**Glued** participates in distinct microtubule-based activities in *Drosophila* eye development

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**SUMMARY**

A C-terminal truncation of Glued, the *Drosophila* homolog of the cytoplasmic dynein activating protein, dynactin, results in a severe and complex retinal phenotype, including a roughening of the facet array, malformation of the photosensitive rhabdomeres, and a general deficit and disorder of retinal cells. We have characterized the developmental phenotype in *Glued*¹ and found defects in multiple stages of eye development, including mitosis, nuclear migration, cell fate determination, rhododerm morphogenesis and cell death. Transgenic flies that express dominant negative Glued under heat-shock control reproduce distinct features of the original *Glued*¹ phenotype depending on the stage of development. The multiple phenotypes effected by truncated Glued point to the multiple roles served by dynactin/dynein during eye development.

Key words: Glued, dynactin, compound eye, morphogenesis, mitosis, nuclear migration, cell death

**INTRODUCTION**

Cytoplasmic dynein, a minus-end directed microtubule motor protein, participates in a host of intracellular functions, including mitosis, directed vesicle transport and axoplasmic transport (Vallee and Sheetz, 1996). Although the roles of dynein and its associated proteins are increasingly well characterized at the cell and molecular level, their participation in multicellular development remains poorly understood. The vital nature of the functions effected by motor proteins complicates a classical genetic analysis based on loss-of-function mutations: cells lacking these proteins die or fail to proliferate normally during development. Dominant negative mutations, alleles encoding mutant proteins which inactivate the multimeric protein complex in which they normally function, offer a useful alternative for the genetic analysis of essential proteins (Herskowitz, 1987). Conditional expression of such ‘poison’ subunits can probe the function of a protein assembly at selective times during development and circumvent the lethality associated with a global loss of the protein during development. We have used this approach to study the roles of cytoplasmic dynein during *Drosophila* compound eye development.

The *Drosophila* gene, Glued, provides an attractive starting point for a developmental analysis of cytoplasmic dynein. Glued encodes the *Drosophila* homolog of vertebrate p150Glued, the large subunit of the dynein-activating complex, dynactin (Gill et al., 1991; Holzbaur et al., 1991; Swaroop et al., 1987). In addition to p150Glued, dynactin includes the 45 kDa actin-related protein (Arp-1), the 50 kDa dynamitin, capping protein and several uncharacterized subunits (Schroer, 1996). Biochemical evidence suggests Arp1 binds to the C terminus of p150Glued (Waterman-Storer et al., 1995), potentially mediating the attachment of cargo to the dynein/dynactin complex (Schroer et al., 1996). Genetic studies suggest dynactin participates in multiple cell functions. p150Glued mutations in filamentous fungi, *Neurospora crassa* disrupt normal nuclear migration along hyphae (Tinsley et al., 1996). Similar nuclear mislocalization is seen in Arp1 mutants of *Neurospora crassa* (Plamann et al., 1994) and budding yeast, *Saccharomyces cerevisiae* (Muhua et al., 1994; Clark and Meyer, 1994). Overexpression of dynamitin, a 50 kDa dynactin complex subunit, in cultured cells arrests mitosis at prometaphase due to misalignment of chromosomes and aberrant mitotic spindles (Echeverri et al., 1996).

Glued was initially identified by the *Drosophila* dominant mutation, Glued¹ (Plough and Ives, 1935), which shows severe visual system defects including disruption of the retina and its connection to the underlying optic lobes (Meyerowitz and Kankel, 1978). Gene dosage studies indicate Glued¹ is dominant due to gain of function rather than haploinsufficiency; a single copy of the normal Glued locus over a deficiency is normal while additional copies of the normal Glued locus diminish the phenotype caused by Glued¹ (Harte and Kankel, 1982). Flies homozygous for a Glued null mutation are embryonic lethal and the failure to recover null clones using somatic recombination suggest it is essential for cell viability (Harte and Kankel, 1982). Molecular characterization of the dominant Glued¹ allele revealed the insertion of a transposable element near the 3’ end of the gene, truncating the C terminus of the protein (Swaroop et al., 1985). Taken together, previous results and those reported here suggest truncated Glued ‘poisons’ normal dynactin complex activity.
The role of Glued in eye development has remained largely unknown. Drosophila eyes develop in an orchestrated program of pattern formation, mitosis, cell fate determination, nuclear movements, cell death and morphogenesis (Waddington and Perry, 1960; Wolff and Ready, 1993). Defects first become evident in Glued eyes shortly after the onset of retinal differentiation and become progressively worse as development proceeds (Renfranz and Benzer, 1989). We have reconstructed the developmental history of Glued eyes using conditional expression of a dominant negative form of Glued protein to dissect the phenotype. The mutant protein impacts nearly every phase of eye development in a stage-specific fashion, and these defects are linked by known or suspected connections to microtubule motor activity.

**MATERIALS AND METHODS**

**Drosophila stocks**

*Drosophila melanogaster, Oregon-R* was used as wild type. Glued/D flies were obtained from the Bloomberg stock center and maintained as a stock with TM3 sb ser. p21 transgenic flies were a gift from Dr. Iswar Hariharan, Massachusetts General Hospital Cancer Center. Flies were raised on standard corn meal-agar media at 25°C.

