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# Mutation of the Apc1 homologue *shattered* disrupts normal eye development by disrupting G1 cell cycle arrest and progression through mitosis

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**Abstract**

The *shattered*$^{1}$ (*shtd*$^{1}$) mutation disrupts *Drosophila* compound eye structure. In this report, we show that the *shtd*$^{1}$ eye defects are due to a failure to establish and maintain G1 arrest in the morphogenetic furrow (MF) and a defect in progression through mitosis. The observed cell cycle defects were correlated with an accumulation of cyclin A (CycA) and String (Stg) proteins near the MF. Interestingly, the failure to maintain G1 arrest in the MF led to the specification of R8 photoreceptor cells that undergo mitosis, generating R8 doublets in *shtd*$^{1}$ mutant eye discs. We demonstrate that *shtd* encodes Apc1, the largest subunit of the anaphase-promoting complex/cyclosome (APC/C). Furthermore, we show that reducing the dosage of either *CycA* or *stg* suppressed the *shtd*$^{1}$ phenotype. While reducing the dosage of *CycA* is more effective in suppressing the premature S phase entry in the MF, reducing the dosage of *stg* is more effective in suppressing the progression through mitosis defect. These results indicate the importance of not only G1 arrest in the MF but also appropriate progression through mitosis for normal eye development during photoreceptor differentiation.


*Keywords:* Anaphase-promoting complex/cyclosome; APC/C; Cdc25; *Drosophila*; *Shattered*; String; CycA; Eye development

## Introduction

Proliferation and differentiation must be regulated coordinately to ensure normal development in multicellular organisms. An important mechanism in the control of these processes is the rapid degradation of proteins by ubiquitin-mediated proteolysis which is regulated by a large family of related ubiquitin protein ligases (Hershko and Ciechanover, 1998). The anaphase-promoting complex/cyclosome (APC/C) is one of the ubiquitin ligases that play a central role during cell cycle progression through the selective ubiquitination of cell cycle regulators that targets them for degradation via the proteasome (Page and Hieter, 1999; Peters, 1999).

The APC/C was originally identified as an activity required for anaphase onset in both yeast and higher eukaryotes (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995). The APC/C is composed of up to thirteen subunits, which are highly conserved from yeast to humans (Yoon et al., 2002; reviewed in Page and Hieter, 1999; Zachariae and Nasmyth, 1999). Mutations in APC/C subunits in yeast lead to a failure to degrade both mitotic cyclins and the anaphase inhibitor securin (Cohen-Fix et al., 1996; Irniger et al., 1995; reviewed in Nasmyth, 2001; Peters, 2002). Stabilization of APC/C substrates in mitosis results in cell cycle arrest at the metaphase–anaphase transition (reviewed in Peters, 2006). Mutations in only a few APC/C subunits have been characterized in *Drosophila*. Animals containing severe mutations in *Apc2/morula* (*mr*) or *Apc5/imaginal discs aberrant* (*ida*) die during development with reduced or absent imaginal discs, the diploid tissue that forms the adult structures of the fly. Mutant cells arrested with high levels of the mitotic cyclin, CycB, and contained aberrantly condensed chromosomes (Bentley et al., 2002; Kashevsky et al., 2002; Reed and Orr-Weaver, 1997).

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Similarly, semi-lethal mutations in *Cdc27/makos* show metaphase arrest of larval neuroblasts, with a corresponding failure to degrade both CycB and CycA (Déak et al., 2003).

APC/C activity is also required for the continued degradation of mitotic cyclins in the subsequent G1 phase. In yeast, accumulation of mitotic cyclins in G1 leads to precocious S phase entry that is independent of G1 phase cyclins (Irniger and Nasmyth, 1997). In higher eukaryotes, the G1 function of the APC/C has been studied using mutations in Cdh1, a G1 phase-specific activator of the complex. A knockout of Cdh1 in vertebrate cells resulted in the accumulation of mitotic cyclins in G1 phase and abrogation of a p21-dependent G1 arrest (Sudo et al., 2001). In *Drosophila* mutations of *fizzy-related* (*fzr*, FlyBase-*rap*), the *Drosophila* ortholog of Cdh1 causes quiescent G1 cells to re-accumulate mitotic cyclins and cells undergo an additional cell division cycle (Jacobs et al., 2002; Sigrist and Lehner, 1997; Pimentel and Venkatesh, 2005). Thus APC/C-Cdh1 regulates stability of mitotic cyclins during G1.

