Eyes closed, a *Drosophila* p47 homolog, is essential for photoreceptor morphogenesis

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SUMMARY

Starting with a mutation impacting photoreceptor morphogenesis, we identify here a *Drosophila* gene, *eyes closed* (*eyc*), as a fly homolog of p47, a protein co-factor of the p97 ATPase implicated in membrane fusion. Temporal misexpression of *Eyc* during rhabdomere extension early in pupal life results in inappropriate retention of normally transient adhesions between developing rhabdomeres. Later Eyc misexpression results in endoplasmic reticulum proliferation and inhibits rhodopsin transport to the developing photosensitive membrane. Loss of *Eyc* function results in a lethal failure of nuclear envelope assembly in early zygotic divisions. Phenotypes resulting from *eyc* mutations provide the first in vivo evidence for a role for p47 in membrane biogenesis.

Key words: *Drosophila*, Photoreceptor, Rhabdomere morphogenesis, p47, p97, Membrane fusion, Nuclear envelope

Movies available on-line

INTRODUCTION

Molecular change is a hallmark of membrane differentiation; developing cells deploy distinct protein assemblies in and around the plasma membrane appropriate for the task at hand. Common tasks during development include reorganization of adhesive contacts between cells and the establishment of well-ordered plasma membrane subdomains. Directed membrane traffic is fundamental to such reorganization, but much remains to be learned of how it contributes.

Starting with the identification of a *Drosophila* eye mutant, *eyes closed* (*eye*)1, in which fragmented rhabdomeres engage in inappropriate contact, we have identified a homolog of mammalian p47, a protein associated with membrane fusion. Temporal misexpression of *Eyc* in developing pupal eyes results in inappropriate retention of adhesive contacts that mediate rhabdomere extension, an early determinant of photoreceptor morphogenesis, and failure to subdivide photoreceptor apical membranes into the normal center-surround domains of the photosensitive rhabdomere and its surrounding stalk domain. Loss of *eye* results in lethal failure of nuclear envelope assembly in early zygotic divisions, a novel role for p47 in cell cycle regulated membrane fusion in higher eukaryotic cells. Phenotypes resulting from *eye* mutations provide the first in vivo evidence for a role for p47 in membrane biogenesis and development.

Membrane fusion is fundamental to virtually all aspects of cell physiology, including vesicle-mediated transport through the secretory pathway and the postmitotic reconstitution of Golgi, endoplasmic reticulum (ER) and nuclear membranes from mitotic vesicles (Guo et al., 2000; Mellman and Warren, 2000; Rothman and Warren, 1994). Substantial evidence suggests fusion is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) integral membrane proteins, assisted by a host of additional cytoplasmic proteins, including two ATPases, NSF (N-ethylmaleimide-sensitive factor) (Block et al., 1988) and p97 (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995). These ATPases disassemble unproductive cis-SNARE complexes, priming them for fusion by making SNAREs available to engage in trans with cognate SNAREs of target membranes and thereby promoting another round of fusion (Mayer et al., 1996; Rabouille et al., 1995). Although exceptions exist, NSF is typically targeted by α-SNAP (soluble NSF attachment protein α) to SNARE complexes that result from heterotypic membrane fusion, while p97 is targeted by p47 to complexes resulting from homotypic membrane fusion (Edwardson, 1998; Mellman, 1995).

p47 was first found complexed with p97 in an in vitro Golgi reassembly assay (Kondo et al., 1997). Loss of p47 results in the failure to reassemble Golgi stacks after their vesiculation during mitosis. Stoichiometry is a crucial determinant of p47/p97 activity; ratios above or below optimal decrease Golgi reassembly (Meyer et al., 1998). Co-operation between NSF and p97 pathways is suggested by the competition of α-SNAP and p47 for a common syntaxin-5 SNARE complex (Rabouille et al., 1998). Both pathways are required to rebuild Golgi cisternae from mitotic Golgi fragments in vitro; alone, each promotes vesicle fusion leading to morphologically distinct cisternae (Acharya et al., 1995; Rabouille et al., 1995). Fusion from mitotic vesicles (Guo et al., 2000; Mellman and Warren, 2000; Rothman and Warren, 1994). Substantial evidence suggests fusion is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) integral membrane proteins, assisted by a host of additional cytoplasmic proteins, including two ATPases, NSF (N-ethylmaleimide-sensitive factor) (Block et al., 1988) and p97 (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995). These ATPases disassemble unproductive cis-SNARE complexes, priming them for fusion by making SNAREs available to engage in trans with cognate SNAREs of target membranes and thereby promoting another round of fusion (Mayer et al., 1996; Rabouille et al., 1995). Although exceptions exist, NSF is typically targeted by α-SNAP (soluble NSF attachment protein α) to SNARE complexes that result from heterotypic membrane fusion, while p97 is targeted by p47 to complexes resulting from homotypic membrane fusion (Edwardson, 1998; Mellman, 1995).