**Transgenic flies expressing dominant negative Glued**

To generate a synthetic dominant negative allele of Glued, a 2897 bp fragment from Glued cDNA clone C39, kindly provided by Dr Alan Garen, was amplified using two primers: 5'-CGATCATCACGGC- GACCTCG-3', 5'-TTAGGGGAGGTGTCCTCGT-3'. The PCR product encodes the N-terminal 922 amino acids of Glued but is missing the C-terminal 379aa. This product deletes an additional 129 aa compared to the original Glued. The PCR fragment was directly cloned into the PCT cloning vector pCRII (Invitrogen, San Diego, CA). The EcoRI fragment was then excised and ligated into the EcoRI site of the pCaSpeR-hs transformation vector (Pirrotta, 1988) to construct phs-tGl. Junctions of all constructs were verified by double-strand DNA sequencing.

Germ-line transformation was performed according to Spradling and Rubin (1982). Briefly, w<sup>1118</sup> embryos were injected with phs-tGl (500 ng/ml) and helper plasmid Δ2-3 (100 ng/ml). Survivors were back crossed with w<sup>1118</sup> flies and w<sup>+</sup> offspring were selected. Expression of the phs-tGl transgene was confirmed using western blots.

**Antibody production, larval protein purification and western blot**

Rabbit anti-Glued antisera was raised against the N terminus of bacterially expressed Glued polypeptide. Two primers (5'-GGGGGAATTCCGCGCCACGACTTTCCA-3', 5'-GGGGCTC- GAGTTCCGATGGCCAGTTCGG-3'), were used to amplify a 945 bp 5' end Glued DNA fragment from the C39 cDNA clone (Swaroop et al., 1987). The PCR product was cloned in frame into the bacterial expression vector, pGEX-KG (Guan and Dixon, 1991). Junction sites of all constructs were verified by double-strand DNA sequencing.

The fusion protein construct was expressed in the bacterial strain XL1-Blue (Stratagene, La Jolla, CA). After induction with IPTG, bacterial cells were harvested and lysed using a French press (twice at 1200 lb/in<sup>2</sup>). The soluble Glued-GST fusion protein was purified using glutathione agarose (Sigma, St Louis, MO) and cleaved from GST using thrombin (Sigma, St Louis, MO). A 40 kDa Glued polypeptide band was excised from SDS-PAGE gels and used as an immunogen. Rabbits received subcutaneous injections of approximately 50 μg of bacterially expressed Glued polypeptide, mixed with complete Freund’s adjuvant. Rabbits were boosted by injection with 100 μg of Glued polypeptide in incomplete Freund’s adjuvant three weeks after the first injection. Antisera were purified by affinity chromatography on protein A sepharose (Sigma, St Louis, MO).

Preparation of larval proteins was based on Hays et al. (1994), with modifications. Briefly, larvae were collected from wild type, Glued or transgenic flies. The larvae were rinsed three times with PMEG buffer (100 mM Pipes, pH 6.9, 5 mM MgSO<sub>4</sub>, 5 mM EGTA, 0.1 M EDTA, 0.5 mM DTT, 0.9 M glycerol) and homogenized on ice in 1.5 vol of PMEG containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 300 μM PMSF, and 1 mM TAME) using a Wheaton glass homogenizer. All subsequent steps were carried out on ice except where noted. The larval homogenate was spun at 57,000 g for 30 minutes and the supernatant was collected for protein analysis.

Larval proteins were separated by SDS-PAGE (Laemmli, 1970). For immunoblots, proteins were transferred to nitrocellulose membrane after electrophoresis. Membranes were rinsed with TBS (100 mM Tris-HCl, pH 7.2, 0.9% NaCl), blocked with 5% BSA in TBS (TBS + 0.1% Tween 20) for 60 minutes at room temperature, incubated overnight at 4°C with anti-Glued antiserum (1:3000) in TBS with 3% BSA. Following incubation with peroxidase-conjugated goat anti-rabbit IgG, blots were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**Histology and immunohistochemistry**

Transmission electron microscopy methods were based on Baumann and Walz (1989), with modifications. Briefly, flies were microinjected with aldehyde fixative (2% paraformaldehyde and 1.75% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4) and dissected after 1 hour. Fixed eyes were then incubated in 1% tannic acid overnight followed by 2% osmium tetroxide in 0.1 M sodium cacodylate for 2 hours. After washing, the eyes were incubated overnight in 2% uranyl acetate. After a serial dehydration with ethanol, eyes were mounted in Epon 812. Tissue was then sectioned using a Reichert ultramicrotome and observed using a Philips 300 electron microscope.