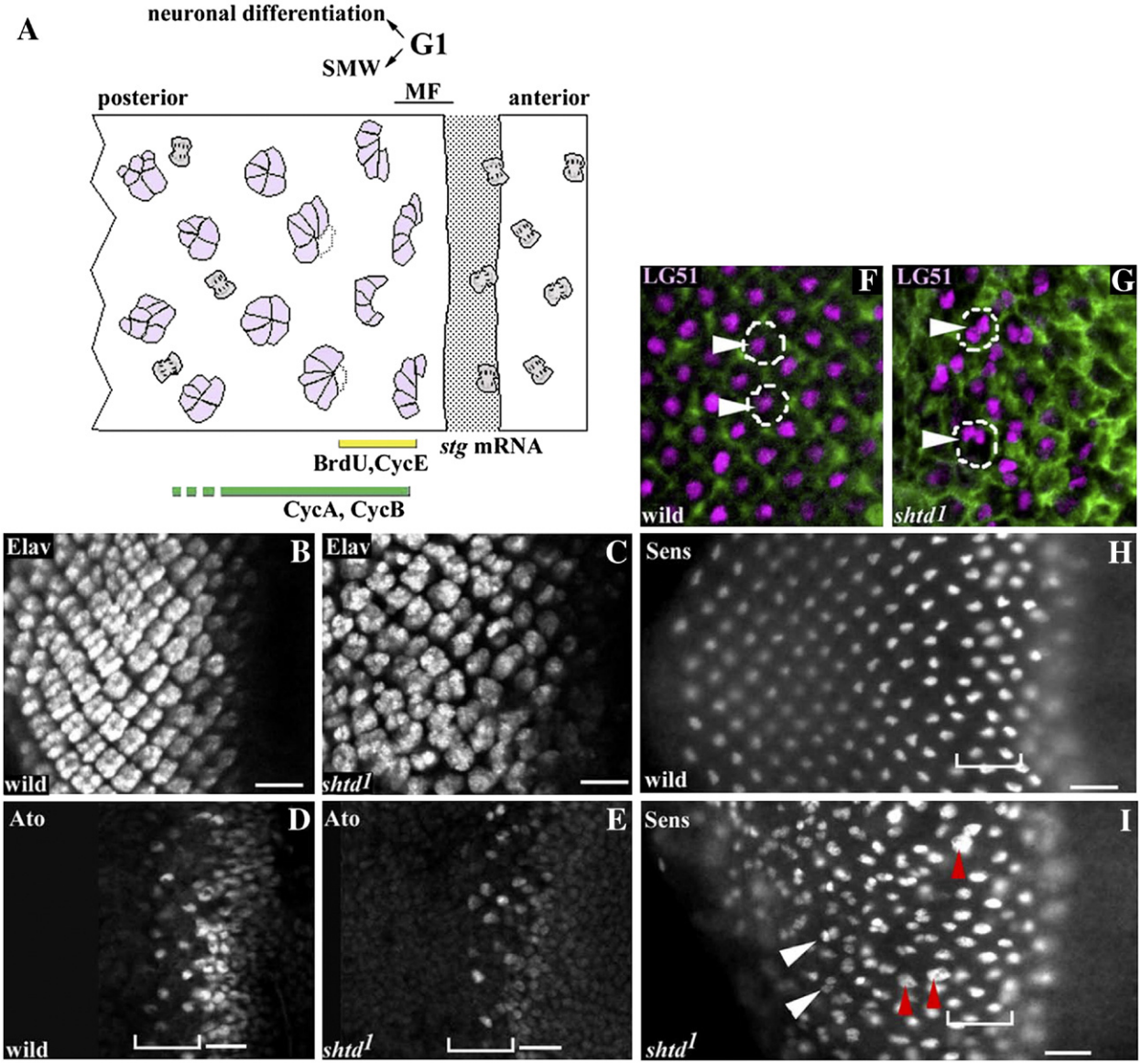



Fig. 1. Abnormal photoreceptor cell differentiation in *shtd*$^{1}$. (A) Schematic representation of cell division and differentiation in the eye disc. Anterior is to the right in these and subsequent panels. Differentiating photoreceptor cell precursors (purple), and mitotic figures (grey) are shown. Synchronous S phase entry domain and G2 domain are indicated by yellow and green bars, respectively. The morphogenetic furrow (MF) is shown by a black bar. Expression of *string* mRNA (dotted) is seen ahead the MF. At the anterior boundary of *string* mRNA expression, cells proceed through mitosis and become synchronized in G1. Behind the MF, cells either exit the cell cycle and differentiate or re-enter a synchronous S phase (SMW). (B, C) Expression of the neuronal marker Elav in differentiating photoreceptor cells in wild-type (B) and the *shtd*$^{1}$ mutant (C). The *shtd*$^{1}$ mutant shows disruptions both in the numbers of neuronal cells in the developing clusters and in spacing of the clusters. (D, E) Ato protein levels were decreased in the MF in *shtd*$^{1}$ (E, white line), however single Ato-positive R8 cells were still observed behind the MF (E, white bracketed line). (F, G) Expression of LG51-*lac*Z in the R8 nucleus (purple) and CycB (green). Two R8-positive nuclei are seen in each photoreceptor cell cluster in *shtd*$^{1}$ (white arrowheads), instead of a single nucleus as in wild-type. (H, I) Sens expression identifies single R8 cells near the MF both in wild-type and *shtd*$^{1}$ mutant. Red arrowheads indicate large mitotic nuclei in *shtd*$^{1}$ mutant (I). More posteriorly, wild-type discs contain single R8 cells (H), while *shtd*$^{1}$ mutant discs contain R8 doublets (white arrowheads) (I). The MF is shown by a white bar.