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of ER membranes from yeast mutant for the p97 homolog, CDC48, is inhibited in an in vitro assay (Latterich et al., 1995). Similarly, a rat liver microsome fusion assay also demonstrates a requirement for p97 in ER assembly (Roy et al., 2000). In addition to its role in membrane fusion, p97 also participates in other activities, including ubiquitin-dependent protein degradation, nuclear transport pathways and DNA unwinding (Meyer et al., 2000; Yamada et al., 2000). p97 activity is directed to different cellular pathways by the regulation of its co-factors. A role for p47 has not been described in development and morphogenesis.

Photoreceptor morphogenesis demands a high level of performance by mechanisms mediating directed membrane traffic. Rhabdomeres, like their vertebrate counterparts, the outer segments of rod and cone photoreceptors, are enormously amplified central subdomains of the photoreceptor apical membrane. Rhabdomere growth during pupal life is driven by delivery of copious photosensitive membrane to the developing rhabdomere. Defects in membrane traffic yield rhabdomere defects, which are plain against the precise, stereotyped and sizeable rhabdomeres of normal flies; Drosophila photoreceptor morphogenesis is a sensitive assay of membrane differentiation.

MATERIALS AND METHODS

Fly strains

The fly strain was provided by E. Fyrberg (Johns Hopkins University). In addition to the mutation in fly that encodes the Drosophila α-Actinin, it contains a second mutation affecting eye development. The fly stocks w; Sco/SM1; TM2/MKR5, Sp1P[2]Pin/SMS, Df(2R)P+2, bl Adh* ch* l(2)*+CyO; ry 106, Sp/Cyo; ry 506 Sh P[y+7.2=∆2-3]/TM6, cn P[ry+7.2=ZP];J(2) 04012/Cyo; ry 506 (P1363), P[y+7.2=neoFRT]42D and y w; P[y+7.2=neoFRT]42D P[y+7.7 ry+2-car20y]A4B P [w=+;GMR-hid] SS2 l(2) CL-R1/Cyo P [w=+;Gal4-e-yH] SS5 P[w+=+; UAS-FLP1.D] J2D (EGUF/hid) were obtained from the Bloomington Stock Center.

Genetics

Flies were raised on standard corn meal-agar media at 25°C. Fly crosses were carried out according to standard procedures. eycl mutant was isolated from the fly strain by crossing fly with w; Sco/SM1; TM2/MKR5. Meiotic mapping of the eyc gene was carried out by crossing eycl to the second chromosome dominant marker strain Sp1P[2]Pin/SMS. Recombination frequency based on deep pseudopupil screening placed the eyc locus in the tip of 2R, very close to Pin (genetic location 2-107.3). Deficiency strains within the mappable region were collected and reared to locate the eyc. One deficiency line, Df(2R)P+2 (break points 60C5-D2), fails to complement eycl. We collected P-element insertion lines, which have been placed in the refined mapping region 60C5-60D2 and found cnP[y+7.2=ZP];J(2) 04012/Cyo; ry 506 (P1363), which maps to 6D01-2 (generated by A. Spradling), failed to complement eycl; P1363/Df(2R) P+2 flies display an eyc-like eye phenotype (Fig. 1D). In order to verify that P1363 is an eyc allele, we remobilized the P-element by crossing P1363 virgins to Sp/Cyo; ry 506 Sh P[y+7.2=∆2-3]/TM6 males. Male progeny cnP[y+7.2=ZP];J(2) 04012/Spcy 506 P[y+7.2=∆2-3] were selected and crossed to b Adh* ch* l(2)*+CyO; ry 506. CyO progeny with eyr- were single paired to Sp/Cyo; ry 506. Sibling CyO, non-Sp progeny were crossed to produce lines homozygous for the P-element excision chromosome. We expected that precise excision lines should generate homozygous viable progeny, but we failed to recover any after screening 250 lines. This is probably due to a deletion we subsequently found in the original P1363 chromosome (see Results). We recombined P1363 onto a wild type background to eliminate this deletion and generated a P1363 homozygous viable line, eycl. We then repeated P-element remobilization. Precise excision of the P-element, determined by PCR assay, restored normal eye morphology. Additional eycl alleles generated by imprecise excision in trans with the original eycl allele show rhabdomeres similar to eycl homozygous.

To generate eycl loss-of-function alleles, the P-element of eycl was remobilized; 27 out of 250 lines screened were homozygous lethal, eycl, which removes the eyc ORF has an intact downstream gene was used as an eyc null allele.

