Defective Pigment Granule Biogenesis and Aberrant Behavior Caused by Mutations in the Drosophila AP-3β Adaptin Gene *ruby*

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ABSTRACT

Lysosomal protein trafficking is a fundamental process conserved from yeast to humans. This conservation extends to lysosome-like organelles such as mammalian melanosomes and insect eye pigment granules. Recently, eye and coat color mutations in mouse (*mocha* and *pearl*) and Drosophila (*garnet* and *carmine*) were shown to affect subunits of the heterotetrameric adaptor protein complex AP-3 involved in vesicle trafficking. Here we demonstrate that the Drosophila eye color mutant *ruby* is defective in the AP-3β subunit gene. *ruby* expression was found in retinal pigment and photoreceptor cells and in the developing central nervous system. *ruby* mutations lead to a decreased number and altered size of pigment granules in various cell types in and adjacent to the retina. Humans with lesions in the related AP-3βA gene suffer from Hermansky-Pudlak syndrome, which is caused by defects in a number of lysosome-related organelles. Hermansky-Pudlak patients have a reduced skin pigmentation and suffer from internal bleeding, pulmonary fibrosis, and visual system malfunction. The Drosophila AP-3β adaptin also appears to be involved in processes other than eye pigment granule biogenesis because all *ruby* allele combinations tested exhibited defective behavior in a visual fixation paradigm.

TRAFFICKING of integral membrane proteins within the eukaryotic cell occurs through transport vesicles that form by the assembly of coat proteins onto the cytoplasmic face of precursor membranes. The coat is required for budding of the vesicle and for cargo selection (Waters *et al.* 1991; Schekman and Orci 1996; Robinson 1997). Clathrin, the first coat to be identified, assembles with adaptor protein complexes that provide the link between the membrane and the clathrin scaffold. The adaptor protein complexes provide membrane specificity for clathrin assembly by recognizing sorting motifs in the cytoplasmic domains of membrane proteins and by interacting with putative docking molecules (reviewed in Heilker *et al.* 1999). Originally, two homologous adaptor protein complexes were identified: AP-1 for sorting at the *trans*-Golgi network (TGN) during biosynthetic transport to the endosomal/lysosomal system and AP-2 for endocytosis at the plasma membrane. More recently, two further complexes, AP-3 (Odorizzi *et al.* 1998) and AP-4 (Dell'Angelica *et al.* 1999b) were characterized. For AP-3, the question of clathrin coat association is controversial (Dell'Angelica *et al.* 1998). AP-4 was not found in association with clathrin-coated vesicles (Dell'Angelica *et al.* 1999b). All AP complexes are heterotetrameric proteins built from homologous subunits. They consist of two large chains (γ, α, δ, and ε, and β1-β4, respectively, of 90–130 kD), one medium chain (μ1–μ4 of ~50 kD), and one small chain (σ1–σ4 of ~20 kD). The interaction of AP-1 and AP-2 with clathrin is mediated by the β subunits (Schröder and Ungewickell 1991; Shih *et al.* 1995).

Evidence for the function of the "classical" complexes AP-1 and AP-2 was predominantly gained from biochemical experiments. In the case of AP-3, genetics is providing information on its role in an apparently conserved transport pathway to lysosome-like organelles (Odorizzi *et al.* 1998). In *Saccharomyces cerevisiae*, AP-3 subunits were identified as factors involved in the transport of alkaline phosphatase from the TGN to the vacuole. This pathway is independent of the function of AP-1 and AP-2 or clathrin (Cowles *et al.* 1997). AP-3 in mouse and humans consists of neuronal and non-neuronal isoforms. The latter complex consists of β3A and μ3A and the ubiquitous δ, σ3A, or σ3B subunits (Dell'Angelica *et al.* 1997a,b; Simpson *et al.* 1997). In neuronal cells, AP-3 contains β3B, μ3B, and the ubiquitous subunits (Newman *et al.* 1995; Pevsner *et al.* 1995; Simpson *et al.* 1996). Mutations in the two large mouse AP-3 subunit genes, AP-3δ in *mocha* (Kantheti *et al.* 1998) and AP-3βA in *pearl* (Feng *et al.* 1999), were recently identified as the cause of a related set of syndromes.

Conditions affecting human AP-3 subunits are of clinical relevance. A patient with autoimmunity against AP-

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3βB suffered from degeneration of the cerebellum, where the protein is prominently expressed (Newman *et al.* 1995). Mutations in AP-3βA were recognized in a subset of patients suffering from the autosomal recessive disease Hermansky-Pudlak syndrome (HPS, Online Mendelian Inheritance in Man no. 203300) (Dell'-Angelica *et al.* 1999a). HPS is characterized by defects in a number of lysosome-related organelles. Patients exhibit subnormal eye and skin pigmentation due to aberrant melanosome development. Associated with this oculocutaneous albinism, patients suffer from impairment in visual acuity, misrouting of optic fibers, nystagmus, and underdeveloped fovea (Summers *et al.* 1988). Clinically more serious is a deficiency of platelet dense granules and a lysosomal malfunction in reticuloendothelial cells, which generally leads to early death. HPS is genetically heterogeneous (Hazelwood *et al.* 1997). In addition to patients affected in the human *pearl* homolog, patients with mutations in the homolog of the mouse *pale ear* gene have been diagnosed with HPS (Oh *et al.* 1996; Gardner *et al.* 1997). A phenotype similar to HPS was described in 14 mouse mutants, all originally isolated by their eye/skin pigmentation defect (Odorizzi *et al.* 1998; Swank *et al.* 1998; Spritz 1999).

Pigmentation mutants of Drosophila offer another approach to the study of lysosome and AP-3 function (Lloyd *et al.* 1998). The eye color genes *garnet* and *carmine* were recently identified as the Drosophila homologs of AP-3δ and AP-3μ, respectively (Ooi *et al.* 1997; Simpson *et al.* 1997; Mullins *et al.* 1999). We report here that the Drosophila AP-3β homolog is encoded by the eye color gene *ruby* (*rb*). *ruby* flies, first described by Bridges in 1914 (Morgan *et al.* 1925), show a reduced number of eye pigment granules. The expression of *rb* throughout ontogenesis and behavioral aberrations of adult flies indicate that the gene plays a role beyond eye pigmentation. *ruby* therefore can provide a model for studying the role of AP-3-mediated vesicular trafficking in development and in adult central nervous system function.

