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A role for the cytoplasmic DEAD box helicase Dbp21E2 in rhodopsin maturation and photoreceptor viability

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Abstract

The *Dbp21E2* (*Dead box protein 21E2*) is a member of a family of DEAD box helicases active in RNA processing and stability. We used genetic mosaics to identify mutants in *Dbp21E2* that affect rhodopsin biogenesis and the maintenance of photoreceptor structure. Analysis of a GFP-tagged Rh1 rhodopsin construct placed under control of a heat shock promoter showed that *Dbp21E2*¹ fails to efficiently transport Rh1 from the photoreceptor cell body to the rhabdomere. Retinal degeneration is not dependent on the Rh1 transport defects. We also show that GFP- and RFP-tagged Dbp21E2 proteins are localized to discrete cytoplasmic structures that are not associated with organelles known to be active in rhodopsin transport. The molecular genetic analysis described here reveals an unexpected role for the Dbp21E2 helicase and provides an experimental system to further characterize its function.

Introduction

Rhodopsins are required for the structure and function of the photoreceptor cells in both humans and *Drosophila* (Chapple et al., 2001; Colley et al., 1991; Dryja et al., 1990; Kumar & Ready, 1995; Kurada & O'Tousa, 1995; O'Tousa et al., 1989; Scavarda et al., 1983; Sung et al., 1991a; Sung et al., 1991b). The Rh1 rhodopsin in *Drosophila*, encoded by the *ninaE* gene, is necessary for the activation of the phototransduction cascade, but also is required for maintenance of the rhabdomere and integrity of the photoreceptor (Kumar & Ready, 1995; Leonard et al., 1992; O'Tousa et al., 1989).

Rh1 maturation requires numerous steps before assuming its final form and localization within the rhabdomere. The Rh1 maturation process begins with the translation of the *ninaE* mRNA on the endoplasmic reticulum (ER) membranes. In *Drosophila*, immature Rh1 is glycosylated within the ER and then is deglycosylated at a later stage of the secretory process (Katanosaka et al., 1998). In the absence of chromophore availability (i.e., when reared in a vitamin A-deficient media), a glycosylated high mass intermediate of Rh1 is found in at higher levels (Ozaki et al., 1993). Additional work showed that N-glycosylation at asparagine-20 is necessary for the maturation of the Rh1 protein (Katanosaka et al., 1998; O'Tousa, 1992) and expression of a Rh1 mutant that lacked the glycosylation site resulted in retention of the immature Rh1 within the ER (Webel et al., 2000). A consequence of Rh1 retention in the ER is an abnormal accumulation of ER membranes and age-dependent retinal degeneration (Webel et al., 2000).

After glycosylation, Rh1 maturation requires the binding of the chromophore, 11-*cis*-3-hydroxyretinal. In *ninaB* mutant flies, which also lack 11-*cis*-3-hydroxyretinal, Rh1 protein was not detectable but transcription levels of *ninaE* mRNA were normal (Gu et al., 2004;

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von Lintig et al., 2001). These results indicate that in the absence of the chromophore, Rh1 is unable to mature and is degraded.

Three ER molecular chaperones have been identified as having a role in the maturation pathway of Rh1. Defects in NinaA, a photoreceptor cell-specific cyclophilin chaperone, result in the retention of glycosylated Rh1 within the ER (Baker et al., 1994; Colley et al., 1991; Webel et al., 2000). Although NinaA interacts with the N-glycosylated form of Rh1, the glycosylation is not the basis of the interaction (Webel et al., 2000). Calnexin is a molecular chaperone for glycoproteins and was shown to physically interact with Rh1. In *calnexin* mutants, photoreceptors failed to transport Rh1 from the ER and exhibited age-related retinal degeneration (Rosenbaum et al., 2006). The third ER chaperone, XPORT, was recently identified as having a key role in Rh1 and TRP channel maturation (Rosenbaum et al., 2011). In *xport*¹ mutants, a high molecular weight immature form of Rh1 is retained within the ER and only a small amount of mature Rh1 is found within the rhabdomere. Interestingly, none of these three chaperones are required for the maturation of the rhodopsins expressed in the R7 and R8 photoreceptors.

In *Drosophila*, several Rab proteins have been identified that affect the trafficking of Rh1 (Satoh et al., 1997; Satoh et al., 2005; Shetty, 1998). A *Rab1* mutant, defective in GTP binding, showed the accumulation of Rh1 in its immature glycosylated intermediate. Examination of the photoreceptor cell morphology showed proliferation of ER membranes and disrupted Golgi indicative of defective vesicle transport between the ER and the Golgi (Satoh et al., 1997). A constitutively active *Rab6* mutant accumulated a glycosylated intermediate form of Rh1 as well as disorganization of the ER and Golgi membranes (Shetty, 1998). A *Rab11* mutant revealed a buildup of large Rh1-filled vesicles in the cytoplasm (Satoh et al., 2005). In all cases, expression of mutant Rab proteins reduced the quantity of mature Rh1 within the rhabdomere and resulted in small rhabdomeric volume and resulted in retinal degeneration (Satoh et al., 1997; Satoh et al., 2005; Shetty, 1998).

The DEAD box family of RNA helicases has been shown to be required in many steps of RNA metabolism, including transcription, pre-mRNA splicing, RNA transport, translation, ribosome formation, organelle gene expression (mitochondrial/ chloroplasts), and RNA degradation (Caruthers & McKay, 2002; Cordin et al., 2006; Fuller-Pace, 2006; Gorbalenya & Koonin, 1993; Rocak & Linder, 2004; Silverman et al., 2003). The DEAD box helicases may act to unwind short intramolecular RNA structures, disrupt RNA-Protein interactions, or as a chaperone assisting in the proper folding of the RNA molecule. In *Drosophila*, several DEAD box helicases have been identified, including *eIF-4A*, *vas*, *abs* and *bel* (Dorn et al., 1993; Irion & Leptin, 1999; Johnstone et al., 2005; Lasko & Ashburner, 1988; Leroy et al., 1989). Here we present the identification of Dbp21E2, a DEAD-box helicase required for rhodopsin maturation and, when mutated, causes retinal degeneration.