The compound eye of *Drosophila* is an excellent system to study the processes regulating cellular differentiation and proliferation. The adult compound eye develops from a monolayer epithelium, the eye imaginal disc. Differentiation and cell cycle synchronization occur coordinately in the morphogenetic furrow (MF), which moves from the posterior to the anterior of the eye epithelium (Fig. 1A). Anterior to the MF, cells are undifferentiated and proliferate asynchronously. At the leading (anterior) edge of the MF, uncommitted cells pass through mitosis within a region that expresses high levels of *string* (*stg*) mRNA (Thomas et al., 1994). String (Stg) is a phosphatase that is required in G2 phase to activate entry into mitosis. Stg protein is rapidly degraded upon exit from mitosis (Edgar et al., 1994). It was previously proposed that the high level of *stg* mRNA is required in this region to drive G2 cells through mitosis, resulting in the accumulation of cells in G1 in the MF (Thomas et al., 1994; Zavitz and Zipursky, 1997). In the MF, *stg* mRNA expression is regulated by the Hedgehog signaling cascade which also initiates patterning and differentiation of ommatidial precursors in the MF (Baonza and Freeman, 2005; Firth and Baker, 2005; Heberlein et al., 1995). Although most, if not all, cells in and ahead of the MF express *stg* mRNA, Stg protein is detected in only a small number of cells (Horsfield et al., 1998), indicating that Stg protein accumulation in this region is regulated post-transcriptionally. Immediately posterior to the MF, differentiating photoreceptor cells remain arrested in G1 while the rest of the cells enter a synchronous round of cell proliferation referred to as the "second mitotic wave" (SMW). Blocking cell proliferation in the SMW or ectopic cell proliferation in the MF generally leads to developmental defects, indicating that precise cell cycle control in the MF and the SMW is critical for normal eye development (de Nooij and Hariharan, 1995; Thomas et al., 1994).

In this report, we characterized the mutant phenotype of *shattered* (*shtd*). The $shtd^1$ allele was identified as a mutation causing visible abnormalities in the compound eye of *Drosophila* (Thomas et al., 1994). We show here that $shtd^1$ mutants fail to undergo G1 cell cycle arrest in the MF and exhibit additional cell divisions of the differentiating photoreceptor cells. In addition, the $shtd^1$ mutant is also defective in progression through mitosis and shows significantly increased apoptosis. We demonstrate that *shtd* encodes Apc1, the largest subunit of the APC/C and show that the developmental defects of $shtd^1$ are, at least in part, due to the cell cycle defects as a consequence of an accumulation of CycA and Stg in the mutants.

## Materials and methods

### *Fly genetics*

Flies were cultured at 25 °C. The EMS-induced *shattered*$^1$ ($shtd^1$) allele was identified as an X-chromosome linked rough eye mutation. The $shtd^1$ mutation was mapped genetically using the non-complementing deficiency *Df(1)M10-A14* (13C7-8; 14A8-9) and the complementing deficiencies *Df(1)M32-C13* (13C3-8; 14B14-18), and *Df(1)M34-C1* (13B7-8; 14B15-18), which localized *shtd* to the cytological interval 13C3-8. The deficiency stocks were gifts from S. Frankel, S. Artavanis-Tsakonas and J. Carlson. The original $shtd^1$ chromosome carried a temperature-sensitive mutation on the X chromosome that increased pupal lethality at 25 °C, but had no effect on cell cycle control. This second site mutation was removed by recombination to $w^{1118}$. For imprecise excision of the EP element, four EP lines that mapped to the 13C region were used: *EP(X)1109* (13C5-6), *EP(X)1593* (13C3), *EP(X)1007* (13C2-3), and *EP(X)491* (13C5-6). A total of 800 chromosomes were examined. The $shtd^{EP\Delta}$ deficiency was recovered from *EP(X)1109*. For gamma-ray mutagenesis, isogenic *y cv* adult males were exposed to gamma irradiation (4000 Rads) using a Shepherd Model 143 $^{137}$Cs irradiator. Two alleles, $shtd^2$ and $shtd^3$, were recovered. The $shtd^2$ deficiency allele was mapped by amplifying genomic DNA fragments by PCR from homozygous mutants and wild-type across a region extending from STS Dm0467 at 13B4 to STS Dm3205 at 13E8. The LG51 and BB02 enhancer trap lines were generated in a screen ("Big M") for insertions showing patterned expression of β-gal (J. Merriam, personal communication). The *m(δ)0.5-lacZ* was used as an R4 marker (Cooper and Bray, 1999). The *CycE* alleles were gifts from H. Richardson, the *stg* alleles from B. Edgar, the *fzy* alleles and *fzy* genomic rescue lines from I. Dawson, the *rap/fzr* alleles from T. Venkatesh, and the EP lines were from Exelixis Pharmaceuticals. All other *Drosophila* stocks were from the Bloomington Drosophila Stock Center at Indiana University, Bloomington, IN.