MATERIALS AND METHODS

Fly stocks and genetic screens: rb^{P2} was generated in this study by γ-irradiation. $Df(1)ovo^{D1rG7}$ was obtained from Dr. A. P. Mahowald (Chicago), rb^{10}, $Df(1)rb^{1}$, $Df(1)rb^{46}$, $In(1)rb^{D1}$, and *Dp(1;2)A1125* from Dr. W. L. Pak (Purdue University), and rb^{S1} and *bi rb cx* from the Mid-American Drosophila Stock Center (Bowling Green, OH). The cytological breakpoints of the deficiencies are listed in Pflugfelder *et al.* (1990). *Dp(1;2)A1125*, also known as $Tp(1;2)rb^{+72g}$ (Lindsley and Zimm 1992), covers the chromosomal region from 3F3 to 5E8. rb^{10} contains a second site lethality not covered by *Dp(1;2)A1125* (Poeck 1992).

Molecular methods: Southern and Northern blot analysis, cloning, and sequencing were performed as described (Pflugfelder *et al.* 1990). cDNAs were isolated from a head cDNA λgt11 library (Zinsmaier *et al.* 1990). More recent sequence analysis employed an ABI Prism Dye terminator cycle sequencing kit (Perkin-Elmer, Norwalk, CT) and an automated ABI 373 sequencer.

***In situ* hybridization:** Frozen sections of pupal and adult tissue were fixed and hybridized as described in Poeck *et al.* (1993). For whole mounts, adult brains were dissected on ice in phosphate-buffered saline (PBS, pH 7.5) and fixed in 4% paraformaldehyde/PBS overnight at 4°. Hybridization was carried out as above in a volume of 50 μl. Whole mounts were stained for 8–16 hr, dehydrated in an ethanol series, and embedded in Epon for 1-μm serial sections.

Tissue sections for light and electron microscopy: Adult heads were prepared for light and electron microscopy as described in Renfranz and Benzer (1989). For light microscopy, 1-μm serial sections were cut and stained with 1% toluidine blue in 1% Borax. Ultrathin Epon plastic sections were poststained with 2% uranyl acetate, followed by Reynolds' lead citrate, and stabilized for transmission electron microscopy by carbon coating. Examination was done with a Philips 201C electron microscope.

Eye pigment spectroscopy: Eye pigments were extracted using a scaled-down variant of the method of Real *et al.* (1985). For the determination of red pigments (drosopterins), 15 longitudinally split heads of male flies were used. For the extraction of brown pigments (xanthommatins), 100 male heads were homogenized. Spectra were measured in a Beckman (Fullerton, CA) DU40 spectrophotometer.

Behavioral analysis: Optomotor response of fixed flies walking on a styrofoam ball, which was floating on an air jet, was determined as in Buchner (1976) (24° pattern wave length, 1 Hz pattern frequency, light intensity 50 lux). The landing response was tested in suspended flight with a spiral stimulus (Braitenberg and Taddei-Ferretti 1966). Fixation in the walking mode was measured by releasing wing-clipped flies into the middle of an illuminated arena (29 cm diameter) that contained an upright and an inverted "T" at opposite sides of the vertical wall (line width 10°, height from bottom 19°, light intensity 3000 lux). The angle of initial approach to these visual stimuli was measured (Wehner 1972). In Buridan's paradigm (Götz 1980), individual flies walked spontaneously between two opposing inaccessible landmarks (vertical black stripes on an otherwise uniformly white surround) on an elevated circular disk of 85 mm diameter surrounded by a water-filled moat. Wings were shortened to about one-third of their normal length under cold anesthesia (4°). At least 4 hr of postoperative recovery time with access to water were given prior to testing. Fly positions were recorded at 5 Hz sampling rate with a video scanning device (Strauss and Pichler 1998).

RESULTS

Genetic and molecular characterization of the *ruby* gene: *ruby* was mapped to cytological position 4C6 proximal to the gene *optomotor-blind* (*omb*) (Banga *et al.* 1986; Pflugfelder *et al.* 1990; Lindsley and Zimm 1992). Essential parts of the *ruby* gene were revealed by the 8.5-kb overlap of the deficiencies $Df(1)rb^{46}$ and $Df(1)ovo^{D1rG7}$ (Figure 1). Flies transheterozygous for these deficiencies were viable and had ruby-colored eyes (Poeck 1992). Probes from the 8.5-kb interval detected two transcripts of 5.4 and 5.2 kb in length in Northern blots (Figure 2). Both transcripts were confined to a 6-kb interval within the deficiency overlap, indicating the lack of large introns. The smaller transcript was only expressed in adult females and in early embryonic stages and is con-

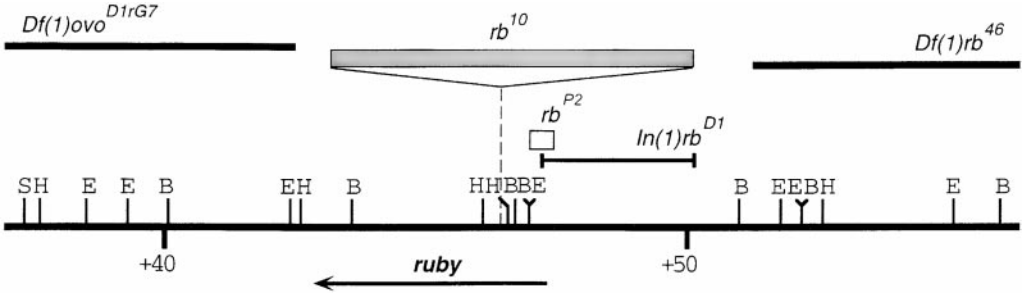

Figure 1.—Genetic map of the *ruby* locus. The breakpoints of *Df(1)rb*^{46} and *Df(1)ovo*^{D1rG7} that uncover rb are shown. The drawn lines correspond to the remaining DNA. Distal is left. The zero origin of the molecular scale is the distal breakpoint of *In(1)omb*^{H31} (Pflugfelder *et al.* 1990). Restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I are symbolized by B, E, H, and S. Position and size of the 7-kb insertion *rb*^{10} and of the small deletion *rb*^{P2} are as indicated. For *In(1)rb*^{D1}, the width of the symbol depicts the interval in which the breakpoint could be localized by Southern analysis. The position of the *rb* transcription unit is shown below.

sidered a maternal variant. Both transcripts were eliminated in *rb* mutant flies *trans*-heterozygous for *Df(1)rb*^{1} and *rb*^{10} [*Df(1)rb*^{1} eliminates the entire region and *rb*^{10} carries a 7-kb insertion in the *rb* transcription unit]. This allele combination is, therefore, null for *rb* function. Transcript size was slightly reduced in *rb*^{P2} flies (data not shown). A 4.2-kb cDNA (pcD4.2) and several smaller cDNAs were isolated from a head cDNA library (Roth 1991). Use of pcD4.2 as a Northern blot probe confirmed the identity of this transcription unit as the *rb* gene (Figure 2). *ruby* transcript abundance was relatively uniform throughout development. The apparent modulation of signal intensity in Figure 2A was mainly caused by unequal RNA load (compare Figure 2B). The direction of transcription was determined as proximal to distal by comparative restriction mapping of genomic and cDNA clones.