To generate homozygous eyc null eyes, eycl was recombined onto the P[ry+7.2=neoFRT]42D chromosome, and balanced over CyO. When crossed to EGUF/hid flies, flipase activity provided by the EGUF chromosome promotes somatic recombination, homozygosing eycl in the eye (Stowers and Schwarz, 1999). Eye clones were examined among non-Cyo progeny.

Molecular analysis

DNA flanking the P1363 insertion site was isolated by plasmid rescue. Briefly, genomic DNA of adult P1363 flies was isolated, digested using Xbal+NheI or HpaI, religated and transformed into DH5α competent cells (Life Technologies) and selected for kanamycin resistant colonies. Genomic DNA flanking the P-element was recovered, restriction enzyme digested and used as hybridization probes to identify transcripts in wild type (Canton S) and eycl flies. One genomic probe, P3, detected a high level of mRNA expression in eycl mutants which was nearly absent in wild type at about 30% pupal development (p.d.). The P3 probe was used to screen a Atl1 adult head cDNA library (provided by W. Pak; original library constructed by E. Buchner). Twelve clones were isolated and all contained a 1.1 kb DNA fragment. This 1.1 kb cDNA was sequenced on both strands.

For Northern hybridization, poly(A)+mRNA from 0.5g mid-pupal stage flies was prepared by using PolyATrac®System 1000 (Promega), fractionated in a 1.2% agarose/formaldehyde gel, and transferred to HybondTM-N+ positive charged nylon membrane (Amersham). Membranes were hybridized to 32P-labeled genomic DNA probes or cDNA probe according to the manufacturer’s instructions.

Germline transformation

A 1112 bp fragment that covers the eyc ORF was excised from an EcoRl digested cDNA clone and cloned into pUAST to construct pUAST-eyc. Germline transformation was performed according to standard procedures (Spradling and Rubin, 1982). Briefly, w1118 embryos were injected with pUAST-eyc and helper plasmid ∆2-3. Transformants with w+ eye color were mapped and homozygosed by crossing to the balancer w; Sco/SM1; TM2/MKR5.

Electron microscopy and immunohistochemistry

Flies were prepared for transmission electron microscopy as described by Baumann and Walz (Baumann and Walz, 1989), with modifications. Fly eyes were dissected and fixed with aldehyde fixative (2% paraformaldehyde and 1.75% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4). Fixed eyes were then incubated in 1% tannic acid overnight followed by post-fixation in 0.1 M sodium cacodylate with 2% osmium tetroxide for 2 hours. After washing, eyes were incubated in 2% uranyl acetate overnight and dehydrated through an ethanol series. The samples were mounted and sectioned as described by Tomlinson and Ready (Tomlinson and Ready, 1987). Samples were observed using a Philips 300 electron microscope.

Phallolidin and immunostaining were performed with whole-mount preparation as previously described (Chang and Ready, 2000), with modifications. For immunohistochemistry, staged eyes were dissected
and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 1 hour. The brain was removed approximately 30 minutes into fixation. Fixed eyes were then given three 10 minute washes in PBST (PBS plus 0.3% Triton X-100). Washed eyes were incubated in primary antibody: anti-Armadillo (1:50), anti-Rh1 (1:50, both obtained from Hybridoma Bank, the University of Iowa), anti-Crumbs (1:300, generous gift from U. Tepass) (Tepass, 1996), or in 2 μg/ml rhodamine-conjugated phalloidin (Sigma) in PBST plus 5% goat serum overnight at 4°C. For detecting DE-Cadherin, staged eyes were fixed in the fixative contains 1 mM CaCl2, the eyes were stained in 1:20 diluted anti-DE-cadherin antibody (a generous gift from T. Uemura) (Uemura et al., 1996). Eyes were given three 10 minutes washes in PBST and were then incubated for 4 hours at room temperature in secondary antibody. After incubation, eyes were given three 10 minutes PBST washes and mounted (0.25% n-propyl gallate, 50% glycerol in PBS, pH 8.6).

For embryo immunohistochemistry, staged embryos were collected from juice plates. Fixation and hand-devitellinization embryo followed protocols of White (White, 1998). Anti-Lamin antibody was a generous gift from P. Fisher (Smith and Fisher, 1989; Stuurman et al., 1995). YOYO-1 was purchased from Molecular Probes (Eugene) a generous gift from P. Fisher (Smith and Fisher, 1989; Stuurman et al., 1995). Antibody inhibition

Stage 2-3 embryos were collected and prepared for microinjection according to standard procedure. Approximately 5/100 egg volume of serum (~0.6 g/ml protein) was injected into the posterior end of the embryo. After 1 hour’s recovery, injected embryos were prepared for immunohistochemistry.