For immunocytochemistry, staged eyes were dissected and fixed in 4% formaldehyde. Fixed eyes were blocked with 10% normal bovine serum in PBST (PBS + 0.2% Triton X-100). After blocking, the eyes were incubated in diluted primary antisera, rat anti-elav (1:50) (Robinow and White, 1991) or rabbit anti-Bar (1:100) (Higashijima et al., 1992) in PBST with 5% bovine serum overnight at 4°C. Eyes were washed three times with PBST and then incubated with secondary antibody labeled with either Texas Red or FITC (Vector, Burlingame, CA) at room temperature for 2 hours. After three washes in PBST, eyes were mounted in mounting medium (0.25% n-propyl gallate, 50% glycerol in PBS, pH 8.6). For microtubule staining, staged eyes were fixed using the pH-shift protocol (Bacalla et al., 1989). Briefly, eyes were dissected in microtubule stabilizing buffer (MTSB) (80 mM K-Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl<sub>2</sub>) and fixed in 4% paraformaldehyde in MTSB (80 mM K-Pipes, pH 6.5, 5 mM EGTA, 2 mM MgCl<sub>2</sub>) for 10 minutes followed by 4% paraformaldehyde in 100 mM Na<sub>2</sub>CO<sub>3</sub> for another 10 minutes at room temperature. After blocking with 10% normal bovine serum, eyes were then incubated in diluted anti-β-tubulin antibody (1:100) (Amersham, Arlington Heights, IL). The remaining procedures followed the immunostaining methods described above. For elav and phalloidin double labeling, the eyes were stained first with the antibody and then with FITC-phalloidin (Sigma, St Louis, MO). Samples were examined and images recorded using a Bio-Rad MRC-1024 confocal microscope.

To detect mitotic figures, third instar eye discs were dissected in Ringer’s solution and fixed in 4% paraformaldehyde in PBS for 20 minutes. After fixation, eye discs were washed three times in PBST. Eye discs were then transferred to PBS with RNase A (2 mg/ml) for 2 hours at 37°C. After three washes with PBST, eye discs were transferred to premixed iodination medium (5 μg/ml in PBST) for 20 minutes. After three washes with PBST, eye discs were mounted in mounting medium and examined using the confocal microscope. The number
of dividing cells in the second mitotic wave for wild-type and mutant flies was counted and compared using the Student’s t-test. An ANOVA for factorial design experiments was used to analyze the number of dividing cells over time. Significant effects were further analyzed using a Newman-Keuls post hoc test (Keppel, 1973).

Acridine orange staining was used to identify cell death. Third instar eye discs were dissected in acridine orange in Ringer’s (10−6 M) and examined immediately on the confocal microscope.

RESULTS

**Glued** eye phenotype

The *Drosophila* eye consists of an orderly, hexagonal array of approximately 750 unit eyes, or ommatidia; small mechanosensory bristles project from alternate vertices over most of the eye (Fig. 1A). Each ommatidium contains eight photoreceptors, which can be identified by the unique position of their photosensitive rhabdomeres (Fig. 1C). Wild-type rhabdomeres are oval in cross-section and are positioned on the longitudinal, optic axis by a smooth adjacent membrane subdomain, the stalk. *Glued* eyes are small and rough with fewer and irregularly positioned bristles (Fig. 1B). Ommatidial structure is severely disturbed in *Glued* eyes (Fig. 1D). As reported by Renfranz and Benzer, 1989, the number of photoreceptors in a *Glued* ommatidium is variable but usually less than normal. Mutant rhabdomeres are usually smaller than wild type. Occasionally, the rhabdomeres are turning away from center of ommatidial cluster (Fig. 1D).

**Mitosis is delayed in the second mitotic wave in Glued eyes**

To understand the origin of the *Glued* eye phenotype, we compared early ommatidial development in wild-type and *Glued* eye discs. Eye discs, the larval stage retinal anlagen, are typical polarized epithelia. The onset of retinal differentiation is marked by a band of apical constriction, the morphogenetic furrow. Posterior to the furrow, five postmitotic cells, the future photoreceptors R2, 3, 4, 5 and 8, organize into an ommatidial precluster (Fig. 2A). Consistent with previous observations (Renfranz and Benzer, 1989), cell outlines in the morphogenetic furrow and preclusters are normal in *Glued* (Fig. 2B).

Posterior to the furrow, cells not incorporated into preclusters undergo a final round of cell division in the second mitotic wave, which generates the remaining population of cells required for ommatidial assembly. When these cells divide, they round up at the apical surface and appear as dark circles.

Fig. 1. *Glued* is required for normal eye development. Scanning electron micrographs show wild-type and *Glued* adult eyes. (A) In wild type, a dome of regular hexagonal lenslets defines the ommatidial field. Small mechanosensory bristles project from alternate facet vertices over most of eye. (B) In *Glued*, the eye is small and rough. Lenslets are malformed and bristles are abnormally arranged and occasionally duplicated. Transmission electron micrographs show internal eye structure. (C) Tangential sections of wild-type eyes show rhabdomeres arranged in a trapezoid. A stereotyped complement of cone and pigment cells, which secrete the lens and provide optical insulation for each ommatidium, surround each group of photoreceptors (not shown). (D) In *Glued*, the number of photoreceptors is usually less than in wild type and rhabdomere profiles are irregular. The arrangement of cone cells and pigment cells is also disrupted. Scale bar, 100 μm in A, B and 10 μm in C, D.