Sequencing of pcD4.2 revealed a 1071-amino-acid (aa) open reading frame (ORF) open at the 5′ end. A search of the computer database showed strong sequence homology over the entire ORF to the human β3A and β3B adaptins and indicated that ~90 aa might still be lacking from the N terminus. Sequencing of genomic DNA upstream of pcD4.2 showed that the ORF extended for another 89 codons, including 6 ATG codons. A 5′ expressed sequence tag clone (Berkeley Drosophila Genome Project GH25915) extended our cDNA by 544 bp and confirmed the continuation of the ORF. On the basis of the sequence homology between the ORFs of *ruby*, β3A, and β3B that extended all the way to the first start codon of the *ruby* ORF and the good fit of the first initiation codon environment to the Cavener consensus (Cavener 1987), we assume that *ruby* is translated from the first AUG codon to yield a 1160-aa protein (Figure 3). A genomic sequence encompassing the *ruby* ORF submitted by Zhang and Broadie under accession no. AJ011778 differs in only three (silent) positions from pcD4.2. Our cDNA extends 629 bp beyond the 3′ end of the AJ01178 sequence and includes a polyA tail preceded by several potential polyadenylation signals, indicating the presence of a 1029-bp 3′ untranslated region. Sequence identity between RUBY and human β3A and β3B is 51 and 54%, respectively, and extends over the entire length of the proteins (Figure 3). In the N-terminal two-thirds (trunk domain) sequence identity is 66 and 70%, respectively. At the beginning of the C-terminal domain, the sequence LLDLDD (aa 875–880 in RUBY), which has been identified as a clathrin-binding motif (Dell'Angelica *et al.* 1998), is fully conserved. Sequence similarity to the mammalian β1, β2, and β4 proteins is much lower and is obvious only in parts of the N-terminal trunk domain. RUBY is similarly related to the rat β2 and to the Drosophila β1/2-type adaptin BAD-1 (Camidge and Pearse 1994) (36% in 572 aa of the N-terminal domain).

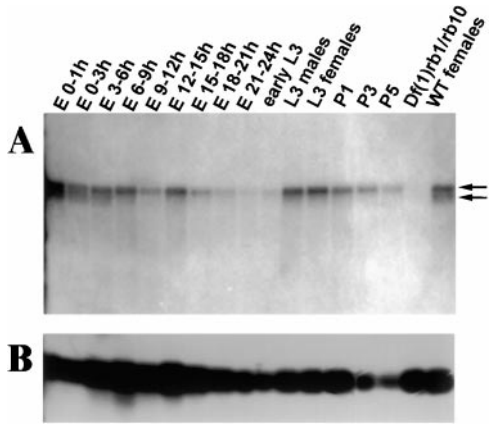

Figure 2.—Northern blot analysis of *rb* expression. A total of 10 μg of poly(A)$^{+}$ RNA was loaded in each lane. Developmental stages are indicated above. The genotype is wild-type Berlin except in lane labeled [*Df(1)rb*^{1}/*rb*^{10}]. Hybridization probe in A was pcD4.2, in B as loading control a cDNA clone encoding ribosomal protein 49 (O'Connell and Rosbash 1984). Arrows indicate the two transcripts of 5.4 and 5.2 kb. For the embryonic RNA preparations (E) the time interval in hours after egg laying is noted. L3 is third larval instar; P1, P3, and P5 are days of pupal development (all at 25°).

***ruby* expression in the developing retina and central nervous system:** For the spatial analysis of the *rb* expression pattern, whole mount and cryostat head sections of stage 13 pupae (Bainbridge and Bownes 1981) and adults were analyzed by *in situ* hybridization. In the pupal eye/lamina complex, *rb* was strongly expressed in a distal zone of the retina extending up to the cornea