Materials and Methods

Fly Stocks

Flies were raised at room temperature on cornmeal-molasses media. The majority of fly strains, including the 2L deficiency kit and Exelixis deficiencies (Thibault et al., 2004) were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The *Rh1-GAL4* transgenic stock was provided by Dr. Hideyuki Okano (Tabuchi, 2000) and the *UAS-Rh1::GFP* stock by Dr. Franck Pichaud (Pichaud & Desplan, 2001). Construction of the *hs-Rh1::GFP*, *P{Dbp21E2⁺}*, *UAS-Dbp21E2::GFP*, and *UAS-Dbp21E2::RFP* stocks are detailed below. Genetic eye mosaics of a single chromosomal arm (Stowers and Schwarz 1999) were generated through the FLP-FRT system (Xu & Rubin, 1993) utilizing the *ey-FLP* driver (Newsome, 2000). The *FRT* chromosome derived from

P{white-un1}30C, *P{neoFRT}40A* with the *P{white-un1}v30C* marker removed (Shetty, 1998), will be referred to here as the *2LW* chromosome. *y w ey-FLP/Y; 2LW/SM1* males were crossed to *y w ey-FLP; P{GMR-hid}G1 P{neoFRT}40A, l(2)CL-L¹/SM1* females to generate the mitotic clones. The expression of *Rh1::GFP* was controlled through the application of the *UAS/GAL4* expression system (Brand & Perrimon, 1993) using the transgenes *Rh1-GAL4* (Tabuchi, 2000), a Rh1-6 photoreceptor specific driver, and *UAS-Rh1::GFP* (Pichaud & Desplan, 2001). The transgenes *Rh1-GAL4* and *UAS-Rh1::GFP*, were recombined on a single 3rd chromosome to create the Rh1GFP reporter chromosome used throughout this study. It was moved into the genetic background of the *2LW* mosaic stocks using standard genetic crosses.

EMS Mutagenesis Screen

Male flies 1–2 days old of the genotype *y w ey-FLP/Y; 2LW/SM1; Rh1-GAL4, UAS-Rh1::GFP/TM2* were collected and starved for 24 hours. The male flies were fed for 24 hours a solution of 25 mM EMS in 1% sucrose. After the EMS treatment, the male flies were then mated en masse to the respective virgins *ey-FLP; GMR-hid FRT40A /SM1; Rh1-GAL4, UAS-Rh1::GFP/TM2* for 3 days. The F1 male mosaic progeny of the genotype *y w ey-FLP/Y; 2LW (EMS treated)/FRT40A GMR-hid; Rh1-GAL4, UAS-Rh1::GFP/TM2* were scored by examination of the fluorescent green pseudopupil (Franceschini & Kirschfeld, 1971; Stark & Thomas, 2004) using a STEMI SV-11 microscope (Carl Zeiss Inc., Oberkochen, Germany). Mutant flies were selected based on a dimmer fluorescent pseudopupil than their heterozygous siblings, *y w ey-FLP; 2LW/SM1; Rh1-GAL4, UAS-Rh1::GFP/TM2*.

Fluorescent Microscopy

Rh1 localization was determined through visual localization of fluorescent markers from newly eclosed mosaic flies which contain the transgenes *Rh1-GAL4* (Tabuchi, 2000) and *UAS-Rh1::GFP* (Pichaud & Desplan, 2001). Another transgene, *UAS-PDI::RFP* (Mecklenburg et al., 2010) was used to identify the endoplasmic reticulum. Transient expression of *hs-Rh1::GFP* was induced by heat shocking newly eclosed flies (less than 4 hours old) for one hour in a 37° Celsius water bath. Ommatidia were prepared for viewing using modifications of the previously described protocols (Hardie, 1991; Hardie et al., 1991). The compound eyes of transgenic flies were dissected in cold 0.5X phosphate-buffered saline (PBS) (pH 7.4). The dissected eyes were treated with a 1:500 dilution of BioStatus Draq5 (Alexis, San Diego, CA) in 1X PBS or 1:1000 dilution of TO-PRO-3 iodide (Molecular Probes, Eugene, OR) in 1X PBS to visualize nuclei. The dissected eyes were transferred to a glass slide and the ommatidia were manually released from the retina in 0.5X PBS. The ommatidia were fixed with 2% paraformaldehyde and an equal volume of Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The GFP and RFP fluorescence in the ommatidia were observed with a Bio-Rad MRC 1024 Scanning Confocal microscope.

The subcellular localization of RFP- and GFP-tagged Dbp21E2 proteins was investigated by creating *cn bw* flies (hence white-eyed) with three different transgenic constructs. These were (1) *Rh1-GAL4* (Tabuchi, 2000), (2) *UAS-Dbp21E::GFP* or *Dbp21E::RFP* (this report), and (3) one of the following: *UAS-Rh1::GFP* (Pichaud & Desplan, 2001), *UAS-PDI::RFP* (Mecklenburg et al., 2010), *UAS-dGrasp65::GFP* (Hagedorn et al., 2006), *UAS-Rab7::GFP* (Satoh et al., 2005), and *UAS-mito::GFP* (*P{mitoGFP.AP}*) (Horiuchi et al., 2005). Flies were prepared for cryosection and imaged as described previously (Mecklenburg et al., 2010).

Electron Microscopy

Electron microscopic analyses were performed as previously described by (Washburn & O'Tousa, 1992) on appropriately aged flies that were maintained in a 12 hour light/12 hour dark cycle unless otherwise indicated.

Protein Blots

Fly heads from each mutant stock and controls were homogenized in Laemmli sample buffer (Boston Bioproducts, Worcester, MA) and incubated at 42° C for 1 hr. The proteins from the head homogenate were separated by electrophoresis in a 10% SDS-polyacrylamide gel (Pierce, Rockford, IL) or 7.5% SDS-polyacrylamide gel for detection of the GFP fusion protein. After electrophoresis, the proteins were transferred overnight onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 25 volts in transfer buffer (0.02 M Tris Base, 0.15 M glycine, 20% methanol). The nitrocellulose membranes were blocked for 1 hr and washed three times in PBS-T, pH7.4. Rh1 protein was probed with the primary monoclonal mouse anti-rhodopsin 4C5 (1:10,000) (De Couet, 1987), or GFP fusion protein was probed with a monoclonal mouse anti-GFP primary antibody (1:10,000 dilution, Living Colors Av. Monoclonal JL-8, Clontech, Mountain View, CA) for 1 hour. The RdgB protein was probed with a primary polyclonal mouse anti-RdgB antiserum (Vihtelic et al., 1993) diluted 1:1000 in PBS-T. Trp protein was probed with a primary polyclonal mouse anti-Trp antibody (from A. Huber) diluted 1:1000 in PBS-T. Rh4 protein was probed with a primary polyclonal mouse anti-Rh4 antibody (from A. Huber) diluted 1:1000 in PBS-T. Actin was probed with a monoclonal mouse anti-actin primary antibody (Calbiochem, San Diego, CA) diluted 1:10,000 in PBS-T. The membranes were washed and then probed with (1:10,000) anti-mouse IgG horseradish peroxidase (Pierce, Rockford, IL) for 1 hour. The membrane was washed and detected with the Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) per the manufacturer's protocol. The chemiluminescent protein bands were either recorded digitally with the Kodak Image Station 2000R using the Kodak 1D 3.6 software (New Haven, CT) or exposed to X-ray films (Life Science Products, Frederick, CO) and developed by a film processor. Antibodies were stripped from the nitrocellulose membranes by incubation at 50° C for 20–30 min in stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) after detection of the first protein. The membranes were then washed twice in PBS-T for 30 min. and then detected for the second protein as previously described.