Fig. 2. The morphogenetic furrow establishes regularly spaced preclusters in *Glued*. Rhodamine-phalloidin-stained eye discs show the morphogenetic furrow (arrows) and the apical profile of precluster cells (small arrowheads) in (A) wild type and (B) *Glued*. Round profiles (large arrowheads) are mitotic cells rounded up to divide in the second mitotic wave. Propidium iodide-stained eye disc revealed the second mitotic wave is wider in *Glued*. (C) In wild type, the second mitotic wave is a narrow band focused on ommatidial rows 3 to 5. (D) In *Glued*, the second mitotic wave expands, extending across rows 3 to 8. The morphogenetic furrow is indicated by arrows. Anterior is to the right. Scale bar, 10 μm.
in phalloidin-stained eye discs (Fig. 2A,B). While all non-precluster cells divide in this wave, since mitosis occurs rapidly, only a subset of cells are in mitosis at any given moment. Glued1 eyes discs contain roughly twice as many cells rounded up to divide as compared to wild type (Fig. 2B).

To count the number of dividing cells in the second mitotic wave, we used propidium iodide to label mitotic figures in wild-type and Glued1 eye discs (Fig. 2C,D). In the second mitotic wave of wild-type eyes, an average of 24±2.5 mitotic figures are seen; in Glued1, this number increases to 48±3.0 (t(38)=−27.1, P<0.001). Furthermore, the spatial pattern of second mitotic wave was expanded in Glued1. In wild type, dividing cells span a width of three rows, forming a tight band between rows 3 and 5 behind the furrow (Fig. 2C). In Glued1, this band widens to about 3-8 rows behind the furrow (Fig. 2D). These mitoses do not appear to be due to cells entering another round of mitosis since labeling of mitotic cells by microinjection of BrdU showed no obvious increase in the number of labeled cells in Glued1 versus wild type (data not shown). The broader second mitotic wave may reflect a mitotic delay in Glued1.

**Fig. 3.** Expression of truncated Glued delays mitosis in the second mitotic wave. Wild-type and transgenic flies were heat shocked for 30 minutes and the number of mitotic cells in the second mitotic wave was counted at various times after heat shock. When analyzed at various time after heat shock, the number of mitotic cells in wild-type and transgenic flies were significantly different (ANOVA: F(5, 108)=16.1, P<0.001). The increase of mitotic figures, is first evident 2 hours after heat shock, reaches a peak at 3 hours post heat shock and returns to normal 6 hours after heat shock.

**Fig. 4.** Glued is required for normal nuclear position in third instar eye discs. Eye discs were double-labeled with anti-elav (red) and FITC-phalloidin (green) and examined using confocal microscopy. A series of optical planes, separated by 2.5 μm was recorded from apical to basal, in wild type (A,C,E,G,I,K) and Glued1 (B,D,F,H,J,L). In wild type, photoreceptor nuclei are regularly ordered at the apical surface, however, Glued1 nuclei are disordered and distributed throughout the depth of the retinal epithelium, with many crowded into the photoreceptor axons where they converge at the optic stalk. Anterior is to the right. Scale bar, 50 μm.
To further characterize the impact of Glued on the second mitotic wave, we expressed truncated Glued during eye development using the hsp 70 promoter. Transgenic and wild-type flies were given a 30 minute heat shock and allowed to recover for varying times before second mitotic wave mitoses were counted. Consistent with previous observations demonstrating heat shocks delay mitosis in embryos (Maldonado-Codina et al., 1993), both wild type and transgenics showed an increase in second wave mitoses. About 24±2.5 mitotic cells were normally observed in non-heat-shock wild-type flies. In contrast, the average number of mitotic figures increased to 34.27 and 43.48 for wild-type and transgenic flies respectively after heat shock. An ANOVA revealed a significant increase in the number of mitotic cells in the heat-shock transgenic flies as compared to the heat-shocked wild-type flies (group main effect, F(187.2)=16.1, \(P<0.001\)). The ANOVA also revealed a significant Group X Time interaction (F(5, 108)=16.1, \(P<0.001\)). As seen in Fig. 3 and supported by the Newman-Keuls post hoc analysis of the interaction, more mitotic cells were seen in transgenic flies at 3, 4 and 5 hours following heat shock as compared to wild type, \(P<0.05\). No significant differences were observed at 1 and 6 hours following heat shock. Assayed at 3 hours, heat shocks of 20 and 15 minutes showed similar increases in mitoses, while 10 minute heat shocks showed no increase (data not shown). The transient increase of mitoses resulting from a pulse of truncated Glued expression resembles that seen in Glued\(^d\) and plausibly shares a similar origin in mitotic delay.

To determine if additional mitotic defects occur in Glued\(^d\), we compared spindle organization in wild-type and Glued\(^d\) eye discs stained with anti-\(\beta\)-tubulin (data not shown). No obvious differences were observed. Moreover, the distribution of different mitotic stages was found to be similar in wild type and Glued\(^d\). Taken together, our results suggest that, although truncated Glued slows down mitosis, it does not alter the frequency of individual mitotic stages or disrupt spindle architecture.