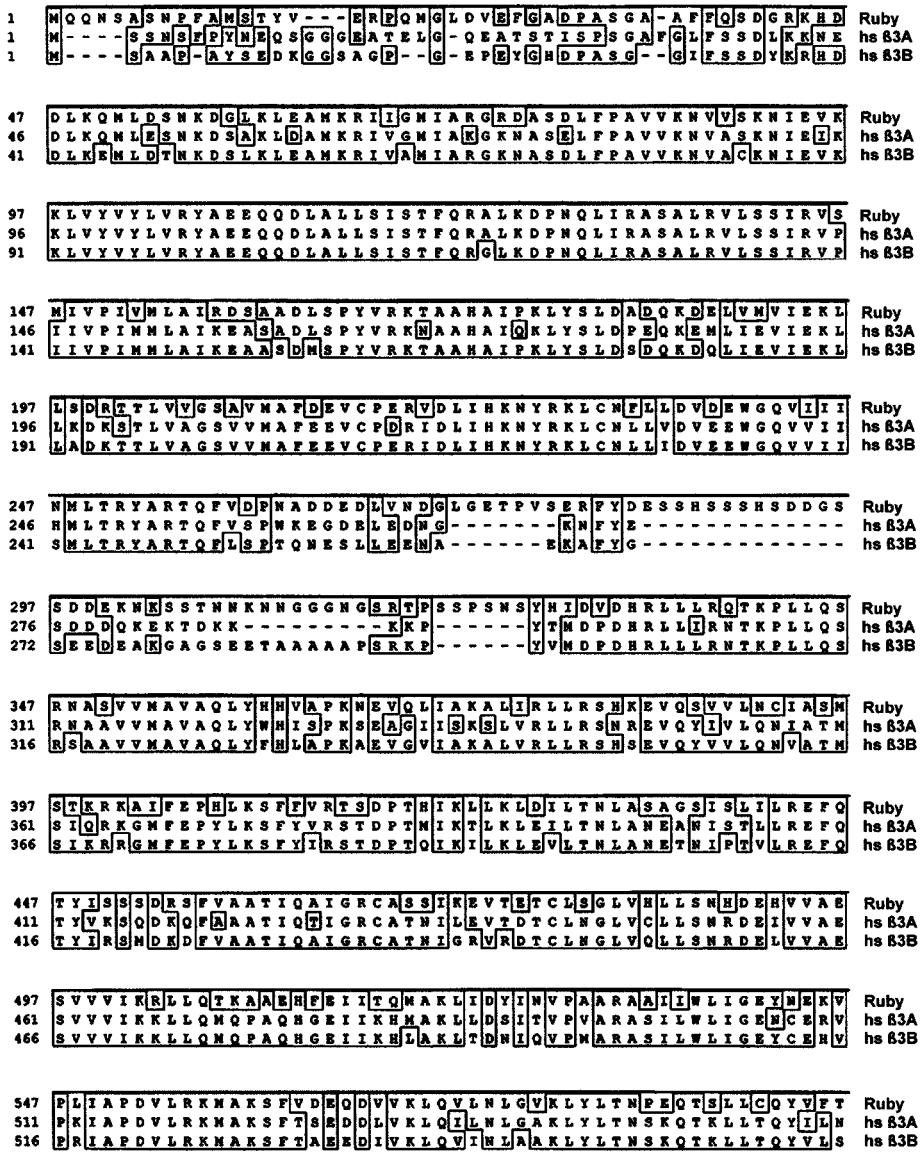

Figure 3.—Predicted amino acid sequence of RUBY (accession no. AF-247194) and the human β3A and β3B proteins (accession nos. U81504 and AF022152). Sequence alignment was performed with the program MegAlign from DNAStar; identical amino acids are boxed.

corresponding to the cell body position of the retinal pigment and outer photoreceptor cells (Figure 4, A and C). Expression in the photoreceptor cells was also visualized by the staining pattern of tangential eye sections, which in each ommatidium outlines the outer rhabdomeres (Figure 4E). In addition, we found expression in the cortex of the entire pupal brain (Figure 4A). This cortical expression declined with pupal maturation and was no longer found in the adult (Figure 4G). This decreasing cortical expression is not an artifact of low probe accessibilty to the adult brain cortex because probes for a less abundantly transcribed gene yield detectable signal under the same conditions (Poeck *et al.* 1993). In the adult visual system, *rb* expression was found in a layer of cells covering the retina basolaterally and within the retina (Figure 4G). The distal staining ensheathed the pseudocone and showed tapering extensions toward the basement membrane at ommatidial periodicity, indicative of expression in pigment cells (Figure 4H). Plastic sections of whole-mount stained head preparations identified the proximal expression in cells lying directly underneath the basement membrane, known as the fenestrated-layer glia (Saint Marie and Carlson 1983) or subretinal glial cells (Cagan and Ready 1989) (Figure 4I).

We investigated the cell autonomy of *rb* function by inducing *rb* mutant eye spots by X-ray irradiating *ruby*/*white* larvae (Stern 1936). Ruby-colored and white twin spots were generated at comparable frequency and appeared not to differ in size (data not shown). While we could not determine *rb* genotype independent of ruby phenotype, the experiment, nonetheless, suggests a cell-autonomous requirement of *rb* function for normal eye pigmentation.

Reduced number of pigment granules and altered retina-brain interface in *ruby*: In the adult fly visual system, pigment granules were described in the retinula cells, the cone cells, the primary, secondary, and tertiary

Figure 3.—(*Continued*).

pigment cells, and in the subretinal glial cells (Cagan and Ready 1989). We have investigated the influence of rb^{S1} on pigment granule number at two levels of the retinal/subretinal complex. The eye color of rb^{S1} or rb^{1} did not differ from that of the presumptive null allele combination $Df(1)rb^{1}/rb^{10}$. rb^{S1} therefore can be considered as null or strongly hypomorphic for pigmentation function. Tangential sections through the eye below the level of the primary pigment cells and above the cone cell endfeet revealed an about twofold reduction in the number of pigment granules in secondary and tertiary pigment cells (Figure 5, A–D). At light microscopic resolution, only the large granules of the pigment cells can be recognized (Stark and Sapp 1988). Below the basement membrane, the reduction in granule number was more than threefold. The mutant granules appeared to have a larger diameter than wild type. The thickness of the pigment containing the fenestrated layer was reduced by ~40% (Figure 5, E and F).

Both red (drosopterins) and brown pigments (xanthommatins) were reduced in *ruby*. In rb^{1} we found a reduction in drosopterin content to 25% of the wild-type level. The drosopterin content was similarly reduced in the g^{1} allele of *garnet* (which encodes the δ subunit of Drosophila AP-3). In the rb^{1} g^{1} double mutant drosopterin content was reduced to 18%. Xanthommatin content was less strongly affected. The single mutants rb^{1} and g^{1} showed a uniform reduction to 58%, the double mutant to 41% of the wild-type value (Figure 6).

Behavioral defects of *ruby* mutants: In *rb* we observed a reduction in thickness of the fenestrated glia layer. It has been shown in other mutants that glial function is necessary for correct development and maintenance of the fly's visual system (Saint Marie and Carlson 1983; Xiong and Montell 1995). Because the brain structure of *ruby* mutants at the resolution of 1-μm serial plastic sections appeared normal otherwise, we conducted an analysis of behavior, which is a more sensitive way to