RESULTS

Genesis of rhabdomere trapezoids

The trapezoid of adult rhabdomeres (Fig. 1A) has its origin early in pupal life with the establishment of a stereotyped set of contacts between photoreceptor apical faces, the future rhabdomeres. While maintaining the zonula adherens (z.a.) junctions formed during pattern formation, inpocketing of photoreceptor apices into the retinal epithelium by closure of the lens-secreting cone cells ‘above’ them, brings photoreceptors ‘face-to-face’ in a trapped apical cavity that is the precursor of the inter-rhabdomeral space (IRS) (see Movie at http://dev.biologists.org/supplemental/). Contacts between photoreceptors R2, R4 and R7 occlude R3 from the center of the ommatidium, displacing its apical membrane to the future ‘point’ of the trapezoid. Between 37% p.d. and 55% p.d., photoreceptor apical surface amplification extends these contacts to the retinal floor, along a ‘core’ of partitions at the center of the ommatidium; photoreceptor apices meet in a manner resembling the sectors of an orange (Fig. 1E,G). During extension, the previously undifferentiated apical membrane is reorganized in center-surround domains dedicated to future development of rhabdomere and stalk membrane. By 55% p.d., face-to-face contacts between photoreceptors are relinquished, opening the IRS (Fig. 1G) (Longley and Ready, 1995).

In order to further characterize contacts between photoreceptor apical faces, we stained developing eyes between 37% p.d. and 55% p.d. using phalloidin and antibodies to the apical membrane protein, Crumbs (Crb) as well as antibodies against Armadillo (Arm, Drosophila β-Catenin) and anti-Drosophila E-cadherin (DE-Cad), proteins associated with adhesive cell-cell contacts that are typically localized to z.a. junctions. Confocal images of wild type 37% p.d. eyes show Crb, Arm and DE-Cad across the entire apical membrane, in addition to strong staining in z.a. junctions (Fig. 2A) (wild type 37% p.d.). By 50% p.d., the stage when apical face contacts are released, Arm and DE-Cad have retreated from the apical surface and stain only the z.a. junctions. These results suggest that the ‘unsticking’ of R cell apical faces by 50% p.d. may be due to reorganization of Arm and DE-Cad in the R cell apical membrane.

Distinct programs of membrane reorganization mark photoreceptor differentiation. Between 55% p.d. and 70% p.d., the apical membrane protein Crb (Tepass, 1996) is relocated from the entire surface (Fig. 2A) to the stalk (U. Tepass and D. Ready, unpublished). This redistribution occurs in eyc mutants.

Isolation of eyc1 and phenotypic characterization

We have found previously that a Drosophila strain, filA4, mutant in α-actinin (Roullet et al., 1992), contains a second mutation that degrades rhabdomere morphogenesis (R. L. Longley, PhD Thesis, Purdue University, 1994). Mutant rhabdomeres are fragmented and inappropriate adhesions join rhabdomeres to rhabdomeres and to stalks of other photoreceptors (Fig. 1B). In occasional planes, all rhabdomeres meet at the ommatidial center, resembling the closed rhabdoms common to most arthropods. We consequently name the gene eyes closed (eyc). It is notable that the external morphology of eyc1 eyes is completely normal (data not shown).

eyc1 photoreceptors are indistinguishable from wild type at 37% p.d.. At this stage, R cell apical membranes contact each other in stereotyped combinations. R2, R4 and R7 contact ‘in front of’ R3, pre-figuring the trapezoid of the adult ommatidium (Fig. 1E,F). The z.a. junctions that join adjacent R cells appear normal. The apical membranes of wild type and mutant R cells are infolded, but do not show regional differentiation.

By 55% p.d., the eyc1 phenotype is plain. The IRS fails to open and R cells contact each other in rhabdomere tip-to-tip and rhabdomere-to-stalk contacts (Fig. 1H). Distinctive stalk and rhabdomere membranes are evident, but they are not organized in a simple center-surround. Islands of trapped stalk make loops in R cell cross-sections. Contacts between R cells
in adult ommatidia commonly include those seen during rhabdomere extension, notably R2/R4/R7; the rhabdomere of R4 frequently bifurcates to contact R2 and R7. Strong R5/R6 contact, not seen between rhabdomeres during extension, is also common in the mutant.

In order to test the hypothesis that persistent contacts between mutant rhabdomeres are due to the inability of R cells to clear adhesive proteins from their apical faces, we examined mutant eyes using phalloidin, anti-Arm, anti-DE-Cad and anti-Crb antibodies. Confocal micrographs of eyc1 eyes show abnormal Arm staining in R cell apical membranes, often in patches localized to abnormal R cell contacts (Fig. 2A) (eyc50% p.d.). DE-Cad staining is similarly localized to abnormal contacts (Fig. 2B). We speculate that eyc1 R cells are unable to reorganize their apical membranes correctly at this crucial stage of morphogenesis.