**Photoreceptor nuclei are mispositioned in Glued mutants**

Nuclear migration is a significant feature of Drosophila eye development; as cells commit to a specific cell fate and begin to differentiate, their nuclei migrate apically (Tomlinson, 1985). To examine the impact of truncated Glued on the migration of photoreceptor nuclei, we double-labeled third instar eye discs with anti-elav antiserum to stain neuronal nuclei and rhodamine-phalloidin to label cortical microfilament cytoskeleton. Nuclear position was determined using serial confocal optical sections (a Z series) to reconstruct wild-type and Glued\(^d\) eye discs. In wild type, photoreceptor nuclei

![Fig. 5](image1.png)

**Fig. 5.** Longitudinal and subapical microtubules in developing wild-type photoreceptors. (A) In third instar eye discs, longitudinal microtubules extend throughout photoreceptors and gather into axon bundles (indicated with arrowheads) before joining the subretinal fan of axons, which converges at the optic stalk at the posterior of the disc. Arrow indicates microtubules concentrated at the photoreceptor apical cytoplasm. (B) Microtubules in an isolated 67% ommatidium are concentrated at the base of the rhabdomere, the apical cytoplasm of the photoreceptor. Developing rhabdomeres lie within the bright boundary. Lighter microtubule densities outline the dark ovals of the photoreceptor nuclei. Scale bar, 10 \(\mu\)m (A) and 5\(\mu\)m (B).

![Fig. 6](image2.png)

**Fig. 6.** Mispositioned nuclei in Glued\(^d\) are substantially rescued in p21/Glued\(^d\) flies. Eye discs were double labeled with anti-elav (red) and phalloidin (green). (A) Mispositioned nuclei in a Glued\(^d\) eye disc shows a deficit of elav-positive nuclei in apical planes (Compare with Fig. 4C). (B) A comparable apical section of a p21/Glued\(^d\) eye disc shows more elav-positive nuclei at apical surface than Glued\(^d\). (C) A basal plane of a Glued\(^d\) eye disc shows many nuclei falling into the fan of axons that converges at the optic stalk. (D) A basal plane of a p21/Glued\(^d\) eye disc shows fewer elav-positive nuclei falling into the fan of axons than Glued\(^d\). Anterior is to the right. Scale bar, 10 \(\mu\)m (A,B) and 25 \(\mu\)m (C,D).
are located in stereotyped clusters near the apical surface of eye discs (Fig. 4A,C,E,G,I,K). In Glued<sup>1</sup>, photoreceptor nuclei were distributed randomly throughout the eye disc; few occupied the normal apical region and many had ‘fallen’ into the optic stalk, having migrated along their axons (Fig. 4B,D,F,H,J,L).

The distribution of microtubules in eye disc cells is consistent with a role for microtubule-based motility in nuclear migration. Similar to wing disc cells (Fristrom and Fristrom, 1975; Eaton et al., 1996), immunostaining of wild-type eye discs using anti-β-tubulin antibody shows two populations of microtubules: an elongated basket of longitudinal microtubules extending through the photoreceptor cell body and enclosing the nucleus and a distinct concentration at the apical pole of the cell (Fig. 5A). No obvious differences were noted between microtubule organization in wild type and Glued<sup>1</sup> (data not shown) suggesting mispositioned nuclei result from defects in motor activity rather than gross defects in the microtubule cytoskeleton.

To explore if mispositioned nuclei are a consequence of defects in second wave mitoses, we crossed transgenic flies in which cell division in the second mitotic wave is blocked by the expression of p21, a cyclin-dependent kinase inhibitor (de Nooj and Hariharan, 1995), to Glued<sup>1</sup>. Substantial reductions in mispositioned nuclei were observed in p21/Glued<sup>1</sup> eye discs (Fig. 6). This result suggests that cell division puts cells at increased risk for nuclear mispositioning in Glued mutants. Cell division at the second mitotic wave is not a prerequisite for nuclear mispositioning, however, since nuclei are also mispositioned in p21/Glued<sup>1</sup> eye discs.

**Glued<sup>1</sup> disrupts cell fates**

Cell cycle regulation and cell fate determination are highly regulated during development (Foe, 1989). The delayed mitoses in the second mitotic wave in Glued<sup>1</sup> led us to investigate the differentiation of photoreceptors and accessory cells born in the second mitotic wave. We examined the differentiation of R1 and R6 using anti-Bar antiserum which stains a homeodomain protein specifically expressed in the nuclei of differentiating R1 and R6 photoreceptors (Higashijima et al., 1992). In wild type, the paired R1, R6 nuclei were stained at the apical surface of the disc, about 10–12 rows behind the furrow (Fig. 7A). The number of Bar-positive cells was dramatically reduced in Glued<sup>1</sup> eye discs (Fig. 7B). This reduction of Bar-stained cells does not appear to be due to the nuclei of R1 and R6 falling into the optic stalk since there are only a few Bar positive cells present in the axons at the retinal floor in Glued<sup>1</sup> eye discs, suggesting the reduction of Bar-stained cells is not simply due to the migration of these nuclei into the optic stalk. Rather, the reduction of Bar positive cells may reflect the failure of photoreceptor R1 and R6 to differentiate normally.