### *Histochemistry*

Eye discs were directly dissected and fixed in iced PLP containing (in 4 ml) 1 ml 16% formaldehyde (Cat #18814, Polysciences, Inc.,) 3 ml 0.1 M lysine pH 7.4, 10 mg sodium meta-periodate for 20 min on ice, and washed in 1× PBS and a balanced salt solution (BSS) containing (in 1 L) 2.21 g NaCl, 3.98 g KCl, 3.07 g $MgSO_4{\cdot}7H_2O$, 0.74 g $CaCl_2{\cdot}H_2O$, 1.79 g tricine, 3.60 g glucose, 17.12 g sucrose and 2.0 g BSA, pH 6.95. Blocking was done with 3% normal goat serum, 0.2% saponin in BSS for 30 min at RT. Primary antibody incubations were performed at 4 °C overnight and secondary and dye-conjugated tertiary antibody reactions were done at room temperature for 4–5 h and 1 h, respectively. For BrdU incorporation, eye discs were dissected in 1× PBS and incubated with 75 μg/ml BrdU in BSS for 45 min at 25 °C in dark. Eye discs were washed twice in 1× PBS and fixed in 4% formaldehyde, 0.6% Tween-20 in 1× PBS for 20 min at RT and washed twice with 1× PBS. For denaturation, tissues were incubated with 100 U of DNaseI (Roche) in 500 μl DNaseI buffer (66 mM Tris pH 7.5, 5 mM $MgCl_2$) for 1 h at 25 °C then washed twice with 1× PBST (0.3% Tween-20). Blocking was done with 3% normal goat serum in 1× PBS for 30 min. Primary antibody dilutions were as follows: mouse anti-CycA, 1:1, mouse anti-CycB, 1:5, rat anti-Elav, 1:10, mouse anti-β-galactosidase JIE7, 1:500, mouse anti-Myc 9E10, 1:5 (Developmental Studies Hybridoma Bank, University of Iowa); rabbit anti-Stg, 1:20 (Edgar et al., 1994); sheep anti-BrdU, 1:1000 (Research Diagnostics, Inc.); rabbit anti-phosphohistone H3, 1:1000, mouse anti-MPM2, 1:500 (Upstate Biotechnology); rabbit anti-β-galactosidase, 1:1000 (ICN); mouse anti-CycE, 1:10 (Richardson et al., 1995), and rabbit anti-ACTIVE® caspase-3, 1:200 (Promega). Dye-conjugated secondary and tertiary antibodies were from Jackson Immunoresearch and were used at 1:500. Optical sections (1.2 μm) were collected on a BioRad MRC1024 confocal microscope. Tissue *in situ* hybridization was done following the E. Bier laboratory protocols (embryos and imaginal discs) using digoxigenin-labeled RNA probes (Roche DIG RNA labeling kit). TUNEL analysis was modified for eye discs from Lisi et al. (2000). Acridine Orange staining was followed Protocol 12.7 in *Drosophila* Protocols (Wolff, 2000). For mosaic clone analysis, $shtd^3$ *FRT18A* chromosomes were generated by recombination. Multiple lines of recombinant chromosomes were examined to yield consistency. To induce mosaic clones, flies were crossed with *FRT18A pπMyc; hs-FLP* (2nd), and 1 h heat shocks at 37 °C were applied at 48 h and 60 h AEL. Prior to dissection, a single 30 min heat shock at 37 °C was applied to induce pπMyc.

### *Molecular biology*

To generate the Shtd rescue construct, a 9.5 kb *Sal*I–*Spe*I genomic DNA fragment from the P1 clone DS08954 was cloned into the *Xho*I–*Spe*I site of pCaSpeR4. P-element-mediated transformation was performed in $w^{1118}$ embryo following standard procedures. Three independent transgenic lines were established and used for rescue experiments. Sequencing was done with

dRhodamin dye termination on an ABI 310 or ABI377 sequencer. Inverse PCR of the $shtd^{EP\Delta}$ allele followed the Berkley Drosophila Genome Project protocol.

## Results

### *R8 photoreceptor cells are frequently twinned in $shtd^1$ mutants*

Flies containing the $shtd^1$ mutation have small eyes with highly disorganized ommatidia. To elucidate the function of Shtd, we set out to characterize the developmental defects of $shtd^1$ mutants. During development of the compound eye, the onset of photoreceptor cell differentiation occurs within the MF as groups of cells form clusters that are competent to assume a neuronal cell fate (Fig. 1A). Immunostaining using an antibody to the pan-neuronal protein Elav showed that differentiating photoreceptor cell clusters in $shtd^1$ displayed aberrant spacing and abnormal numbers of photoreceptor neurons as compared to wild-type (Figs. 1B and C). To further characterize $shtd^1$ eye phenotype, we examined specific differentiation markers that express in distinct developing photoreceptor neurons. We first examined R8 cell markers as R8 is the first cell to be specified in each ommatidial cluster and is required for the subsequent photoreceptor cell recruitment (Brennan and Moses, 2000; Frankfort and Mardon, 2002). Specification of the R8 cell fate is induced in the MF by expression of the proneural gene *atonal* (*ato*). Ato is first expressed in a broad stripe in the MF. Its expression is gradually restricted to a group of cells called the R8 equivalence group and finally to the R8 photoreceptors (Fig. 1D) (Baker et al., 1996; Dokucu et al., 1996; Jarman et al., 1994). *ato* loss-of-function mutations cause failure of both R8 differentiation and subsequent photoreceptor recruitment (Jarman et al., 1994; Jarman et al., 1995). In $shtd^1$ mutant eye discs, Ato protein as well as mRNA levels were slightly decreased in the MF (Figs. 1D and E, white line; data not shown). However, Ato-positive R8 cells were still observed behind the MF with a slightly disorganized pattern in the $shtd^1$ mutants (Figs. 1D and E, white bracketed line).