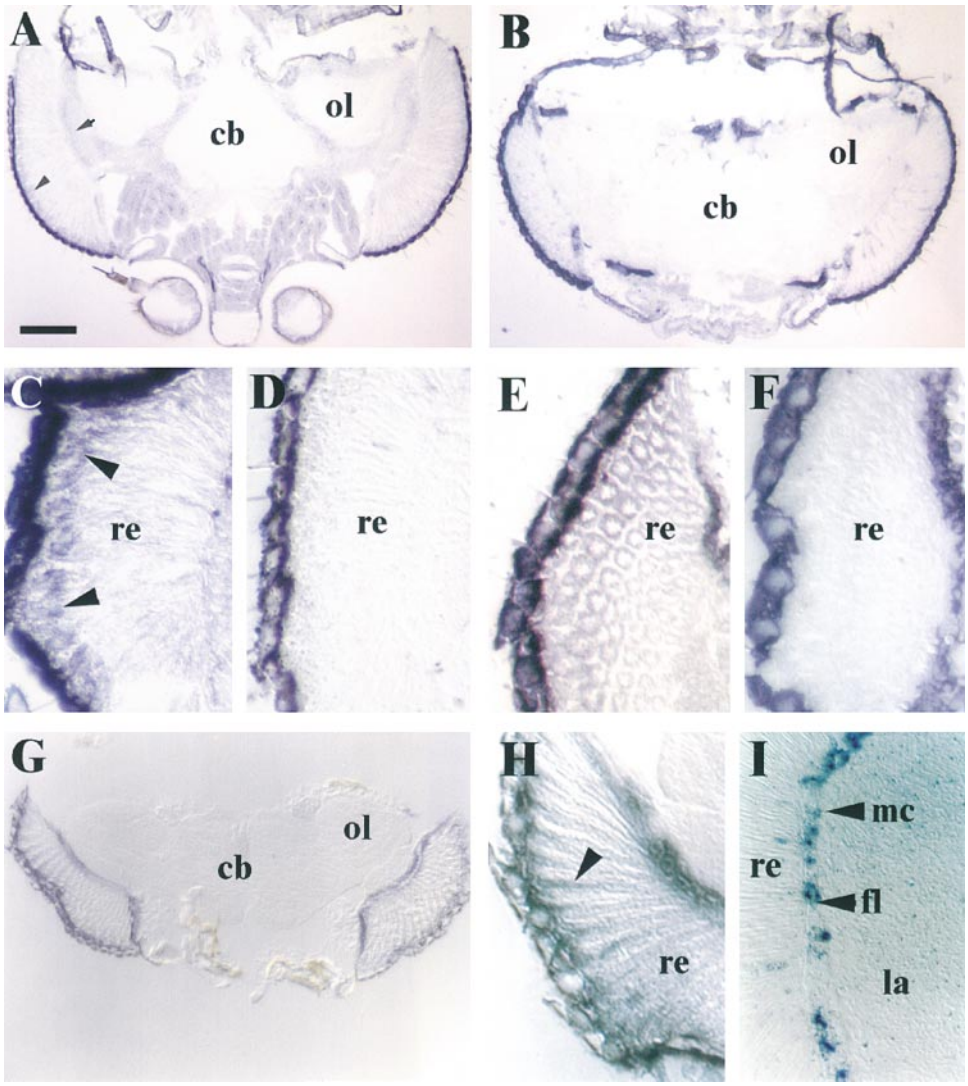

Figure 4.—*ruby* expression. Hybridization with sense and antisense RNA probes derived from pcD4.2 was performed on frontal cryostat sections (A–H) and on whole mounts (I). The latter were subsequently Epon-embedded and sectioned. Hybridization in A, C, E, G, H, and I was performed with antisense; in B, D, and F, with sense RNA. Both probes caused a strong background signal over the cornea tissue (compare A and B). (A–F) *rb* expression in the visual system of stage 13 pupae. A general *rb* expression was found in the entire brain cortex. The strongest signal was detectable in a layer of cells between the optic lobes and the retina (arrow in A) and in the distal retina (arrowheads in A and C). In the tangential section in E and F, subsequent ommatidial rows are cut at an increasingly proximal level (also evidenced by their decreasing diameter), revealing *rb* transcripts in the direct vicinity of the rhabdomeres. In the adult visual system, expression is most prominent in the pigment cells ensheathing the ommatidia (arrowhead in H) and around the basement membrane that separates the retina from the underlying brain tissue (G–I). The higher magnification in I shows hybridization predominantly brain-proximal to the basement membrane. Bar in A, 100 μm in A and B; in C–F and H, 50 μm; in G, 90 μm; in I, 30 μm. cb, central brain; ol, optic lobes; re, retina; la, lamina; mc, monopolar cells; fl, fenestrated layer.

detect brain malfunction. Four allelic combinations of *rb* were tested [$In(1)rb^{D1}/rb^{S1}$, rb^{10}/rb^{S1}, $Df(1)rb^{1}/rb^{S1}$, and $rb^{10}/Df(1)rb^{1}$] and responded normally to moving (landing and optomotor response) and stationary visual stimuli (pattern orientation; data not shown). The same mutants showed, however, abnormal behavior in Buridan's paradigm, a test for alternating pattern fixation. Fixation of visual landmarks was significantly impaired in *rb* mutants. Unlike that in blind flies, this behavior was not random in *rb*. *ruby* mutations also caused a highly significant increase in walking speed between the landmarks [17.6 +/− 0.9 (*rb*) *vs.* 13.4 +/− 0.5 (control) cm/sec; $P < 0.001$]. Importantly, introduction of the duplication *Dp(1;2)A1125* into the *rb* mutant background rescued both the eye color and the behavioral defects (Figure 7). The one tested *g* allele g^{1} was similarly disturbed in both orientation behavior and walking speed (data not shown). As will be argued below, the altered eye pigmentation is unlikely to account for the highly specific behavioral phenotype.

DISCUSSION

We have determined by genetic and molecular means the identity of the Drosophila *ruby* gene. High sequence homology throughout the protein coding region to the mammalian β3 adaptins and phenotypic similarity to mammalian β3A mutants suggests that *ruby* encodes a functional β3 adaptin homolog. In humans, two β3 variants with 61% aa sequence identity were described. The β1 and β2 adaptins constitute another pair of related proteins (83% aa sequence identity) (Dell'Angelica *et al.* 1997b; Simpson *et al.* 1997). Sequence conservation between the three human gene groups β1/2, β3A/B, and β4 lies between 21 and 28% aa sequence identity (Dell'Angelica *et al.* 1999b). RUBY is about equidis-