Despite the severity of the rhabdomere phenotype, eyc1 eyes show the expression of rhodopsin in outer photoreceptors at the appropriate developmental stage; there is no obvious morphological phenotype beyond rhabdomere malformation (data not shown).

**Eyc encodes a Drosophila p47 homolog**

Cloning of the eyc gene was initiated from an eyc P-element allelic P1363, which failed to complement eyc1 (see Materials and Methods). Plasmid rescue of P1363, recovered ~16 kb genomic DNA flanking the P-element, A ~2 kb genomic probe (P3), 3’ to the P-insertion site revealed a ~1.1 kb transcript which is elevated in eyc1 and P1363/Df(2R)Px2 flies relative to wild type at ~30% p.d. (Fig. 3A). Eyc protein levels are likewise elevated relative to wild type at this stage (Fig. 3B).

P3 was then used to screen a Drosophila adult head cDNA library and identified a single class of cDNA clone, representing a transcript of 1.1 kb. This 1112 bp cDNA was sequenced on both strands to obtain the gene sequence. Genomic sequence of a P1 clone (DS02336) indicates eyc is intronless. Conceptual translation of the longest ORF predicts a 353 amino acid protein with 46% similarity and 31% identity extending over the entire protein to rat p47, a co-factor that regulates the activity of the AAA ATPase (Patel and Latterich, 1998), p97. Two additional p47 homologs, human p47 and yeast SHP1 have been reported. A ClustalW multiple sequence alignment of the p47 family is shown in Fig. 3D. In addition to eyc, the Drosophila Genome Project genome predicts a potential p47 homolog, CG11139, localized to 43C4-5; no mutant phenotype or characterization of this gene has been reported.
The site of P-element insertion in P1363 was found 139 bp downstream of the eyc gene stop codon (Fig. 3C). In addition, the P-element also carried a 137 bp DNA fragment from 44D1-D2 placed proximal to eyc; a fragment of undetermined size is located on the other side of P-element. This suggests the original P1363 strain may contain a deletion resulting from a previous imprecise excision and could account for its homozygous lethality. Recombination of P1363 onto a wild-type second chromosome generated a homozygous viable P1363, eycP, ommatidia show the eycI eye phenotype (Fig. 1C). Additionally, this strain shows mild fusion of abdominal tergites.

eyc genomic DNA was examined to determine mutant molecular defects. Three independent clones sequenced on both strands showed no alterations in the eyc ORF. Two point mutations, an A to G transition 162 bp downstream of the stop codon and a single base deletion 486 bp downstream of the stop codon, were found in eyc genomic DNA (Fig. 3C). Since the parental chromosome exposed to ethylmethane sulphate (EMS) mutagenesis is no longer available, it cannot be ruled out these changes are polymorphisms. Nevertheless, the location of these changes and the site of P1363 insertion suggest the observed misexpression of eyc may be due to changes in 3’ regulatory elements, as observed for other genes (Zhou et al., 1999).

That eycI and eycP are recessive appears at odds with a hypothesis suggesting transcript elevation causes the eye phenotype: should they not be dominant? Regulation of transcription by interallelic interactions, known in Drosophila and elsewhere (Lewis, 1954; Henikoff, 1997), could account
for our current observations (Table 1). We consider the possibility that a 3' suppressor sensitive to epigenetic influences contributes to eyc regulation. Analogous to the trans activity of the yellow enhancer (Morris et al., 1998), pairing a wild-type homolog with an eyc mutant may allow recruitment of a suppressor that downregulates transcription of both copies, over-riding decreased binding of a repressive factor by 3' changes in eyc1 and eycP. Further genetic and molecular analysis of eyc will be required to explore this possibility.

**Temporal misexpression of Eyc produces an eyc-like phenotype**

As the temporal misexpression of eyc coincides with the developmental stage at which the rhabdome/stalk organization of the photoreceptor apical surface is established, we hypothesized that Eyc misexpression at this stage might produce an eyc-like eye phenotype. In order to test this, we used the GAL4/UAS system (Brand and Perrimon, 1993) to drive Eyc expression at mid-pupal stages and examined its effect on rhabdome development.

Developmentally staged pupae carrying one copy of UAS-eyc and one copy of Hs-GAL4 were given six cycles of 45 minutes 37°C heat shock and 5 hours 15 minutes 25°C recovery, starting at 30% p.d. Eyes were dissected from freshly eclosed adults and examined using confocal and electron microscopy. We found eyc-like defects in the eyes of heat-

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shocked Hs-GAL4/UAS-eyc flies (Fig. 4A). In the EM, approximately 23% (21/89) of ommatidia showed abnormal contacts between rhabdomeres. Eyc misexpression started later in pupal development did not generate an eyc eye phenotype (data not shown). These results support the hypothesis that the eyc phenotype is due to mid-pupal Eyc misexpression.