Electroretinograms

The electrophysiological response was measured in newly eclosed or one day old flies as previously described (Larrivee et al., 1981). The ERG response was digitally recorded using the Instrunet 100 Network Device and SuperScope II software (GW instruments, Somerville, MA).

Generation of transgenic flies

The *hs-Rh1::GFP* transformation vector was constructed by retrieving the *Rh1::GFP* coding sequence from the *P[UAS-ninaE::EGFP]* vector (Pichaud & Desplan, 2001) following digestion with *NotI* and *XbaI* restriction enzymes and ligating into *PCaSpeR-hs* (Pirrotta, 1988).

To construct the *P{Dbp21E2⁺}* rescue P-element transformation vector, a 2932 bp genomic region, containing the coding sequence of *Dbp21E2* and a 808 bp promoter region was amplified from the BAC clone *BACR30G21* (Hoskins et al., 2000) using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the primers 5'-CA GCG GCC GCT CTT GAG TCG TGT GCC TCC ATC -3' and 5'-CGA TGA CTA GTT CAA

ACC GAA GCC TAC GCT CTG -3'. The 2932 bp PCR product was ligated into the *pCR-2.1 TOPO* sequencing vector (Invitrogen, Carlsbad, CA) and subsequently into *pCaSpeR-4* vector (Pirrotta, 1988).

To create the GFP and RFP tagged versions of the *Dbp21E2* gene under UAS promoter control, the 1608 bp open reading frame of CG3561 without the stop codon was amplified from the cDNA clone LD21669 with the primers 5'-ATGCTGAACTTGTCCGTGGTGCTC-3' and 5'-AAAGGCTTCTTCCTGAGGCGTATG-3'. The isolated PCR product was ligated into the Gateway entry vector ENTR D-TOPO (Invitrogen, Carlsbad, CA), and the plasmid construction verified by DNA sequencing. This entry plasmid was then subjected to LR Clonase (Invitrogen, Carlsbad, CA) reaction with the plasmids pTWG and pTWR to create the P element transformation vectors.

All constructed P element transformation vectors were transformed into *Drosophila* embryos using standard protocols (Spradling & Rubin, 1982)

Results

Isolation of the *Dbp21E2*¹ mutant

We executed a genetic screen to detect novel mutations that affected *Drosophila* Rh1 expression. The *FLP/FRT* system (Golic & Lindquist, 1989; Xu & Rubin, 1993) was utilized to create genetic mosaics in which only the adult eye was homozygous for a mutagenized chromosome arm (Stowers and Schwarz 1999). This approach allowed for the recovery of recessive lethal mutations that are not identified in conventional F2 schemes. To assess the Rh1 levels within the EMS mutagenized progeny, we placed a GFP-tagged Rh1 transgene (*Rh1::GFP*) in the genetic background of these flies. The head of a control mosaic fly is shown in Fig. 1A and a corresponding fluorescent image shown in Fig. 1B. Rh1GFP is readily detected in the pseudopupil (Franceschini & Kirschfeld, 1971) of the control mosaic eye as six prominent spots arranged in a trapezoidal structure, corresponding to the properly organized rhabdomeres of the R1-6 photoreceptor cells.

We used this approach to isolate an EMS-induced mosaic mutant showing weak fluorescence and pronounced disorganization of the pseudopupil shown in Fig. 1D and the corresponding head shown in Fig. 1C. We show in this report that this mutation occurs in the *Dbp21E2* gene, so named because this gene codes for a Dead-box containing protein (Boudet et al., 2001) and is found at the chromosome position 21E2. The EMS induced mutation is therefore named *Dbp21E2*¹. The compound eye of the *Dbp21E2*¹ mutant is roughened and modestly reduced in size (Fig 1C).

Genes involved in rhodopsin maturation or phototransduction often exhibit retinal degeneration when affected. Using electron microscopy, we examined the cellular ultrastructure of retinal cells. In wild type flies Rh1 is found within the densely packed but high organized membrane structure of the rhabdomere (Fig. 1E). The rhabdomere of each photoreceptor lie within the center of each ommatidium and are organized in their distinctive trapezoidal shape. In one day old *Dbp21E2*¹, the photoreceptor cells have reduced R1-6 cell rhabdomeric volume and show classic indicators of apoptotic cell death such as dark cytoplasmic condensation (Fig. 1F, black arrow), proliferation of endoplasmic reticulum membrane within the R1-R6 photoreceptor cells, numerous vacuoles. The R7 photoreceptor (Fig. 1F, asterisk) is less affected. In 3 days old *Dbp21E2*¹ photoreceptors, retinal degeneration has progressed, which is evident by the increased number of photoreceptor cells showing signs of degeneration (Fig 1G). Rearing the *Dbp21E2*¹ mutants in 24 hr dark conditions did not alter the time progression of the retinal degeneration (data not shown).

Molecular genetics of Dbp21E2

The genetic identification of *Dbp21E2¹* mutation was accomplished by various genetic methods. First, the low fluorescent GFP pseudopupil phenotype of *Dbp21E2¹* was mapped to the distal end of the 2L chromosome by recombination of the *Dbp21E2¹* with a homozygous viable *al b pr FRT40A* chromosome, thereby creating *Dbp21E2¹ b pr FRT40A*. Subsequent experiments utilized this refined chromosome. The genetic location of the *Dbp21E2¹* mutant was determined utilizing the 2L deletion kit from the Bloomington stock center. In particular, the deficiency *Df(2L)ast4* mapped the lethality to cytological region 21D1-E2. This cytological region was further refined by the use of 3 molecularly defined deficiencies (Parks et al., 2004). Two of the deficiencies, *Df(2L)Exel6002* and *Df(2L)Exel7005*, partially overlap within the 21E2 cytological region and uncover the lethality of *Dbp21E2¹*. In contrast, the deficiency *Df(2L)Exel6003* complemented *Dbp21E2¹* (Fig. 2A). This narrowed cytological region of 21E2 encompassed the 13 genes starting with *CG13947* and ending within the gene *dreadlocks*. Sequencing of the 13 intact genes that lie within this 49 kb region identified *CG3561* as the strongest candidate with a change of cytosine to a thymine at base pair position 173 which resulted in an early termination of the protein at amino acid 58 of the protein sequence (Fig. 2A), and the gene was hence renamed *Dbp21E2*. The coding sequence of *Dbp21E2* contains 2 exons and encodes a protein of 536 amino acids.

To support that the defective *Dbp21E2* gene was responsible for the low fluorescent phenotype, a second allele was generated. A P-element mutagenesis screen was initiated by mobilizing the nearby P-element *P{lacW}dock^{k13421}* (Bier et al., 1989) and testing for lethal complementation with *Dbp21E2¹*. An allele was generated, named *Dbp21E2²*, and was recombined onto the *al b pr FRT40A* chromosome and combined with *Rh1-GAL4, UAS-Rh1::GFP*. Pseudopupil analysis of the mosaic *DBP21E2²* mutants revealed a similar low fluorescent pseudopupil phenotype (data not shown). Subsequent sequencing analysis of *Dbp21E2²* revealed the unexpected insertion of a Copia element in base position 531 of *Dbp21E2* (Fig. 2A).