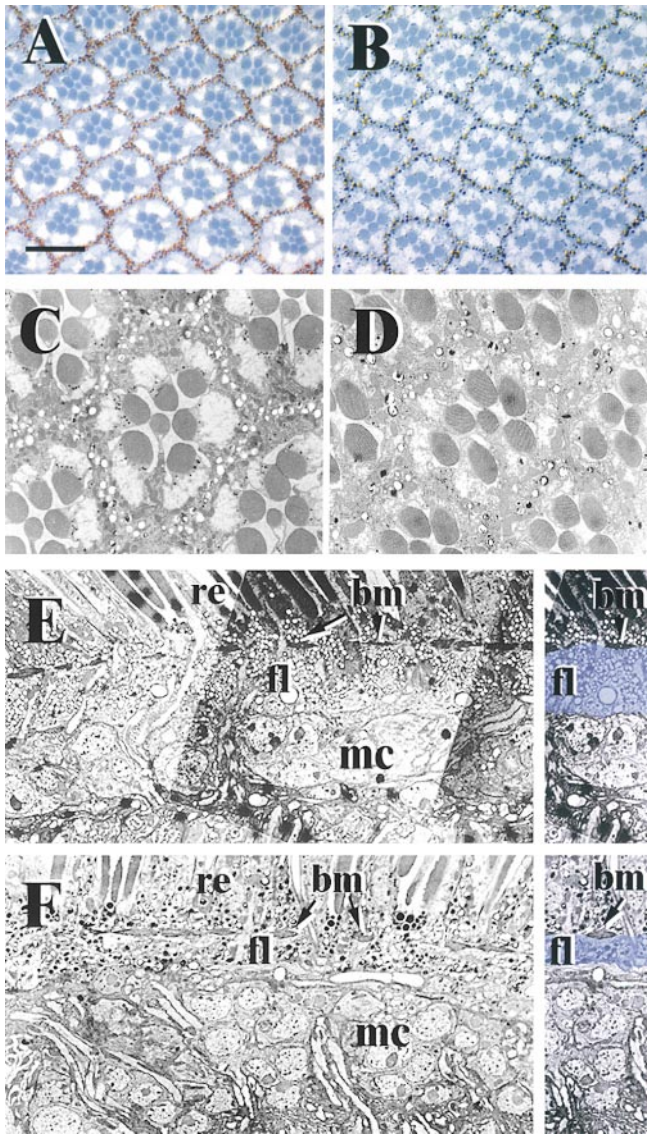

Figure 5.—*ruby* influence on pigment granule levels in the retina (wild type in A, C, and E; rb^{S1} in B, D, and F). (A and B) Tangential sections of Epon-embedded eyes stained with toluidine blue for light microscopic visualization. At the level of sectioning, the pigment granules of the secondary and tertiary pigment cells can be seen to encircle each ommatidium. (C and D) Electron micrographs of ommatidial cross sections. The number of pigment granules is reduced about twofold in rb^{S1}; their size appears to be slightly enlarged. (E and F) Pigment granules in subretinal glial cells. Ultrathin sections from plastic-embedded heads were chosen in which the plain of section lay approximately orthogonal to the plain of the basement membrane (arrows). Similar to the findings in ommatidia, there are fewer but larger pigment granules in the fenestrated layer. The width of the fenestrated layer between the basement membrane and the cellular cortex of the lamina monopolar cells is reduced. In the electron microscopic preparations, fixation and sectioning often led to loss of the black pigment granule content that therefore generally appears as white holes. Bar (in A), 5 μm in A and B; 2.5 μm in C and D; and 1 μm in E and F. re, retina; bm, basement membrane; fl, fenestrated layer; mc, monopolar cells.

tant to human β3A and β3B (51 and 54%), but is clearly more closely related to these proteins than to β1/2 or β4 where sequence similarity is confined to N-terminal regions. Another Drosophila AP-β protein has previously been described (BAD-1, β-adaptin-Drosophila; Camidge and Pearse 1994). Its sequence is equidistant to human β1 and β2 (73% homology). We found no evidence (from low stringency Southern hybridizations or from the sequence database) for the existence of more β3-related genes in Drosophila. It appears, therefore, that the evolutionary split into β1 and β2 and into β3A and β3B took place after the separation of protostomes and deuterostomes and only in the deuterostome lineage. RUBY contains a putative clathrin-binding motif. In Drosophila, several mutations in clathrin heavy chain gene were described (Bazinet *et al.* 1993). The significance of the clathrin-binding motif can now be assessed in transgenic animals expressing mutant versions of the *rb* gene and by testing for genetic interactions.

ruby is transcribed throughout development. Two transcripts differing only slightly in size were found in young embryos and in adult females. The smaller transcript appears to be a purely maternal form, not found in later embryonic stages or in adult males. Surprisingly, the larger transcript also was found during the first hour of embryonic development before the onset of zygotic transcription. In contrast to *garnet* where female sterility and to *carmine* where lethality was reported for some alleles, all *rb* mutants and allele combinations were fertile. *garnet* also differs from *rb* in that strong alleles have an orange eye color (Lindsley and Zimm 1992).

If all subunits of a multiprotein complex were equally necessary for its function, the phenotype of mutants in all subunit genes should be the same. This appears to be the case for yeast AP-3 (Cowles *et al.* 1997; Panek and Stepp 1997). The difference in the severity of strong rb, g and cm phenotypes could mean that β3, δ, and μ3 differ in the degree with which their function can be substituted by related proteins. There are observations that support this hypothesis. In mammalian AP-3 subunits, a higher constraint on δ *vs.* β3 function is evident from the sequence conservation between mouse and humans: 98% for δ and 88% for β3A (Kantheti *et al.* 1998; Feng *et al.* 1999). Mutation of AP-3δ in the *mocha* mouse leads to a nearly complete loss of the three other subunits of the complex; mutation of AP-3βA in the *pearl* mouse has a more subtle effect on the complementary subunits (Kantheti *et al.* 1998). In transfected Cos cells, the β1/2-type Drosophila BAD-1 protein can be accommodated in AP-1 and AP-2 complexes without disturbing their targeting to the TGN or plasma membrane (Camidge and Pearse 1994). Similarly, in the mammalian AP-3 complex, one δ subunit can interact with different isoforms of β, μ, and σ (Odorizzi *et al.* 1998).