More severe defects were observed when heat shocks were continued into later pupal stages; rhabdomeres displayed abnormal adhesions and their size was reduced in almost all R cells (Fig. 4B). Moreover, anti-Rh1 immunostaining using 4C5 showed these animals were also deficient in rhodopsin delivery to outer R cell rhabdomeres. Most contained little or no rhodopsin; instead, rhodopsin was concentrated in the photoreceptor cytoplasm (Fig. 4D). Control Hs-GAL4 flies given the same heat shock regimen did not show a phenotype. Scale bars: in A, 1 μm in A,B; in C, 5 μm in C,D.

Fig. 5. Eyc misexpression increases R cell ER and inhibits rhodopsin delivery to the rhabdomere. In parallel confocal (A,B) and electron microscope (C,D) preparations, Hs-GAL4/UAS-eyc flies (A,C) and Hs-GAL4/+ (B,D) were given three pulses of 1 hour 37°C heat shock with 5 hours 25°C recovery starting at ~70% p.d. After the last recovery, animals were dissected and processed for parallel experimental preparations. For confocal microscopy, retinal whole-mounts were stained using rhodamine-phalloidin (red) and 4C5 anti-Rh1 antibody (green). (A) Eyc overexpression results in rhodopsin accumulation in the cytoplasm and diminished delivery to the rhabdomere. (B) Rhodopsin localization is normal in heat-shocked controls; the central R7 rhabdomere does not express Rh1 and consequently only stains with phalloidin. (C) ER accumulates in photoreceptors overexpressing Eyc. (D) ER is normal in parallel heat-shocked Hs-GAL4/+ controls. Scale bars: in A, 5 μm in A,B; in D, 2 μm in C,D.

induces eyc transgene expression starting immediately behind the furrow in third instar larvae, also generated an eyc-like phenotype (Fig. 4C).

Eyc overexpression results in endoplasmic reticulum expansion

p97 has been shown essential for the budding of vesicles from transitional ER (Zhang et al., 1994) and we explored the possibility that rhodopsin accumulation in the photoreceptor cytoplasm was related to ER defects. We made parallel
preparations for both anti-Rh1 immunostaining (Fig. 5A,B) and electron microscopy (Fig. 5C,D). *Hs-GAL4/UAS-eyc animals received three 1 hour heat shocks separated by 5 hours recovery starting at 70% p.d.; eyes were dissected and prepared for observation at approximately 85% p.d. In accordance with results obtained with earlier heat shocks (Fig. 4D), overexpression of Eyc inhibits Rh1 transport to the rhabdomere (Fig. 5A,B). Using electron microscopy, we found abundant ER accumulation in photoreceptor cytoplasm, which is not seen in control animals (Fig. 5C,D).

In order to quantify this difference, we collected tangential sections approximately 25 μm below the corneal surface and photographed approximately 20 ommatidia. ER stacks in R cells were counted manually on prints. In *Hs-GAL4/+ controls, the majority of R cells had two to three ER stacks, similar to normal *Hs-GAL4/+ eyes (data not shown). In *Hs-GAL4/UAS-eyc flies, R cells showed more ER stacks, ranging from three to 10 stacks (Fig. 6A). We speculate that the cytoplasmic rhodopsin staining observed in the confocal microscope results from disruption of vesicle traffic to the rhabdomere, possibly owing to rhodopsin becoming trapped in the ER.

**Eyc overexpression inhibits rhodopsin delivery**

We investigated the impact of Eyc overexpression on rhodopsin synthesis and maturation. In normal photoreceptors, rhodopsin synthesis and core glycosylation in the ER yields immature forms that are deglycosylated in the Golgi before transport of the mature 35 kDa form to the rhabdomere (Colley et al., 1995). If Eyc overexpression disrupts vesicle-mediated protein trafficking, we might expect an increase in immature rhodopsin in *Hs-GAL4/UAS-eyc eyes. To test this, we heat shocked pupae as before assessed rhodopsin maturation using western blots. Mature rhodopsin levels were decreased in *Hs-GAL4/UAS-eyc retinas relative to *Hs-GAL4 controls (Fig. 6B), consistent with the reduced stain in rhabdomeres observed using immunohistochemistry. Immature, higher molecular weight rhodopsin was also increased relative to mature rhodopsin in these eyes, suggestive of defects in rhodopsin processing, potentially arising from disturbance of intracellular membrane traffic.

**eyc loss-of-function mutations disrupt nuclear envelope assembly**

In order to further define the function of Eyc, we used imprecise excision of P1363 to generate loss-of-function *eyc alleles. We screened 250 lines and recovered 27 homozygous lethal excisions. Embryo development was observed in progeny of 10 balanced excision heterozygotes. In all 10 lines, approximately one quarter of the embryos, later confirmed as lacking the *eyc ORF using PCR, arrested before cellularization. Three of the lines (*eyc120, *eyc127 and *eyc139) were selected for molecular characterization and rescue experiments (below).