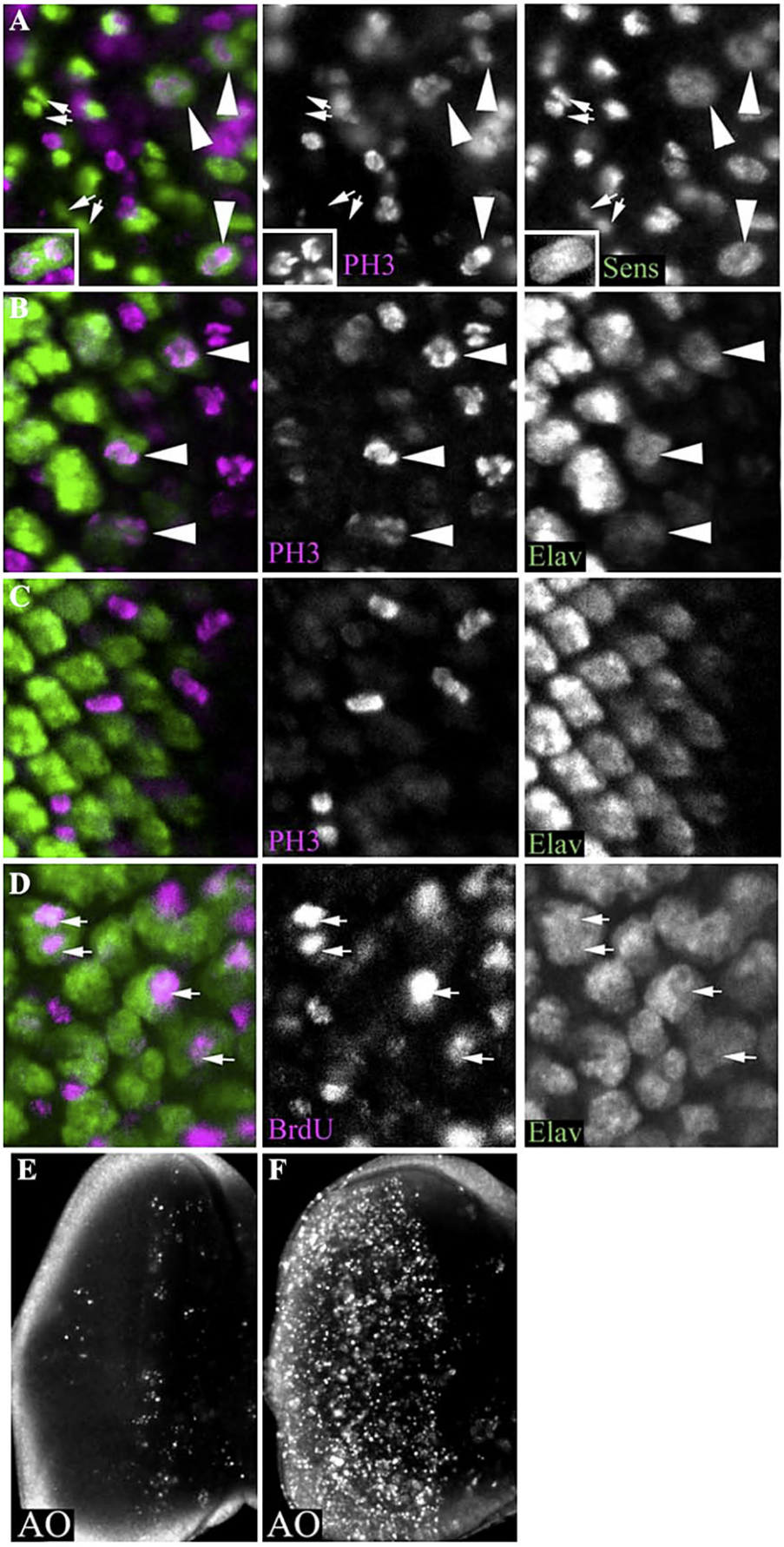
Since *ato* expression persists only in the first 3–4 rows of differentiating R8 cells, the R8-specific enhancer trap lines, LG51 and BB02 were used to monitor R8 cells in the posterior of the eye disc. Interestingly, R8 doublets were observed with the R8 enhancer trap lines in $shtd^1$ eye discs even though only single R8 cells were specified in the first 3–4 rows as shown by anti-Ato staining. Double staining with Elav and CycB showed that the twinned R8 cells were in the same clusters (Figs. 1F and G, clusters are circled with a white broken line, white arrowheads indicate R8). To further characterize the R8 twinning phenotype, we used Senseless (Sens) to mark the R8 cells. Sens is a good marker to identify R8 cells throughout third instar eye disc development, because Sens expression begins in the R8 equivalence group and later its expression is restricted and maintained only in the R8 cells (Frankfort et al., 2001) (Fig. 1H). We found Sens expressed in the R8 equivalence groups in $shtd^1$ with a slightly disorganized spacing pattern (data not shown, out of focus in Figs. 1H and I). Interestingly, single Sens-positive R8 cells were observed immediately behind the MF (Figs. 1H and I, white bracketed line), while immediately posterior to this region, large single nuclei of Sens-positive cells were observed, followed by twinned R8 cells more posteriorly (Fig. 1I, red and white arrowheads, respectively). In contrast, Sens staining in the same region in wild-type eye discs did not detect either the large nuclei or the R8 doublets (Fig. 1H).