To show that the *Dbp21E2¹* low fluorescent Rh1GFP pseudopupil correlated to reduced levels of native Rh1, we removed the *Rh1::GFP* transgene from the genetic background and assayed the levels of the native Rh1 protein by protein blots. Detection with an anti-rhodopsin antibody, showed that the Rh1 levels of *Dbp21E2¹* and *Dbp21E2²* were dramatically lower than the controls (Fig 2B).

To confirm that pseudopupil phenotype was the result of the *Dbp21E2¹* and *Dbp21E2²* mutations, a *Dbp21E2⁺* rescue transgene was constructed. For this purpose, a 2.9 kb rescue construct was cloned from a genomic DNA BAC clone that contained the entire coding sequence of *Dbp21E2*. Inclusion of genomic rescue transgene of *P{Dbp21E2⁺}* in the genetic background of *Dbp21E2¹* and *Dbp21E2²* mosaic mutants restored Rh1 protein levels (Fig. 2B). Furthermore, the inclusion of *P{Dbp21E2⁺}* restored the viability and Rh1 protein levels of the *Dbp21E2¹ / Df(2L)Exel7005* genotype (Fig. 2B).

To determine if the native Rh1 in *Dbp21E2¹* *Drosophila* was functionally active, electroretinograms were recorded from the mosaic mutant. Newly eclosed, 4 hour old *Dbp21E2¹* mutants show a small depolarization and a small recovery phase (Fig 4c, upper middle trace) when compared to the strong depolarization response observed in wild type flies (Fig. 2C, left trace). In the slightly older one day *Dbp21E2¹*, no photoreceptor response could be elicited when stimulated (Fig. 2C, middle lower trace). When the rescue transgene *P{Dbp21E2⁺}* was included in the genetic background of *Dbp21E2¹*, the depolarization response was restored to wild type levels (Fig. 2C, right trace). These data confirm that the

mutation we isolated on the basis of the low Rh1GFP fluorescent pseudopupil maps to the gene *Dbp21E2*.

Dbp21E2 affects Rhodopsin trafficking

To assess the role of *Dbp21E2* in rhodopsin trafficking, a modified pulse-chase experiment was done using an inducible *Rh1::GFP* transgene. We generated transgenic *Drosophila* that contained the open reading frame of Rh1 fused to GFP (Pichaud & Desplan, 2001) under the control of an inducible heat shock promoter. In wild type *Drosophila*, expression of native Rh1 begins during late pupal stage and highly expressed through 1 day post eclosion (Kumar & Ready, 1995). We heat-pulsed *Rh1::GFP* transcription in newly eclosed *Drosophila* (during this active time period of rhodopsin production) and observed the fluorescent expression within the photoreceptors at several subsequent time points. At four hours post heat-shock, Rh1GFP protein is observed within the cell bodies of the photoreceptor cells (Fig 3A, micrograph 1 and 4) of both the *Dbp21E2^l* mutants and heterozygous control. At the later time points, the Rh1GFP signal remains heavily within the cell bodies of *Dbp21E2^l* mutants and has only minor progression to the rhabdomeres (Fig. 3A, micrograph 2 and 3). In contrast, at 10hrs and 14 hrs post heat shock, the heterozygous control *Dbp21E2^{l/+}* localized Rh1GFP predominantly within the rhabdomeres and little Rh1GFP was present in the photoreceptor cell body (Fig. 3A, micrograph 5 and 6).

To assess whether the *Dbp21E2^l* mutation had an effect on the maturation of the rhodopsin protein, we utilized a GFP antibody to detect Rh1 protein at different times after heat shock (Fig. 3B). In these blots, more protein extract (five heads) from the *Dbp21E2^l* mutant was loaded than from the *Dbp21E2^{l/+}* control (one head) since steady state blots showed the mutant retained much lower levels of rhodopsin. Figure 3B shows that both the *Dbp21E2^l* mutants and the heterozygous *Dbp21E2^{l/+}* controls produced the immature glycosylated form of Rh1GFP (and a higher weight dimerized form (Colley et al., 1995)) at 4 hrs post heat shock. At 14 hrs post heat shock, more mature non-glycosylated form of Rh1GFP (marked by asterisk) is detected in the *Dbp21E2^{l/+}* controls than in the *Dbp21E2^l* mutant. These results show that *Dbp21E2* mutants are competent to synthesize the immature forms of Rh1 but are defective in a subsequent step required for Rh1 accumulation within the rhabdomere.

To determine if *Dbp21E2* mutants affected production of other photoreceptor proteins, we used protein blots to compare the levels of Rh4, RdgB, Trp, and NorpA in *Dbp21E2^l* mutants and control strains. Figure 3C shows all of these proteins are reduced in *Dbp21E2* mutants. The extent of the reduction appears unique to each protein, with NorpA and RdgB being more affected than TRP and Rh4. To confirm this interpretation, following detection of Rh4, we reprobbed the same membrane for Rh1 levels. These results are also shown in Figure 3C, and indicate that Rh1 levels are impacted to a greater extent than Rh4 levels by the *Dbp21E2^l* mutant.

***Dbp21E2^l* Induced Retinal Degeneration is Independent of Rh1**

Previous work showed that the inability to mature Rh1 causes retinal degeneration (Baker et al., 1994; Colley et al., 1991; Rosenbaum et al., 2006; Webel et al., 2000). This raises the possibility that degeneration seen in *Dbp21E2^l* results from the accumulation of immature Rh1. To test this possibility, we examined the ability of *Dbp21E2* to induce retinal degeneration in an Rh1 null background (*ninaE¹¹⁷*). Electron micrographs of ommatidial sections show that in the absence of Rh1, the progression of retinal degeneration was not altered. In one day old *Dbp21E2^l* flies, several photoreceptor cells in each ommatidia show signs of cytoplasmic condensation and vacuole formation in the presence (Fig. 4A) or absence of Rh1 (Fig. 4B). In the Rh1 null photoreceptors, the rhabdomeres are reduced in size

(Fig. 4C, arrow) and show unraveling of the rhabdomeric membranes into the cell body (Fig. 4C, asterisk). The R7 photoreceptor cell (labeled), which does not express Rh1, maintains its rhabdomeric volume. At three days post-eclosion, the majority of the R1-R6 cells had dark cytoplasmic condensation and the rhabdomeres were greatly reduced in size or absent, either with (Fig. 4D) or without Rh1 (Fig. 4E). Photoreceptors lacking only Rh1 at this age (Fig. 4F) show the expected reduction in rhabdomere volume but the cell bodies do not show cytoplasmic condensation. These results show that the rapid progression of retinal degeneration in the *Dbp21E2^l* mutants is not caused by the loss of mature Rh1.