The fates of cone and pigment cells were studied in rhodamine-phalloidin-stained pupal eyes. Wild-type eyes contain four cone cells and two primary pigment cells (Fig. 7C). In Glued<sup>1</sup>, the number of cone cells per ommatidium is reduced from the normal four to two or three and the number of primary pigment cells is occasionally reduced from the normal two to one (Fig. 7D). The number and organization of secondary and tertiary pigment cells is also abnormal in Glued<sup>1</sup> (Fig. 7E,F).

**Cell death in Glued<sup>1</sup> eye discs**

The observation that adult Glued<sup>1</sup> eyes contain fewer than normal cells prompted us to ask if excessive cell death occurred during eye development. We used acridine orange staining to visualize dying cells in third instar eye discs of wild type and Glued<sup>1</sup>. In wild-type eye discs, acridine orange staining revealed scattered cell death ahead of the morphogenetic furrow and little or none behind it (Fig. 8A). In Glued<sup>1</sup> eye discs, a broad band of dying cells is seen a few rows behind the furrow (Fig. 8B).

The coexistence of cell death, mitotic delay and mispositioned nuclei behind the morphogenetic furrow led us to ask if these phenotypes are independent or related to each other. Acridine orange staining of p21/Glued<sup>1</sup>, in which the second mitotic wave is blocked, reveals substantial cell death, suggesting a delay of mitosis or other events associated with cell division in the second mitotic wave are not an exclusive
Glued in eye development

cause for cell death (Fig. 8C); cell death is not observed in p21
eye discs (data not shown). Furthermore, cell death is also not
likely to be the principal cause of nuclear mispositioning since
nuclear mispositioning was significantly rescued in p21/Glued
eye discs with little change in cell death (compare Figs 8B and
6C with Figs 8C and 6D). Consistent with the idea that cell
death occurs independent of mitotic delay and nuclear mis-
positioning, ectopic expression of truncated Glued by brief
heat shocks can result in cell death with few misplaced nuclei
(see below).

Functional dissection of multiple Glued functions in
eye development using ectopic expression of
dominant negative Glued protein

The pleiotropic phenotypes seen in Glued eyes may represent
multiple sites for Glued action or, alternatively, the progressive
accumulation of defects following an early, unitary disturbance
of normal development. In order to further dissect the Glued
phenotype, we expressed truncated Glued at defined stages of
development using transgenic flies in which the mutant protein
is expressed under control of the hsp 70 promoter.

Immunoblots were used to establish the expression of
truncated Glued in transgenic flies. 1 hour after heat shocks of
varying duration, larval proteins were prepared from normal
and transgenic larvae and probed with anti-Glued antiserum.
Blots of wild-type flies showed a band at 150 kDa (Fig. 9A,
lane 1); in heterozygous Glued, which contains one copy of
normal Glued and a copy of truncated Glued, the 150 kDa plus
an additional band at 125 kDa, the predicted molecular weight
of the Glued truncated protein, was observed (Fig. 9A, lane
2). Non-heat-shocked transgenic flies show the wild-type 150
kDa band (Fig. 9A, lane 3). After heat shock, they show an
additional band at 110 kDa, the predicted molecular weight of
our synthetic truncated Glued (Fig. 9A, lane 4). Longer heat
shocks resulted in stronger expression of truncated Glued (Fig.
9B).

To assay the impact of truncated Glued on eye development,
we heat shocked transgenic third instar larvae for 1 hour and

Fig. 8. Truncated Glued causes cell death in the eye disc. (A) In wild
type, there is scattered cell death ahead of and little or none behind
the furrow. (B) In Glued, a broad band of cell death occurs behind
the furrow. (C) Substantial cell death occurs in p21/Glued, in which
the second mitotic wave is blocked. (D) Transgenic flies, which
received a 30 minute heat shock and were allowed to recover for 4
hours, show a band of cell death behind the furrow. The
morphogenetic furrow is indicated by arrows. Anterior is to the right.
Scale bar, 50 μm.

Fig. 9. Glued expression in wild-type, Glued and
transgenic flies. Samples from larval proteins,
approximately 20 μg of protein was loaded in each
lane, separated on a 8.5% SDS-PAGE gel and
transferred to nitrocellulose membrane. The
membrane was probed with anti-Glued antiserum.
In wild type, anti-Glued antiserum revealed a
band at 150 kDa (A, lane 1). In Glued heterozygous,
anti-Glued antiserum revealed a
150 kDa band and an additional 125 kDa
truncated Glued (A, lane 2). Transgenic flies that
do not receive heat shock express the 150 kDa
protein (A, lane 3). Transgenic flies that received
heat shock express a 150 kDa and an additional
110 kDa truncated Glued (A, lane 4). The level of
truncated Glued expression increases with
increasing heat-shock duration (B, lane 1, 10
minutes shock; lane 2, 15 minutes shock; lane 3,
20 minutes shock; lane 4, 30 minutes shock; lane
5, 60 minutes shock). Wild-type flies that received
60 minutes heat shock express only the normal
150 kDa Glued (B, lane 6).
examined their eyes after varying recovery times. Adult eyes of these animals showed a dorsal-ventral band of defective ommatidia two to three rows wide (Fig. 10A). This band moved anteriorly with later heat shocks, paralleling the posterior-to-anterior progress of retinal differentiation. Animals heat shocked at early third instar and allowed to recover for 24 hours showed a band of defective ommatidial clusters two to three rows wide located approximately 16 rows behind the furrow (Fig. 10C). Elav-positive nuclei were mispositioned in axons at the retinal floor in these defective clusters (Fig. 10D). Anti-Bar staining also indicates differentiation of R1 and R6 is abnormal in these clusters; one and occasionally both Bar-positive nuclei are missing in defective ommatidia (Fig. 10B). Serial optical reconstruction of the defective ommatidia did not locate mispositioned Bar-positive nuclei at the floor of the retina, suggesting the missing photoreceptors failed to differentiate. 1 hour heat shocks also resulted in a band of cell death behind the furrow (Fig. 8D).