### *Additional cell division of the developing photoreceptor cells in $shtd^1$ mutants*

R8 twinning could be caused by a defect in the specification of R8 or by an additional round of cell division of already specified single R8 cells. The observation that single Sens-positive R8 cells were specified before the appearance of R8 doublets suggests the possibility of an additional round of cell division. To directly test this idea, we performed double labeling using Sens and the mitotic marker phosphorylated Histone H3 (PH3) (Goto et al., 1999). Double labeling showed that the large nuclei were also stained with PH3 antibody, indicating that these large R8 nuclei were in mitosis (Fig. 2A). Occasionally, Sens-positive anaphase cells were also observed (Fig. 2A, inset), indicating that the twinned R8 cells in the posterior were derived from an additional cell division from the already specified single R8 cells. To determine if other photoreceptor types also undergo additional round of cell division, we carried out double staining of Elav and PH3. As shown in Fig. 2B, a subset of Elav-positive cells colocalized with PH3 in $shtd^1$, but not in wild-type eye discs (Figs. 2B and C). Each cluster of Elav-positive cells in $shtd^1$ mutants frequently contains 1–2 cells labeled with PH3, suggesting that other types of photoreceptor cells also undergo an additional round of mitosis. In support of this observation, we found a subset of R2/R5 cells and R3/R4 cells were also labeled with mitotic marker PH3 in $shtd^1$ mutants when R2/R5, R3/R4 or R4 specific enhancer traps were used (data not shown). Therefore, multiple types of photoreceptor cells undergo additional rounds of cell division in $shtd^1$ mutants. Interestingly, despite the observed additional mitosis, decreased number of R3 and R4 photoreceptor cells was observed in $shtd^1$ mutants. For example, there are only $41.5 \pm 6$ ($n=6$) R4 cells (identified with *m(δ)0.5-lacZ*) observed in $shtd^1$ eye discs as compared to $275.3 \pm 23$ ($n=6$) observed in wild-type discs. The decreased R3 and R4 cells in $shtd^1$ mutants could be due to increased apoptosis or due to defects in R cell recruitment (see Discussion).

### *Premature S phase entry of G1 arrested cells and increased apoptosis in $shtd^1$ mutants*

We found multiple R cell types undergo additional mitosis in $shtd^1$; therefore, we were interested to examine the effect of the $shtd^1$ mutation on the pattern of S phase. The MF spans about 8–9 cell widths and cells are arrest in G1 phase in this domain. Upon exiting the MF, the clusters of five photoreceptor precursor cells remain arrested in G1 while all cells that are not incorporated into preclusters re-enter S phase synchronously behind the MF, which is often referred to as the SMW (Fig. 3A)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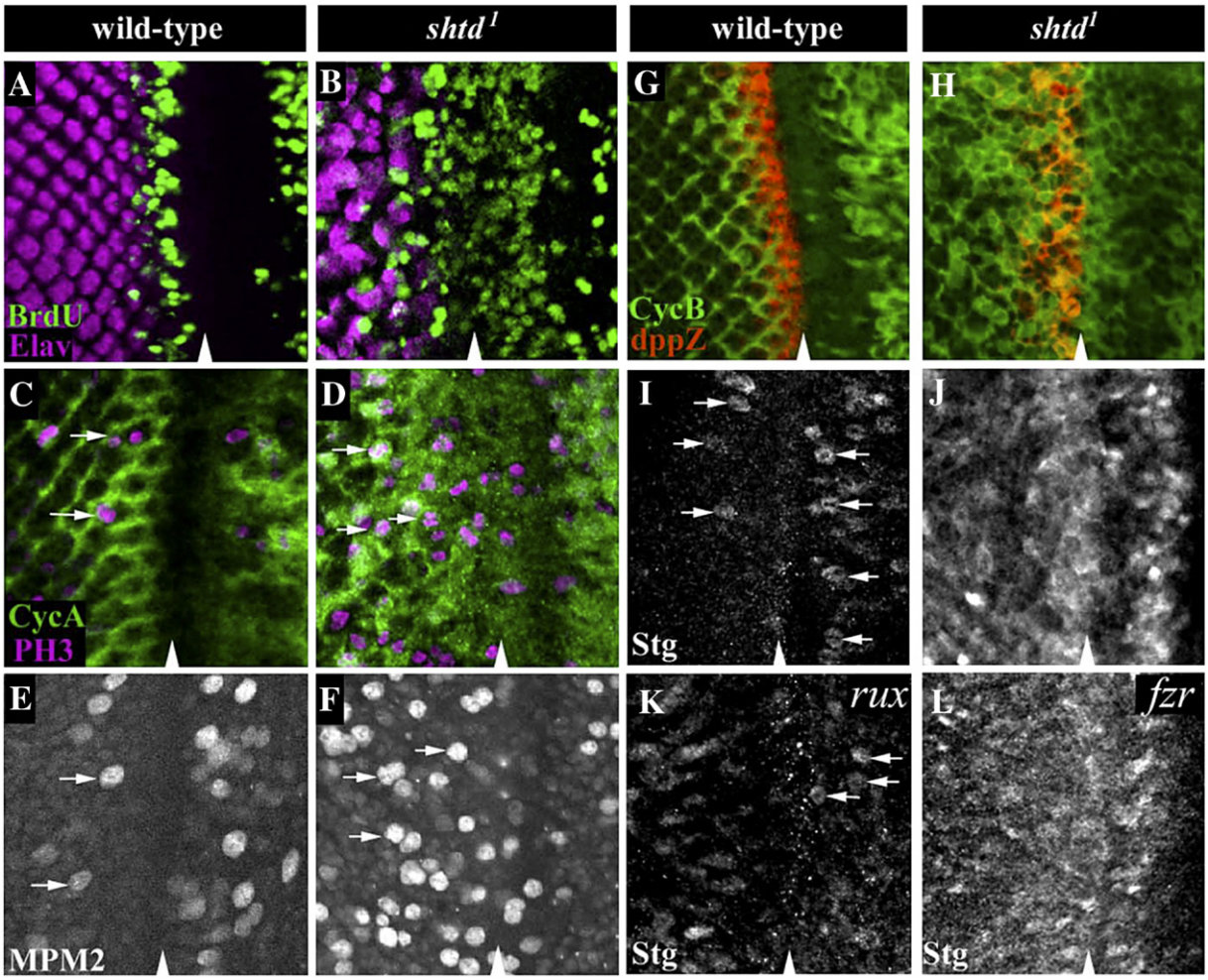