The *Dbp21E2* Protein Localizes to Cytoplasm

To discern the cellular location of the Dbp21E2 protein, we created two transgenes in which the UAS promoter element controlled expression of a *Dbp21E2::GFP* open reading frame and a *Dbp21E2::RFP* open reading frame. Figure 5A shows the retinal structure of animals simultaneously expressing *Dbp21E2::RFP* and *Rh1::GFP* under control of the *Rh1-GAL4* driver. Rh1GFP, as expected, localized to the photoreceptor rhabdomere. Dbp21E2::RFP, however, was localized to discrete cytoplasmic compartments. Similar localization was seen for Dbp21E2::RFP (Fig. 5B). Fig. 5B also shows that the Dbp21E2 compartment does not correspond to the endoplasmic reticulum labeled by a *PDI::RFP* transgene. With a similar approach, localization to the Golgi (Fig. 5C) endosomal compartments (Fig. 5D) and mitochondria (Fig. 5E) was discounted. Thus, the Dbp21E2 fluorescent reporters suggest that tagged Dbp21E2 is localized to discrete cytoplasmic regions but the identity and function of these regions is not known.

Discussion

We carried out an EMS mutagenesis screen to identify novel components required for Rh1 maturation. The genetic screen employed genetic mosaics created by FLP/FRT technology to allow lethal mutations to be examined for effects within the adult eye (Stowers & Schwarz, 1999). The effort resulted in the identification of the mutations in *Dbp21E2*. The Dbp21E2 protein contains both DEADc and HELICc domains, two conserved domains that have been shown to have a role in ATPase and helicase activity. (Linder et al., 1989; Tanner et al., 2003). In the *Drosophila* genome, the DEAD box helicase most closely related to *Dbp21E2* is *EIF4a* (Lasko, 2000), responsible for unwinding the 5' mRNA secondary structures to enable ribosome binding (Rogers et al., 1999; Rozen et al., 1990). In mammals, the closest relative is the DEAD-box protein DDX28, a protein that localizes to both the nucleus and the mitochondria (Valgardsdottir et al., 2001; Valgardsdottir & Prydz, 2003).

Our genetic screen relied on *in vivo* fluorescence of an Rh1GFP reporter to rapidly assess differences in rhodopsin content. In subsequent analysis, we showed the *Dbp21E2^l* mutant also decreased the levels of the native Rh1 protein. Further, a *Dbp21E2* transgene rescued this mutant phenotype, confirming that the *Dbp21E2* mutation was solely responsible for the low rhodopsin phenotype. A second phenotype associated with the *Dbp21E2* mutant is retinal degeneration. Analysis of mutants lacking Rh1 protein showed that retinal degeneration is not a consequence of *Dbp21E2*'s effect on Rh1 levels. This result suggests that the *Dbp21E2* requirement in the photoreceptor is more widespread than its role in maintaining rhodopsin content, consistent with the lethal phenotype of non-mosaic Dbp21E2 homozygous mutants.

We explored possible roles of the Dbp21E2 RNA helicase in rhodopsin maturation. We found no compelling evidence for a role in Rh1 mRNA production prior to protein synthesis, nor in the primary translation of the Rh1 mRNA. First, Rh1 mRNA was assessed by RT-PCR and found to be ~40% of the wild type level (data not shown). We reasoned that the reduction in Rh1 protein is more severe, hence could not be attributed solely to the mRNA

reduction and further the RNA reduction may be consequence of retinal degenerative processes occurring in the *Dbp21E2* mutant. More compelling evidence against a role of Dbp21E2 in Rh1 mRNA processing was obtained from Rh1GFP protein production following heat shock-induced gene activation. These experiments showed that in the *Dbp21E2* mutant there is robust production of immature Rh1GFP protein following heat shock-induced expression. Thus the effect of *Dbp21E2* mutations cannot be solely due to an effect on Rh1 mRNA synthesis, Rh1 mRNA transport, or in the translation process. Dbp21E2 must be required in a posttranslational process, as the ability to both mature and localize Rh1GFP within the rhabdomere was impaired in the *Dbp21E2* mutant. Further, the localization of GFP and RFP-tagged version of the Dbp21E2 protein to a cytoplasmic compartment also suggests it is required in a posttranslational process. Additional work is needed to ascertain if the GFP and RFP-tagged versions of the Dbp21E2 protein are localized to the same compartment as native Dbp21E2.

The genetic screen that led to the isolation of mutants in the RNA helicase *Dbp21E2* gene was designed to identify novel proteins affecting the maturation or trafficking of rhodopsin. These are not easily identified in traditional screens because the gene products' roles are not specific to rhodopsin expression and therefore severe mutants are lethal. Our analysis shows that *Dbp21E2* is indeed an essential gene. While it is required in photoreceptors to maintain high levels of rhodopsin expression, the finding that *Dbp21E2* mutants trigger retinal degeneration in the absence of rhodopsin expression shows a more global role in photoreceptor biology. Our analysis further showed the Dbp21E2 protein is localized to, and acts within, the cytoplasm. The results suggest the Dbp21E2 protein has a different role than closely related RNA helicases, and provides an experimental system to further probe its function.

References

- Baker EK, Colley NJ, Zuker CS. The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *EMBO J.* 1994; 13:4886–4895. [PubMed: 7957056]
- Bier E, Vaessin H, Shepherd S, Lee K, McCall K, Barbel S, Ackerman L, Carretto R, Uemura T, Grell E, et al. Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. *Genes & Development.* 1989; 3:1273–1287. [PubMed: 2558049]
- Boudet N, Aubourg S, Toffano-Nioche C, Kreis M, Lecharny A. Evolution of Intron/Exon Structure of DEAD Helicase Family Genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. *Genome Res.* 2001; 11:2101–2114. [PubMed: 11731501]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 1993; 118:401–415. [PubMed: 8223268]
- Caruthers JM, McKay DB. Helicase structure and mechanism. *Curr Opin Struct Biol.* 2002; 12:123–133. [PubMed: 11839499]
- Chapple JP, Grayson C, Hardcastle AJ, Saliba RS, van der Spuy J, Cheetham ME. Unfolding retinal dystrophies: a role for molecular chaperones? *Trends Mol Med.* 2001; 7:414–421. [PubMed: 11530337]
- Colley NJ, Baker EK, Stamnes MA, Zuker CS. The cyclophilin homolog *ninaA* is required in the secretory pathway. *Cell.* 1991; 67:255–263. [PubMed: 1913822]
- Colley NJ, Cassill JA, Baker EK, Zuker CS. Defective Intracellular-Transport Is the Molecular-Basis of Rhodopsin-Dependent Dominant Retinal Degeneration. *Proc Natl Acad Sci USA.* 1995; 92:3070–3074. [PubMed: 7708777]
- Cordin O, Banroques J, Tanner NK, Linder P. The DEAD-box protein family of RNA helicases. *Gene.* 2006; 367:17–37. [PubMed: 16337753]
- De Couet HG. Monoclonal antibodies provide evidence that rhodopsin in the outer rhabdomeres of *Drosophila melanogaster* is not glycosylated. *Eur J Cell Biol.* 1987; 44:50–56.