As p97 is sensitive to alkylation by N-ethyl-maleimide (NEM), and as in vitro reassembly of *Xenopus* nuclear membrane is NEM sensitive (Macaulay and Forbes, 1996; Marshall and Wilson, 1997), we speculated that p47/Eyc might be essential for nuclear envelope fusion. We stained embryos of *eyc139/Cyo* inter se crosses with an antibody to Lamin, a nuclear envelope protein that has been shown to play an essential role in nuclear envelope assembly (Burke and Gerace, 1986; Dabauvalle et al., 1991; LenzBohme et al., 1997; Lourim and Krohne, 1993; Chaudhary and Courvalin, 1993; Moir et al., 2000; Newport et al., 1990).

During the first half hour after egg deposition, we observed no differences in the nuclear envelopes of embryos from wild-type or *eyc139/Cyo* crosses, suggesting early nuclear division in *eyc* nulls proceeds normally using maternally supplied Eyc (data not shown). At 1.5 to 2 hours, wild-type embryos stained with anti-Lamin show two staining patterns: Lamin is either concentrated around the separated M-phase chromosomes (Fig. 7A), consistent with an association between Lamin and chromatin (Moir et al., 2000), or the interphase nuclear envelope is highlighted (Fig. 7B). In *eyc139/Cyo*-derived progeny, approximately one quarter of the embryos show no nuclear envelopes at the same stage (Fig. 7C); occasional embryos showed Lamin aggregates (Fig. 7D), which may be similar to the annulate lamellae observed in *Drosophila lamin Dmo* (LenzBohme et al., 1997).

Concomitant with loss of the nuclear envelope, the normally dense nuclear DNA seen in zygotic nuclei becomes irregularly fragmented and dispersed. It seems likely that absence of the nuclear envelope results in abnormal chromosome segregation. Probably as a result of destructive mitosis and chromosome dispersal, *eyc* null nuclei do not migrate to the surface of the
embryo and organize the normal hexagonal actomyosin staining pattern at the embryo membrane; cellularization fails in mutant embryos (Fig. 7C).

### Injection of anti-Eyc antiserum in syncytial blastoderm embryos disrupts cell cycle progression

To further examine Eyc loss-of-function phenotypes, we microinjected anti-Eyc antiserum into the posterior of stage 2-3 embryos and observed its effects on nuclear division using confocal microscopy 1 hour after injection (~ stage 4-5). We found a gradient of cortical nuclear organization across injected embryos: nuclei were dense and regularly arrayed at anterior ends; nuclei were sparse and poorly organized at the posterior (Fig. 7F). Occasional clustered nuclei could be found in the affected region. Control embryos injected with an equal volume of pre-immune serum did not show nuclear disarray (Fig. 7E).

Side views reveal disruption of cellularization in the posterior of anti-Eyc injected embryos. In normal stage 4-5 embryos, and in embryos injected with pre-immune serum, syncytial nuclei are closely packed and cylindrical (Fig. 7G). By contrast, the posterior region of anti-Eyc injected embryos show fewer nuclei...
with a spherical shape, presumably a more relaxed shape in the less crowded environment (Fig. 7H). We speculate that the nuclear divisions are slowed in the Eyc-inhibited cytoplasm, perhaps by delays of postmitotic nuclear envelope reassembly.

**Eyc is required for cell viability**

Our results shown above indicate Eyc is fundamental to cell cycle progression during embryogenesis. To determine whether Eyc is generally used at different developmental stages and tissues, we applied the EGUF/hid method (Stowers and Schwarz, 1999) to generate homozygous eyc null eyes. Eyes are absent in flies in which eyc is homozygosed early in the development of the eye primordium (data not shown), supporting a cell-essential role for Eyc.

A cell-essential role for p47/p97 is also suggested by our failure to obtain ter94 (a *Drosophila* p97 homolog) null eye clones (data not shown), as well as the failure to obtain ter94 loss-of-function germline clones (Leon and McKeain, 1999).

**An eyc transgene rescues eyc lethality**

In order to define the deletion resulting from imprecise excision, we synthesized primers to test the integrity of genes neighboring eyc. One eyc null allele (eyc<sup>139</sup>) contained a complete downstream neighbor, CG3419, and deleted an upstream gene, CG15873, predicted endopeptidase and possible additional undefined upstream sequence.