Fig. 3. Loss of *shtd* results in ectopic mitotic cyclins and String accumulation and failure to arrest in G1. (A, B) S phase cells are labeled with BrdU (green), and differentiating cells are labeled with the neuronal marker Elav (purple). BrdU incorporation in the SMW is seen just behind the MF and anterior to the Elav expressing neuronal cells in wild-type (A). In $shtd^{1}$, precocious BrdU incorporation is seen in many cells in the MF (B). For wild-type, basal BrdU and medial Elav optical sections were taken separately and two images merged. For $shtd^{1}$, a basal optical section is shown. (C–F) Mitotic cells were marked with PH3 (purple) (C, D) or MPM2 (E, F) (arrows). A large increase in PH3 and MPM2-positive cells is seen in and behind the MF in $shtd^{1}$ (D, F) compared to wild-type (C, E). CycA is shown in green (C, D). (G, H) CycB (green) prematurely accumulates in the MF (*dpp-lac*Z, red) in $shtd^{1}$ (H), but not in wild-type (G). (I) In wild-type, String protein is expressed transiently in metaphase cells just ahead of and behind the MF (arrows). (J) In $shtd^{1}$, String protein is highly expressed in cells in a band which corresponds to those cells expressing high levels of *stg* mRNA in wild-type. (K) String accumulation is not significantly increased in the $rux^{8}$ null mutant. A modest accumulation of String protein is seen posterior to the MF. Metaphase cells are identified in the MF (white arrows). (L) *fzr* mutants show an increased String protein levels. Arrowheads indicate the MF in all panels.

(Baker and Yu, 2001; Wolff and Ready, 1993). In $shtd^{1}$ eye discs, BrdU labeling showed that cells enter S phase precociously in the G1 arrest domain (Figs. 3A and B). We found that some Elav-positive neuronal cells incorporated BrdU posterior to the MF (Fig. 2D), indicating that differentiating photoreceptor cells enter an additional round of S phase. In contrast we did not observe BrdU incorporation into Sens-positive R8 cells (data not shown), indicating that the Sens-positive twinned R8 cells did not re-enter S phase prior to mitosis. These observations suggest that by the time R8 photoreceptors were determined and expressed Sens in $shtd^{1}$, the R8 cells had already completed S phase.

As $shtd^{1}$ mutant flies have much smaller eyes than wild-type, we also examined whether there is increased apoptosis in $shtd^{1}$ eye discs. Using Acridine Orange staining, there is a dramatic increase in the number of cells undergoing apoptosis in the posterior of the developing eye discs (compare Figs. 2E and F). Increased apoptosis is also observed in a pupal lethal allele of *shtd*, $shtd^{3}$. Larvae mutant for $shtd^{3}$ have greatly reduced imaginal tissues. We generated $shtd^{3}$ mutant clones in imaginal

Fig. 2. Loss of $shtd^{1}$ overrides G1 arrest of developing photoreceptor cells. (A) $shtd^{1}$ mutant eye discs double stained with Sens (green) and PH3 (purple). White arrowheads indicate Sens-positive metaphase cells. Anaphase or telophase cells are occasionally seen (inset). Behind the band of metaphase cells, Sens-positive R8 doublets are seen (white arrows). (B, C) Elav-labeled (green) neuronal cells colocalized with PH3 (purple) in the $shtd^{1}$ mutant (B), but not in wild-type (C). (D) Elav-stained cells (green) co-labeled with BrdU in $shtd^{1}$ (purple, white arrows). (E, F) Acridine orange staining showed a few apoptotic cells in wild-type (E). In contrast, a dramatic increase of apoptotic cells was seen in the posterior of the eye disc in the $shtd^{1}$ mutant (F).

discs using the FLP/FRT method. Increased expression of activated Caspase-3 was found in the *shtd*$^{3}$ mutant clones but not in the adjacent wild-type tissues (Fig. S2C). These data indicate that increased apoptosis also contributed to the *shtd*$^{1}$ mutant phenotype.