Since the furrow advances at a rate of approximately one row every 1.5 hours, the position of the defective ommatidia suggests they were in the early stages of cluster formation when the heat shock began; it appears that the furrow and/or second mitotic wave are most sensitive to the expression of truncated Glued.

1 hour heat shocks resulted in phenotypes comparable to Glued<sup>1</sup> but were restricted to a narrow band behind the furrow. In an attempt to separate the Glued<sup>1</sup> phenotypes, we decreased the duration of the heat shocks given to transgenic larvae. Shorter heat shocks produced milder defects; 20 minutes was the shortest shock that resulted in mispositioned nuclei and missing Bar-positive cells (data not shown), suggesting these two phenotypes either share a common origin or their separate causes are equally sensitive to truncated Glued. Surprisingly, cell death is more sensitive than other phenotypes to short duration heat shocks. A 15-minute heat shock shows a clear strip of cell death behind the furrow (data not shown). A few dying cells were found behind the furrow in shocks as short as 10 minutes, but not at shorter times.

Cell death as a consequence of truncated Glued expression appears to be tissue and stage specific. Wing or leg discs from Glued<sup>1</sup> or heat-shocked transgenic animals do not show increased cell death, nor do pupal eyes heat shocked at 37% of pupal development (pd), a stage at which pattern formation is complete and morphogenesis has begun (data not shown).

**Glued is required for rhabdomere assembly**

The malformed rhabdomeres of Glued<sup>1</sup> photoreceptors might be due to a direct role for Glued in rhabdomere morphogenesis, for example in the targeted delivery of the membrane vesicles that build the rhabdomeres or, alternatively, to the distorted cellular environment in which they develop. In order to distinguish between these possibilities, we heat shocked transgenics at 37% pd, before rhabdomeres are formed, and examined their eyes at 67% pd and as adults. At 67% pd, the developing rhabdomeres of heat-shocked wild-type and unshocked transgenic flies occupy a stereotyped, well-defined subdomain of the photoreceptor (Fig. 11A). However, transgenic flies that received identical heat shocks showed wide, irregular rhabdomeres not restricted to their normal subdomains (Fig. 11B). Rhabdomere defects persist in adult eyes. The normal trapezoid of rhabdomeres is seen in heat-shocked wild-type (Fig. 11C) and unshocked transgenic flies (not shown). In heat-shocked transgenic flies, rhabdomeres are often fused and bifurcated (Fig. 11D). Consistent with the idea that microtubules participate in rhabdomere morphogenesis, anti-β-tubulin immunostaining showed a dense meshwork of microtubules at the base of the developing rhabdomere (Fig. 5B). Taken together, the genetic and immunostaining evidence suggests that microtubules and dynactin/dynein may participate in apical vesicle transport during rhabdomere morphogenesis.

**DISCUSSION**

Retinal defects resulting from the expression of truncated Glued resemble those caused by the original, dominant Glued<sup>1</sup> mutation. Both proteins act as dominant negatives and are sensitive to gene dosage. Additional copies of the normal gene
reduce the Glued\(^1\) phenotype (Harte and Kankel, 1982). Expression of additional truncated Glued using the transformation vector pGMR, which drives expression only behind the morphogenetic furrow (Hay et al., 1994), increases the severity of the defects (Fan and Ready, unpublished). Beyond indicating a requirement for the protein’s C terminus, the ‘gain-of-function’ dominance of truncated Glued suggests the mutant protein poisons the dynein complex.

Genetic studies from lower eukaryotic cells suggest a role for the dynactin complex in mitosis. Mutations of Arp1 in budding yeast show defects in spindle orientation and nuclear migration but not in chromosome segregation (Muhua et al., 1994; Clark and Meyer, 1994). In Cos-7 cells, overexpression of p50 results in aberrant mitotic spindles and arrest at prometaphase (Echeverri et al., 1996). Consistent with these results, immunolocalization of cytoplasmic dynein, p50 and p150\(^{\text{Glued}}\) to mitotic spindles and kinetochores in cultured cells also indicates a role for dynein/dynactin in mitosis (Steuer et al., 1990; Pfarr et al., 1990; Echeverri et al., 1996; Waterman-Storer et al., 1996). Our observation of prolonged second wave mitoses provides evidence for a functional requirement for p150\(^{\text{Glued}}\) in mitosis. Unlike the defective spindles observed upon p50 expression, spindles appear normal in dividing cells in Glued mutant eye discs. Truncated Glued protein slows down but does not block mitosis in the eye disc.