Eye pigmentation in Drosophila proceeds through

many steps. Low-molecular-weight pigment precursors are synthesized in the fat body and Malpighian tubules and are taken up by the future pigmented cells during pupal development. Precursor transport across the pigment cell membrane is accomplished by heteromeric ABC transporters encoded by *white*, *brown*, and *scarlet* (Dreesen *et al.* 1988; Tearle *et al.* 1989). In contrast to mutations in genes encoding precursor-synthesizing enzymes, mutations affecting uptake functions or pigment granule biogenesis are autonomous. Autonomy of the ruby eye color phenotype is suggested by our mosaic experiments, by the existence of the variegating allele rb^{m48}, and by the pigment autonomy of *rb* eye disks transplanted into wild-type hosts (Beadle and Ephrussi 1936; Lindsley and Zimm 1992). *ruby* reduces the level of both types of eye pigments, the drosopterins and ommochromes (Nolte 1950, 1952; our data). This property is shared by many of the more than 85 eye pigmentation mutants known in Drosophila, most of which have not yet been cloned. In addition to *rb*, the sequence and mutant phenotype of four pigmentation genes currently suggests their involvement in vesicle transport: *garnet*, *deep orange*, *light*, and *carnation* (Lloyd *et al.* 1998; Sevrioukov *et al.* 1999). *carnation* was recently shown to be the constituent of a protein complex unrelated to AP but also involved in protein transport to lysosomes and pigment granules (Sevrioukov *et al.* 1999). *carnation*, *carmine*, *g*, and *rb* were collectively termed "*ruby* group" based on the similarity of their pigmentation phenotype (Nolte 1952). *Carmine*, *g*, and *rb* have by now been identified as genes encoding AP-3 components. Since *carnation* does not encode the σ3 subunit, mutations in the σ3 gene will probably differ in eye phenotype from the members of the "ruby group."

Allele combinations null for *rb* function still contain pigment granules and have ruby-colored eyes. This can be explained by various hypotheses. As discussed above, Drosophila β-type adaptin function may be partially redundant. Another possibility is that alternative pathways for pigment granule biogenesis can be activated in *rb* or are constitutively active at a low level. This is in agreement with observations in yeast, where inactivation of AP-3 only partially eliminates transport of alkaline phosphatase to the vacuole (Cowles *et al.* 1997; Stepp *et al.* 1997). Lysosomes and melanosomes can receive proteins through both the biosynthetic route from the TGN and via endocytosis from the plasma membrane (Lloyd *et al.* 1998). Inactivation of AP-3 by mutations in β3A or with antisense oligonucleotides leads to an increased expression of lysosomal proteins on the surface of mammalian cells (LeBorgne and Hoflack 1998; Dell'Angelica *et al.* 1999a). A similar effect was observed in AP-3 mutants in yeast (Cowles *et al.* 1997; Stepp *et al.* 1997). The coexistence of alternative pathways appears to be a general characteristic of membrane trafficking (Mostov *et al.* 1999). Our observation of an increased granule size in *rb* (Figure 5) may be an indication of an alternative biogenic pathway. Similar observations have previously been made in several Drosophila pigmentation mutants, including *rb* (Nolte 1950; Shoup 1966; Stark and Sapp 1988). Redundancy at the β-adaptin or pathway level also appears to exist in the mouse where the *pearl* and *mocha* mutants have dilute, but not albino, pigmentation (Kantheti *et al.* 1998). As in Drosophila, altered melanosome size or shape was observed in several mouse pigmentation mutants, including the two HPS models *pearl* and *pale ear* (Mangini *et al.* 1985; Gardner *et al.* 1997).

In *rb*, the thickness of the pigmented layer underneath the retinal basement membrane was found to be reduced (Figure 5). Similar findings were described by Nolte (1950) at the resolution of light microscopy for *rb* and several other pigment mutants. Since pigment granules appear to take up a considerable fraction of

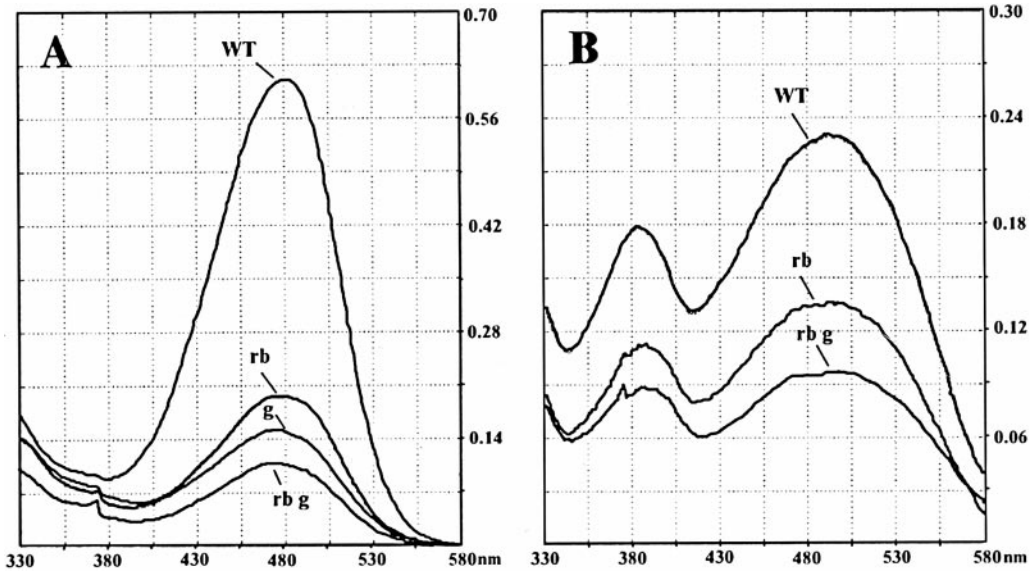

Figure 6.—Spectral analyis of pigment content. Drosopterin (A) and xanthommatin (B) were extracted from heads of flies reared under identical conditions. The genotypes are indicated next to the curves. The small deflection in the curves below 380 nm is caused by the ultraviolet to visible wavelengths transition of the spectrophotometer. In B, the curves for rb^1 and g^1 were nearly superimposable. Only the *rb* curve is therefore shown. WT, wild type; rb, *ruby*; g, *garnet*.

the cellular volume, the volume reduction could be a consequence of the reduction in granule number. There are similarities in the structural organization of the interfaces between the vertebrate photoreceptor cells and the retinal pigment epithelium and between the dipteran fly retina and the optic lobes. In both cases pigmented glia cells extend apical processes in close contact with the photoreceptor cells (Saint Marie and Carlson 1983; Bok 1985; Williams *et al.* 1985). The pigment granules/melanosomes in these glial cells appear to be similarly affected in the *pearl* mouse and the *rb* fly. They are reduced in number and increased in size (Mangini *et al.* 1985; Williams *et al.* 1985).

