prism (GraphPad Software, Inc, USA) and Microsoft Excel. For protein sequence visualisation, Illustrator of Biological Sequences (IBS; Liu et al., 2015) software package was used.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

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