To determine if embryonic lethality was due to the loss of eyc, rather than CG15873 or additional upstream genes, we crossed a UAS-eyc transgene and an Hs-GAL4 driver into three different homozygous lethal lines, eyc<sup>20</sup>, eyc<sup>27</sup> and eyc<sup>19</sup> (eyc<sup>1</sup>), to generate UAS-eyc; eyc<sup>20</sup>; Hs-GAL4/+, eyc<sup>27</sup>; eyc<sup>19</sup>; Hs-GAL4/+ lines. The transgene rescued the lethality of all three lines. PCR confirmed the rescued flies lacked the endogenous eyc gene. Rescue did not require heat shock, suggesting the basal level of *eyc* transgene expression suffices for viability. Indeed, crosses including constitutive actin- or tubulin-GAL4 drivers were lethal. Rescued eyc<sup>1</sup> homozygous hold their wings at an abnormal, droopy angle and appear less active than wild type.

**DISCUSSION**

We have identified and characterized *Drosophila eyc*, a rat p47 homolog. Gain and loss of Eyc function results in developmental phenotypes that share a common focus of membrane biogenesis. The original eyc allele, recovered as a second hit in a strain selected as flightless in an EMS screen, is a gain-of-function mutation that results in Eyc misexpression at the time when photoreceptor apical surfaces must reorganize to relinquish the face-to-face contacts that guide rhabdomere extension to the retinal floor and to establish definitive rhabdomere and stalk subdomains. eyc mutants fail to release these contacts and to generate the normal center-surround organization of rhabdomere and stalk membrane. Temporal misexpression in the mutant coincides with a stage in which photoreceptor apical surfaces are undifferentiated, consistent with the participation of both stalk and microvillar membrane in adhesions. We speculate that normally directed membrane traffic is necessary for photoreceptor membrane reorganization, including removal of proteins that promote adhesion, such as Armadillo and DE-Cadherin.

A requirement for Eyc in nuclear envelope reassembly adds a novel role for p47. The failure of nuclear envelope reassembly in *eyc* loss-of-function mutants, and the inhibition of nuclear divisions by anti-Eyc antiserum is consistent with observations that pretreatment of nuclear membrane vesicles with NEM, known to inactivate p97, prevents fusion (Macaulay and Forbes, 1996). The continuity of outer nuclear membranes with the ER, where a role for p47/p97 in membrane fusion is well established, offers a plausible basis for a shared fusion mechanism.

One attractive possibility for the proliferation of ER in response to elevated Eyc is suggested by observations in yeast that excess Sec17p, the yeast α-SNAP homolog, inhibits membrane fusion by stabilizing unproductive cis-SNARE pairing (Wang et al., 2000). SNAREs nucleate assembly of the COP II coats that mediate ER-Golgi transport (Springer et al., 1999). As transport vesicle budding must endow the vesicle with SNAREs that are competent to mediate fusion, coat assembly may reject fusion-incompetent cis-SNARE complexes. Excess Eyc may shift the balance in favor of fusion-incompetent cis-SNARE complexes, hindering budding, increasing the size of the ER and disrupting normal rhodopsin traffic.

Alternate scenarios for an effect of Eyc overexpression include diversion of p97 activity from other tasks. For example, p97 also participates in ubiquitin-dependent protein degradation and nuclear transport pathways, and is targeted to these activities by a protein complex, Ufd1/Npl4, which competes with p47 for binding to p97 (Meyer et al., 2000). Excess Eyc might diminish the availability of p97 for these roles. p47/p97 activity in vitro is stoichiometry dependent (Meyer et al., 1998) and it is possible that excess Eyc produces a sub-optimal ratio, which compromises normal membrane fusion in vivo. Excess Eyc may also impact NSF-mediated pathways as α-SNAP and p47 compete for Golgi syntaxin-5 SNARE complexes (Rabouille et al., 1998).

Rhabdomere defects of eyc mutants are provocative in light of the report that anti-SNP and anti-α-SNAP antibodies do not inhibit MDCK apical membrane delivery (Ikonen et al., 1995). SNAREs participate in apical membrane transport and presumably require disassembly after fusion (Low et al., 1998). p47/p97 is an attractive candidate to mediate such disassembly.

It is interesting to consider *Drosophila* photoreceptor morphogenesis against the background of Arthropod eye development generally. Higher Dipterans and some Coleopterans are unusual in having ‘open’ rhabdoms in which separate rhabdomeres individually face the IRS. Rhabdomeres of most insects and crustaceans adhere on the central axis to form a multicellular ‘closed’ rhabdom. As in *Drosophila*, closed rhabdoms develop ‘down’ from the distal, corneal surface of the eye to the retinal floor (Eisen and Youssef, 1980; Hafner and Tokarski, 1998). Rhabdomere contacts of closed rhabdoms display diverse and intriguing patterns which may offer clues to the adhesive rules guiding rhabdomere extension (Paulus, 1979). Perhaps an only small change in the ‘machine language’ of photoreceptor development, regulation of targeted membrane delivery, for example, might allow fly rhabdomeres to unstick after extension, opening the IRS.

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