The connection between second wave mitoses and nuclear mispositioning is notable; blocking the second mitotic wave using the cyclin-dependent kinase inhibitor, p21 (de Nooij and Hariharan, 1995), substantially rescues nuclear mispositioning in Glued\(^1\). The cortical migration of syncytial blastoderm nuclei is coordinated with the cell cycle (Baker et al., 1993). If nuclear migration in photoreceptors is keyed to the cell cycle, mitotic delays in the second wave in Glued\(^1\) may disrupt this coordination.

The basal displacement of nuclei in cells expressing truncated Glued may be related to the polarity of the longitudinal microtubules spanning the cells. While microtubule polarity remains to be determined in eye disc cells, microtubules in Drosophila wing disc cells (Mogensen et al., 1989) and epithelial Madin-Darby canine kidney cells (Bacallao et al., 1989) are oriented with their minus ends toward the apical cell surface. If nuclear position depends upon minus-end directed microtubule motor activity, compromise of this system could lead to a failure of nuclei to achieve their normal apical position.

Nuclear position has been suggested to depend on a balance of microtubule-dependent tensions (McKerracher and Heath, 1986); UV microbeam disruption of microtubules on either side of the nucleus in the growing hyphal tip of the fungus, Basidobolus magnus results in the movement of nuclei away from the site of microtubule depolymerization (McKerracher and Heath, 1986). In photoreceptors expressing truncated Glued, it is possible to see if nuclear migration along axons is suppressed by mutations in plus end directed motors responsible for axonal transport (Hurd and Saxton, 1996).

Mispositioning of photoreceptor nuclei is also observed in marbles mutants (Fischer-Vize and Mosley, 1994); similar to Glued, marbles mutant photoreceptor nuclei fall below the fenestrated membrane and crowd into the fan of axons exiting the retina. A second notable similarity between marbles and Glued are abnormally shaped rhabdomeres (Fischer-Vize and Mosley, 1994), suggesting both genes may participate directly in rhabdomere morphogenesis. Glued and marbles differ in their role in cell-fate determination. Photoreceptors adopt their normal fate in marbles eyes (Fischer-Vize and Mosley, 1994) but cell-fate determination is aberrant in Glued.

Rhabdomere defects caused by the expression of truncated Glued may be related to a disturbance of directed vesicle transport. Rhabdomeres are apical cell membranes which are massively amplified by the polarized delivery of membrane vesicles late in pupal life (Longley and Ready, 1995). Intracellular vesicle transport in polarized epithelial cells depends on microtubules (Cole and Lippincott-Schwartz, 1995) and microtubule motors, with a distinctive role for dynein in apical transport (Lafont et al., 1994). Immunolocalization shows dynein and dynactin concentrated in the apical cytoplasm of intestinal epithelial cells, whose microvillar brush borders share numerous similarities with rhabdomeres (Fath et al., 1994). The failure of photoreceptors expressing truncated Glued to establish orderly rhabdomere and stalk subdomains may be due to an inability of transport to ‘focus’ vesicle delivery. Alternatively, decreased dynactin/dynein activity may

![Fig 11. Rhabdomere morphogenesis is disrupted by truncated Glued.](Image)

Starting at 37% pd, transgenic flies received two 1 hour heat shocks separated by 4 hours and were allowed to recover to 67% pd and adulthood. (A) At 67% pd, wild-type eyes stained with rhodamine-phalloidin show typical trapezoidal rhabdomeres. (B) In transgenics, rhabdomeres lose their specific domains; usually fused or extend wider than wild type. (C) Adult rhabdomeres of flies heat shocked at 37% pd showed no changes in wild type but (D) the rhabdomeres in transgenics show aberrant morphologies, often fused and bifurcated. Scale bar, 50 μm (A,B); 2μm (C,D).
hinder a cell’s ability to send or receive the signals that localize these subdomains.

In addition to its roles in mitosis and nuclear migration, dynein has been implicated in retrograde axonal transport (Schnapp and Reese, 1989). It has been suggested that the Glued eye phenotype may be due to a disruption of retrograde transport that prevents the delivery of an essential signal back to the developing retina (McGrail et al., 1995). Our observations of defects as early as the preclaster stage, well before axons extend to the brain, suggest that the Glued eye phenotype is independent of innervation of the optic lobes. Photoreceptors differentiate normally in disconnected mutants, in which the optic nerve fails to innervate the brain, again suggesting an independence of early photoreceptor survival and differentiation from target innervation. Only late in pupal development do disconnected photoreceptors degenerate (Steller et al., 1987).

The distinctive, temporally separable defects caused by truncated Glued reflect the multiple roles of dynactin in development; the pleiotropic phenotype of Glued mutants is an amalgam of several failures of cellular development. The well-characterized cellular landscape of the developing fly eye and the developmental separation of critical events such as pattern formation and morphogenesis makes the Drosophila eye an attractive system for the study of microtubule motors in development.

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