pearl mice show a defective optokinetic nystagmus, a defect *pearl* shares (with variation) with several mouse hypopigmentation mutants. This defect appears to correlate with a reduced numerical density of melanosomes in the retinal pigment epithelium. *pearl*, like many other hypopigmented mammalian mutants, displays a reduced ipsilateral retinal projection, the developmental origins of which are not yet clear (Marcus *et al.* 1996). We did not observe pathfinding defects in the visual system of *rb* flies. Indeed, *ruby* flies responded normally to moving visual stimuli (optomotor and landing response). They were, however, abnormal in alternating pattern fixation in Buridan's paradigm. In a different pattern fixation paradigm, in which only the first target approach was evaluated, *rb* flies performed normally. In agreement with previous investigations of various eye color mutants (Hengstenberg and Götz 1967), the normal performance of *rb* mutants in several visual tests indicates that the altered eye pigmentation has little effect on the processing of visual signals in the distal periphery of the visual system. The pattern fixation test in which *rb* flies performed normally requires a slightly higher visual acuity than Buridan's paradigm, indicating that the behavioral defect in the latter is not caused by an influence of eye pigmentation on visual acuity. This suggests that the development or function of a higher coordination center is disturbed in *rb*. The two behavioral defects, affecting orientation and walking speed, may map to different foci. Intriguingly, in a *mocha* mouse mutant an altered neocortical activity was observed (Noebels and Sidman 1989). Our *in situ* hybridization data showed *rb* expression in the central nervous system (CNS) cortex during pupal development but not in the

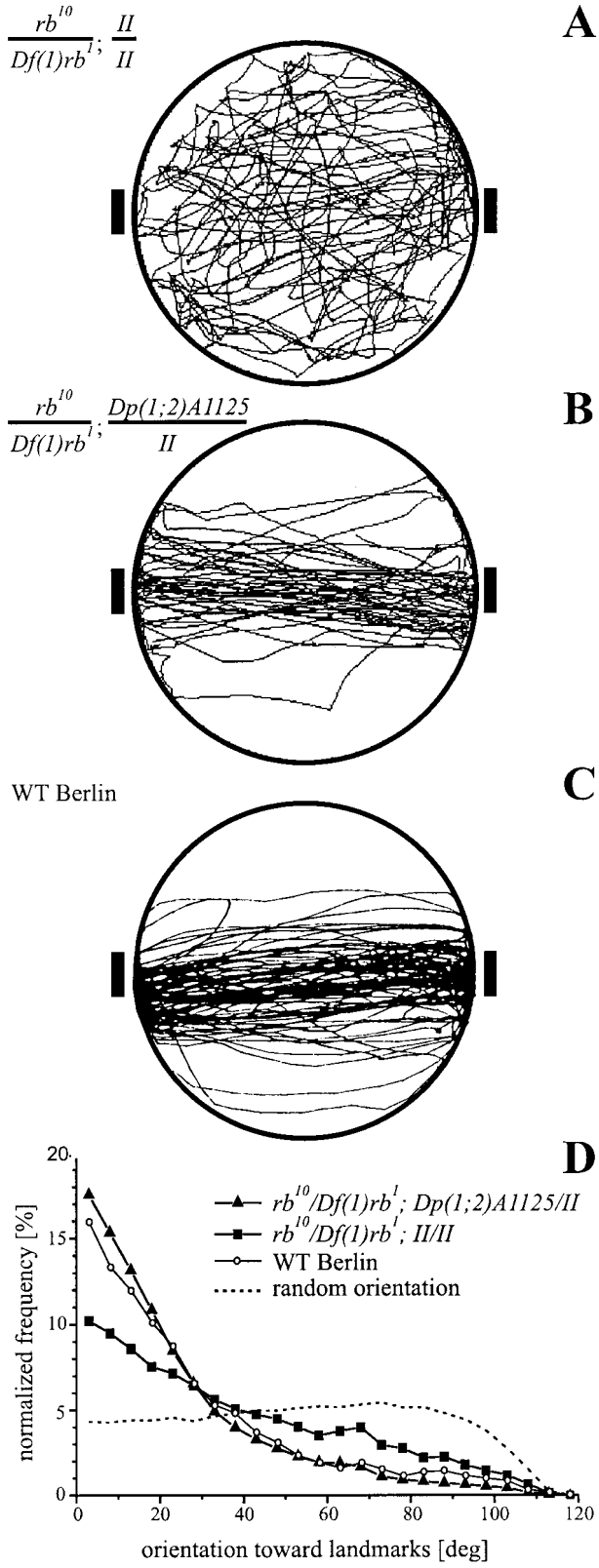

Figure 7.—Behavior analysis in Buridan's paradigm. (A–C) Traces of single flies walking for 15 min between inacessible visual landmarks (symbolized by solid bars flanking the circles). The genotypes are as indicated. In B, the rb phenotype is rescued by the duplication *Dp(1;2)A1125*. The wild-type trace in C is taken from Strauss and Pichler (1998). (D) Quantitative evaluation of 12 flies for each genotype. The angle of orientation between fly and approached target was measured every 0.2 sec (4500 recordings per fly). Angles between 0° and 5°, 5° and 10°, etc., were pooled and plotted against the normalized frequency with which they were observed. The error bars are smaller than the symbols. The difference in orientation behavior between the two genotypes is highly significant (*t*-test, two-tailed, $P < 10^{-4}$). The curve for random orientation was calculated as in Strauss and Pichler (1998).

adult stage. This indicates a developmental role of *rb* in the CNS, rather than a function in neuronal activity. An adult neuronal function is fulfilled by an AP-2-related Drosophila adaptor complex that is involved in synaptic vesicle recycling from the presynaptic cell membrane (González-Gaitan and Jäckle 1997), possibly in conjunction with another clathrin adaptor protein (Zhang *et al.* 1998, 1999). *In vitro* experiments have implied mammalian AP-3 in synaptic vesicle recycling from endosomes (Faundez *et al.* 1998). Whether AP-3 has a synaptic function in Drosophila can now be studied electrophysiologically in mutants of the identified AP-3 subunit genes.

There is indirect evidence for the developmental function of two other Drosophila eye pigmentation genes in the brain. The combination of the two viable mutations *carnation* and *light* leads to synthetic lethality. By mosaic analysis, the focus of this lethal interaction was localized to the brain, which is abnormal in double-mutant larvae (McCarthy 1980). The high penetrance of the aberrant behavior revealed in Buridan's paradigm will allow an investigation of AP-3's neurobiological function. The spatial and temporal requirements of rb function for normal behavior can be determined using Gal4 (Brand and Perrimon 1993) and flippase (Golic and Lindquist 1989) technology.

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