- Dorn R, Morawietz H, Reuter G, Saumweber H. Identification of an essential *Drosophila* gene that is homologous to the translation initiation factor eIF-4A of yeast and mouse. *Molecular and General Genetics*. 1993; 237:233–240. [PubMed: 8455559]
- Dryja TP, McGee TL, Reichel E, Hahn LB, Cowley GS, Yandell DW, Sandberg MA, Berson EL. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature*. 1990; 343:364–366. [PubMed: 2137202]
- Franceschini N, Kirschfeld K. Pseudopupil phenomena in the compound eye of *Drosophila*. *Kybernetik*. 1971; 9:159–182. [PubMed: 5134358]
- Fuller-Pace FV. DEXD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. *Nucleic Acids Res*. 2006; 34:4206–4215. [PubMed: 16935882]
- Golic KG, Lindquist S. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell*. 1989; 59:499–509. [PubMed: 2509077]
- Gorbalenya AE, Koonin EV. Helicases: amino acid sequence comparisons and structure-function relationships. *Curr Opin Struct Biol*. 1993; 3:419–429.
- Gu G, Yang J, Mitchell KA, O'Tousa JE. *Drosophila* ninaB and ninaD act outside of retina to produce rhodopsin chromophore. *J Biol Chem*. 2004; 279:18608–18613. [PubMed: 14982930]
- Hagedorn EJ, Bayraktar JL, Kandachar VR, Bai T, Englert DM, Chang HC. *Drosophila melanogaster* auxilin regulates the internalization of Delta to control activity of the Notch signaling pathway. *J Cell Biol*. 2006; 173:443–452. [PubMed: 16682530]
- Hardie RC. Voltage-sensitive potassium channels in *Drosophila* photoreceptors. *J Neuroscience*. 1991; 11:3079–3095.
- Hardie RC, Voss D, Pongs O, Laughlin SB. Novel potassium channels encoded by the Shaker locus in *Drosophila* photoreceptors. *Neuron*. 1991; 6:477–486. [PubMed: 2001287]
- Horiuchi D, Barkus RV, Pilling AD, Gassman A, Saxton WM. APLIP1, a kinesin binding JIP-1/JNK scaffold protein, influences the axonal transport of both vesicles and mitochondria in *Drosophila*. *Curr Biol*. 2005; 15:2137–2141. [PubMed: 16332540]
- Hoskins RA, Nelson CR, Berman BP, Lavery TR, George RA, Ciesiolka L, Naeemuddin M, Arenson AD, Durbin J, David RG, Tabor PE, Bailey MR, DeShazo DR, Catanese J, Mammoser A, Osoegawa K, de Jong PJ, Celniker SE, Gibbs RA, Rubin GM, Scherer SE. A BAC-Based Physical Map of the Major Autosomes of *Drosophila melanogaster*. *Science*. 2000; 287:2271–2274. [PubMed: 10731150]
- Irion U, Leptin M. Developmental and cell biological functions of the *Drosophila* DEAD-box protein abstrakt. *Current Biology*. 1999; 9:1373–1381. [PubMed: 10607561]
- Johnstone O, Deuring R, Bock R, Linder P, Fuller MT, Lasko P. Belle is a *Drosophila* DEAD-box protein required for viability and in the germ line. *Dev Biol*. 2005; 277:92–101. [PubMed: 15572142]
- Katanosaka K, Tokunaga F, Kawamura S, Ozaki K. N-linked glycosylation of *Drosophila* rhodopsin occurs exclusively in the amino-terminal domain and functions in rhodopsin maturation. *FEBS Lett*. 1998; 424:149–154. [PubMed: 9539140]
- Kumar JP, Ready DF. Rhodopsin plays an essential structural role in *Drosophila* photoreceptor development. *Development*. 1995; 121:4359–4370. [PubMed: 8575336]
- Kurada P, O'Tousa JE. Retinal degeneration caused by dominant rhodopsin mutations in *Drosophila*. *Neuron*. 1995; 14:571–579. [PubMed: 7695903]
- Larriave DC, Conrad SK, Stephenson RS, Pak WL. Mutation that selectively affects rhodopsin concentration in the peripheral photoreceptors of *Drosophila melanogaster*. *J Gen Physiol*. 1981; 78:521–545. [PubMed: 6796648]
- Lasko P. The *Drosophila melanogaster* genome: translation factors and RNA binding proteins. *J Cell Biol*. 2000; 150:F51–56. [PubMed: 10908586]
- Lasko PF, Ashburner M. The product of the *Drosophila* gene vasa is very similar to eukaryotic initiation factor-4A. *Nature*. 1988; 335:611–617. [PubMed: 3140040]
- Leonard DS, Bowman VD, Ready DF, Pak WL. Degeneration of photoreceptors in rhodopsin mutants of *Drosophila*. *J Neurobiol*. 1992; 23:605–626. [PubMed: 1431838]

- Leroy P, Alzari P, Sassoon D, Wolgemuth D, Fellous M. The protein encoded by a murine male germ cell-specific transcript is a putative ATP-dependent RNA helicase. *Cell*. 1989; 57:549–559. [PubMed: 2720782]
- Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, Schnier J, Slonimski PP. Birth of the D-E-A-D box. *Nature*. 1989; 337:121–122. [PubMed: 2563148]
- Mecklenburg KL, Takemori N, Komori N, Chu B, Hardie RC, Matsumoto H, O'Tousa JE. Retinophilin is a light-regulated phosphoprotein required to suppress photoreceptor dark noise in *Drosophila*. *J Neuroscience*. 2010; 30:1238–1249.
- Newsome TPA, Bengt, Dickson, Barry J. Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development (Cambridge)*. 2000; 127:851–860.
- O'Tousa JE. Requirement of N-linked glycosylation site in *Drosophila* rhodopsin. *Vis Neurosci*. 1992; 8:385–390. [PubMed: 1534022]
- O'Tousa JE, Leonard DS, Pak WL. Morphological defects in oraJK84 photoreceptors caused by mutation in R1-6 opsin gene of *Drosophila*. *J Neurogenet*. 1989; 6:41–52. [PubMed: 2528612]
- Ozaki K, Nagatani H, Ozaki M, Tokunaga F. Maturation of major *Drosophila* rhodopsin, ninaE, requires chromophore 3-hydroxyretinal. *Neuron*. 1993; 10:1113–1119. [PubMed: 8318232]
- Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS, Edgar KA, Howie E, Killpack K, Milash B, Norton A, Thao D, Whittaker K, Winner MA, Friedman L, Margolis J, Singer MA, Kopczynski C, Curtis D, Kaufman TC, Plowman GD, Duyk G, Francis-Lang HL. Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet*. 2004; 36:288–292. [PubMed: 14981519]
- Pichaud F, Desplan C. A new visualization approach for identifying mutations that affect differentiation and organization of the *Drosophila* ommatidia. *Development*. 2001; 128:815–826. [PubMed: 11222137]
- Pirrotta V. Vectors for P-mediated transformation in *Drosophila*. *Biotechnology*. 1988; 10:437–456. [PubMed: 2850048]
- Rocak S, Linder P. DEAD-box proteins: the driving forces behind RNA metabolism. *Nat Rev Mol Cell Biol*. 2004; 5:232–241. [PubMed: 14991003]
- Rogers GW Jr, Richter NJ, Merrick WC. Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J Biol Chem*. 1999; 274:12236–12244. [PubMed: 10212190]
- Rosenbaum EE, Hardie RC, Colley NJ. Calnexin is essential for rhodopsin maturation, Ca²⁺ regulation, and photoreceptor cell survival. *Neuron*. 2006; 49:229–241. [PubMed: 16423697]
- Rosenbaum EE, Brehm KS, Vasiljevic E, Liu CH, Hardie RC, Colley NJ. XPORT-dependent transport of TRP and rhodopsin. *Neuron*. 2011; 72:602–615. [PubMed: 22099462]
- Rozen F, Edery I, Meerovitch K, Dever TE, Merrick WC, Sonenberg N. Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol Cell Biol*. 1990; 10:1134–1144. [PubMed: 2304461]
- Satoh A, Tokunaga F, Kawamura S, Ozaki K. In situ inhibition of vesicle transport and protein processing in the dominant negative Rab1 mutant of *Drosophila*. *J Cell Sci*. 1997; 110:2943–2953. [PubMed: 9359879]
- Satoh AK, O'Tousa JE, Ozaki K, Ready DF. Rab11 mediates post-Golgi trafficking of rhodopsin to the photosensitive apical membrane of *Drosophila* photoreceptors. *Development*. 2005; 132:1487–1497. [PubMed: 15728675]
- Scavarda NJ, O'Tousa J, Pak WL. *Drosophila* locus with gene-dosage effects on rhodopsin. *Proc Natl Acad Sci U S A*. 1983; 80:4441–4445. [PubMed: 16593338]
- Shetty KM. Rab6 regulation of protein transport in *Drosophila* photoreceptor cells. 1998
- Silverman E, Edwards-Gilbert G, Lin RJ. DExD/H-box proteins and their partners: helping RNA helicases unwind. *Gene*. 2003; 312:1–16. [PubMed: 12909336]
- Spradling AC, Rubin GM. Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science*. 1982; 218:341–347. [PubMed: 6289435]

- Stark W, Thomas C. Microscopy of multiple visual receptor types in *Drosophila*. *Molecular Vision*. 2004; 10:943–955. [PubMed: 15616481]
- Stowers RS, Schwarz TL. A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics*. 1999; 152:1631–1639. [PubMed: 10430588]
- Sung CH, Davenport CM, Hennessey JC, Maumenee IH, Jacobson SG, Heckenlively JR, Nowakowski R, Fishman G, Gouras P, Nathans J. Rhodopsin mutations in autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 1991a; 88:6481–6485. [PubMed: 1862076]
- Sung CH, Schneider BG, Agarwal N, Papermaster DS, Nathans J. Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 1991b; 88:8840–8844. [PubMed: 1924344]
- Tabuchi KSK, Suzuki E, Ozaki K, Sone M, Hama C, Tanifuji-Morimoto T, Yuasa Y, Yoshihara Y, Nose A, Okano H. GAL4/UAS-WGA system as a powerful tool for tracing *Drosophila* transsynaptic neural pathways. *J Neurosci Res*. 2000; 59:94–99. [PubMed: 10658189]
- Tanner NK, Cordin O, Banroques J, Doere M, Linder P. The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Mol Cell*. 2003; 11:127–138. [PubMed: 12535527]
- Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, Singh CM, Buchholz R, Demsky M, Fawcett R, Francis-Lang HL, Ryner L, Cheung LM, Chong A, Erickson C, Fisher WW, Greer K, Hartouni SR, Howie E, Jakkula L, Joo D, Killpack K, Laufer A, Mazzotta J, Smith RD, Stevens LM, Stuber C, Tan LR, Ventura R, Woo A, Zakrajsek I, Zhao L, Chen F, Swimmer C, Kopczynski C, Duyk G, Winberg ML, Margolis J. A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat Genet*. 2004; 36:283–287. [PubMed: 14981521]
- Valgardsdottir R, Brede G, Eide LG, Frengen E, Prydz H. Cloning and characterization of MDDX28, a putative dead-box helicase with mitochondrial and nuclear localization. *J Biol Chem*. 2001; 276:32056–32063. [PubMed: 11350955]
- Valgardsdottir R, Prydz H. Transport signals and transcription-dependent nuclear localization of the putative DEAD-box helicase MDDX28. *J Biol Chem*. 2003; 278:21146–21154. [PubMed: 12663657]
- Vihtelic TS, Goebel M, Milligan S, O'Tousa JE, Hyde DR. Localization of *Drosophila* retinal degeneration B, a membrane-associated phosphatidylinositol transfer protein. *J Cell Biol*. 1993; 122:1013–1022. [PubMed: 8354691]
- von Lintig J, Dreher A, Kiefer C, Wernet MF, Vogt K. Analysis of the blind *Drosophila* mutant *ninaB* identifies the gene encoding the key enzyme for vitamin A formation *in vivo*. *Proc Natl Acad Sci U S A*. 2001; 98:1130–1135. [PubMed: 11158606]
- Washburn T, O'Tousa JE. Nonsense Suppression of the Major Rhodopsin Gene of *Drosophila*. *Genetics*. 1992; 130:585–595. [PubMed: 1551579]
- Webel R, Menon I, O'Tousa JE, Colley NJ. Role of asparagine-linked oligosaccharides in rhodopsin maturation and association with its molecular chaperone, *NinaA*. *J Biol Chem*. 2000; 275:24752–24759. [PubMed: 10811808]
- Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*. 1993; 117:1223–1237. [PubMed: 8404527]

Figure 1.

Phenotypic characterization of the *Dbp21E2* mutant. (A–D). Images of the compound eye and fluorescent pseudopupil images of the control *2L FRT40A* mosaic (A, B) and the *Dbp21E2¹* mutants (C, D) are shown. The pseudopupil of the *Dbp21E2¹* mutant is dimmer than parental strain and has been digitally enhanced in this image to allow visualization of the loss of the distinctive trapezoidal shape of the pseudopupil resulting from the rough surface of compound eye. (E–G). Electron micrographs show cross-sectioned ommatidia at the level of the R1-6 photoreceptor nuclei. The one day old control *2L FRT40A* mosaic is shown in (E). The one-day old *Dbp21E2¹* mutant is shown in (F). Some photoreceptors in the *Dbp21E2¹* mutant show apoptotic cell death of the photoreceptor cells as indicated by the electron dense cell body (arrow). The R7 cell is marked with an asterisk. At three days post-eclosion (G), all R1-R6 cells show cytoplasmic condensation and vacuole formation indicative of retinal degeneration. The rate of degeneration in the R7 photoreceptor is slower.

Figure 2.

Identification of the *Dbp21E2* gene. (A) The *Dbp21E2*¹ lethal mutation is cytologically mapped to the 21E2 region. The deficiencies *Df(2L)Exel6002* and *Df(2L)Exel7005* uncover the *Dbp21E2*¹ lethal mutation. *Df(2L)Exel6003* complemented *Dbp21E2*¹. Sequencing of the EMS derived mutant identified *Dbp21E2* (CG3561) as a candidate gene due to the base change of cytosine to thymine at base position 172 within the first exon creating a nonsense codon at this site. Sequencing of a second mutant allele, *Dbp21E2*², revealed the insertion of a Copia transposon at base position 531 in the first exon. The rescue transgene of *Dbp21E2*, *P{Dbp21E2⁺}* was constructed from genomic DNA, including 0.8 kb upstream and 0.4 kb downstream of the coding sequence. (B) Native Rh1 protein levels were assessed in the *Dbp21E2*¹ and *Dbp21E2*² mutant allele in a non-GFP background. Rh1 antibodies detected low Rh1 protein levels in *Dbp21E2*¹ and *Dbp21E2*² (lanes 3 and 8). Addition of the rescue construct, *P{Dbp21E2⁺}*, to *Dbp21E2*¹ and *Dbp21E2*² restored Rh1 protein to comparable wild type levels (lanes 4 and 9). The *P{Dbp21E2⁺}* construct also rescued the lethal phenotype of *Dbp21E2*¹/*Df(2L)Exel7005* and the Rh1 protein was at wild type levels in these flies (lane 5). Wildtype and the Rh1 null mutant were positive and negative controls respectively (lanes 1, 2, 6, and 7). (C) Electrophysiological analysis showed that *P{Dbp21E2⁺}* restored the activation of the phototransduction cascade in the *Dbp21E2*¹ mutant. The

phototransduction cascade was activated with a stimulus of blue light for 5 seconds. Wild type responds as expected to the light stimulus (left trace) but newly eclosed *Dbp21E2^l* show a slight response to the pulse of light (second trace) and by one day, *Dbp21E2^l* no longer responds to the pulse of light (third trace). Addition of the rescue construct, P{*Dbp21E2⁺*}, to the genome of the *Dbp21E2^l* mutant restored the light response (right trace).

Figure 3.

The trafficking of Rh1GFP is impeded in *Dbp21E2¹* mutants. An *Rh1::GFP* fusion gene was expressed under the control of a *heat shock* promoter and Rh1GFP examined in *Dbp21E2¹* mutants and *Dbp21E2¹* heterozygous siblings at specific times after heat shock. (A) Confocal images of single ommatidial bundles at specific time point following induction of Rh1GFP. At four hours after heat shock, Rh1GFP was visible in the cell body in both the *Dbp21E2¹* mutant and the *Dbp21E2^{1/+}* control. At fourteen hours after heat shock, the *Dbp21E2¹* mutant retained the majority of the Rh1GFP within the cell body while the *Dbp21E2^{1/+}* controls showed most of the Rh1GFP within the rhabdomeres (R) and little within the cell body (CB). (B) Protein blot analysis of the heat shock-induced Rh1GFP levels in *Dbp21E2¹* mutants and *Dbp21E2^{1/+}* controls. In the SDS-PAGE experiment, the mutant lanes are loaded with a

5-fold excess of protein extract to facilitate detection of Rh1. At 4 hours after heat shock induction, both dimer form (~134 kDa, black arrowheads) and a glycosylated immature form of Rh1GFP (~67 kDa, white arrowheads) is easily detected in both mutant and controls (lanes 4 and 9). For the *Dbp21E2^{l/+}* control, the 6, 10, and 14 hour time points show that the immature Rh1GFP forms gradually diminish and the mature non-glycosylated form of Rh1GFP (~61 kDa, asterisk) accumulates. Even though the lanes of the *Dbp21E2^l* mutant contained 5-fold more protein, the mature non-phosphorylated form of Rh1GFP (asterisk) is found at lower levels than seen in the controls.

(C) Protein blots analyses of Rh4, RdgB, Trp, and NorpA in *Dbp21E2^l* mutants and control strains. In *Dbp21E2* mutants, these 4 eye-specific proteins all show reduced protein levels. The reduction in protein amounts appear unique to each protein, with NorpA and RdgB being more affected than Trp and Rh4. Following detection of Rh4, the same membrane was reprobbed for native Rh1 levels. The Rh1 levels in the *Dbp21E2* mutant are impacted to a greater extent than Rh4. This blot was reprobbed with an actin antibody to confirm similar loading of protein, as were the 3 other protein blots (data not shown).

Figure 4.

Dbp21E2 induces retinal degeneration in the absence of Rh1. *Dbp21E2^l* mutants were reared in Rh1⁺ and a null Rh1 (Rh1⁻) genetic background. Ommatidial cross sections were imaged at 1 (A-C) and 3 (D-F) days of age. There was no observable difference in the rate of retinal degeneration of *Dbp21E2^l* mutants in the presence or absence of Rh1 (A vs. B, D vs. E). (C) The Rh1 null mutant at 1 day has small rhabdomeres (white arrow) and cytoplasmic extension of the rhabdomeric membranes (asterisk). The R7 photoreceptor is marked. (F) By 3 days, the R1-R6 rhabdomeres of the Rh1 null mutant are further decreased in size, but R1-6 cell bodies remain intact (one is marked by a star).

Figure 5.

Cytoplasmic localization of GFP and RFP-tagged Dbp21E2 proteins. (A) Image of a cryosectioned retina from flies expressing both Rh1GFP and Dbp21E2RFP under control of the *Rh1-GAL4* driver. Rh1 localizes to rhabdomeric membranes and Dbp21E2RFP is found in discrete cytoplasmic regions. (B) Image of a cryosectioned retina from flies expressing both PDIRFP and Dbp21E2GFP. Dbp21E2GFP is also found in a small number of cytoplasmic regions. These do not colocalize with PDIRFP, a protein expected to decorate the endoplasmic reticulum. (C–E.) Images of retina from flies expressing GFP and RFP-tagged Dbp21E2 with the cytoplasmic organelle markers Grasp65GFP, to label the Golgi compartment, Rab7GFP, to label endomembrane organelles, and mitoGFP, to label the mitochondria. For A–E, the GFP and RFP images are shown separately below the composite image.