### *Accumulation of mitotic cyclins and String protein in shtd mutants*

To further characterize the observed cell cycle defect in *shtd*$^{1}$ mutants, we examined the expression pattern of cell cycle regulators. In the MF of the developing eye, the levels of mitotic cyclins CycA and CycB were low in wild-type eye discs (Figs. 3C and G, Fig. S1A). In *shtd*$^{1}$ eye discs, cells showed premature accumulation of CycA and CycB in the G1 arrest domain (Figs. 3D and H, Fig. S1B). Double labeling showed that ectopic accumulation of CycA correlated with the appearance of S phase cells in the mutant eye discs (Fig. S1B). In higher eukaryotes, entry into S phase normally requires CycE. In *Drosophila*, CycE is sufficient to trigger quiescent cells to enter S phase and CycE activation often led to the accumulation of the mitotic cyclins, CycA and CycB (Knoblich et al., 1994). In the eye disc, CycE accumulation begins in a subset of cells in the MF (Fig. S1C) (Richardson et al., 1995). Surprisingly, neither premature CycE enhancer activation nor ectopic CycE protein accumulation was observed in the MF of *shtd*$^{1}$ mutant eye discs (Fig. S1D and data not shown), suggesting that ectopic S phase entry in the mutant is independent of *CycE*. In *Drosophila*, high-level expression of CycA triggers the G1/S transition in wild-type embryos and eye discs (Dong et al., 1997; Sprenger et al., 1997) and this effect is also observed in mutant embryos lacking *CycE* (Sprenger et al., 1997). Thus, ectopic S phase entry of G1 arrested cells in *shtd*$^{1}$ correlated with the accumulation of the mitotic cyclin CycA.

In wild-type eye discs, *stg* mRNA expression is induced to high levels adjacent and anterior to a band of dpp-*lac*Z (BS3.0) in the MF and in a lattice pattern posterior to the furrow (Baonza et al., 2002; Blackman et al., 1991; Heberlein et al., 1995; Thomas and Zipursky, 1994). However, Stg protein is only observed transiently in metaphase cells in the proliferating population of cells anterior and posterior to the MF (Fig. 3I). By contrast, *shtd*$^{1}$ mutants accumulate high levels of Stg protein. Anterior to the MF, Stg accumulation corresponds to the band of *stg* mRNA expression (Fig. 3J and data not shown). The increased levels of Stg protein anterior to the MF are not due to an increase in *stg* transcription, since *stg* mRNA expression in this region is not significantly elevated in *shtd*$^{1}$ mutants as compared to wild-type (data not shown). Stg accumulation is also consistently observed in the *shtd*$^{3}$ allele. FLP/FRT-mediated mosaic clone analysis revealed that Stg protein accumulated in mutant clones that spanned the MF (Fig. S2A). In addition, increased CycB was also observed in *shtd*$^{3}$ mutant clones in the MF (Fig. S2B). These data indicate that Shtd regulates Stg protein level in addition to mitotic cyclins CycA and CycB.

The observation that *shtd*$^{1}$ mutant eye discs exhibit ectopic BrdU incorporation and CycA accumulation in the MF is similar to that of the previously characterized *roughex* (*rux*) mutants (Thomas et al., 1994). Rux was shown to be required for the G1 arrest in the MF by preventing the accumulation and/or activity of the mitotic cyclin, CycA (Avedisov et al., 2000; Foley et al., 1999; Thomas et al., 1997). Interestingly, *shtd*$^{1}$ and *rux* are distinct in their ability to regulate Stg protein. As shown in Fig. 3K, there are only a few Stg-positive cells observed near the MF in *rux* null mutants (Fig. 3K, white arrows) as compared to the large number of cells that accumulate Stg in *shtd*$^{1}$ mutants.

### *Mitotic delay in the shtd*$^{1}$ *mutants*

In wild-type eye discs behind the MF, large populations of cells between each developing cluster accumulate mitotic cyclins, CycA and CycB, indicating that cells are in G2 (Figs. 3C, G in green). All of these G2 cells eventually undergo mitosis, which is regulated in part through EGFR signaling (Figs. 3C, E white arrows; Baker and Yu, 2001; Baonza et al., 2002). In wild-type eye discs stained with the anti-PH3 antibody, cells displaying anaphase-like chromosomal configurations can readily be observed (Fig. 4B). In the *shtd*$^{1}$ mutants, however, many more PH3-positive cells were observed in and behind the MF and many cells displayed a condensed chromosomal morphology. In contrast, anaphase-like chromosomal configurations were quite rare (Fig. 4D). Double labeling of PH3 and CycA showed that many cells have stabilized CycA (Fig. 3D and data not shown). These observations suggest that the *shtd*$^{1}$ mutants have a defect in progression through mitosis, in particular during the transition from metaphase to anaphase. To further characterize the mitotic phenotype of *shtd*$^{1}$, we used the anti-MPM2 antibody to visualize and quantify mitotic cells (Ryo et al., 2001). It is expected that a mitotic delay will significantly increase the number of cells in mitosis. An average of 59±5 mitotic cells was seen in and behind the MF in *shtd*$^{1}$ as compared to 17±5 in wild-type (Figs. 3E and F, $n=6$ for both mutant and wild-type). These observations support the idea that there is a mitotic delay in the *shtd*$^{1}$ mutants. Interestingly, the number of mitotic cells anterior to the MF was also slightly increased in *shtd*$^{1}$ mutant as compared to that in wild-type (18±6 versus 10±2, respectively) while no difference was seen in the number of S phase cells in the mutant as compared to wild-type anterior to the MF (24±9 for *shtd*$^{1}$, $n=8$, versus 24±12 for wild-type, $n=9$). These observations suggest that *shtd*$^{1}$ may also cause a delay in progression through mitosis in the anterior asynchronously proliferating cells even though it did not affect the rate of S phase entry there.

### *The shtd gene encodes Drosophila Apc1*

In order to further characterize the gene corresponding to *shtd*, the *shtd* locus was mapped to the 13C-D region by deficiency mapping (Fig. 5A, see Materials and methods). Imprecise excision of EP1109 (Fig. 5B and Materials and methods) generated one allele, *shtd*$^{EP\Delta}$, in addition, two

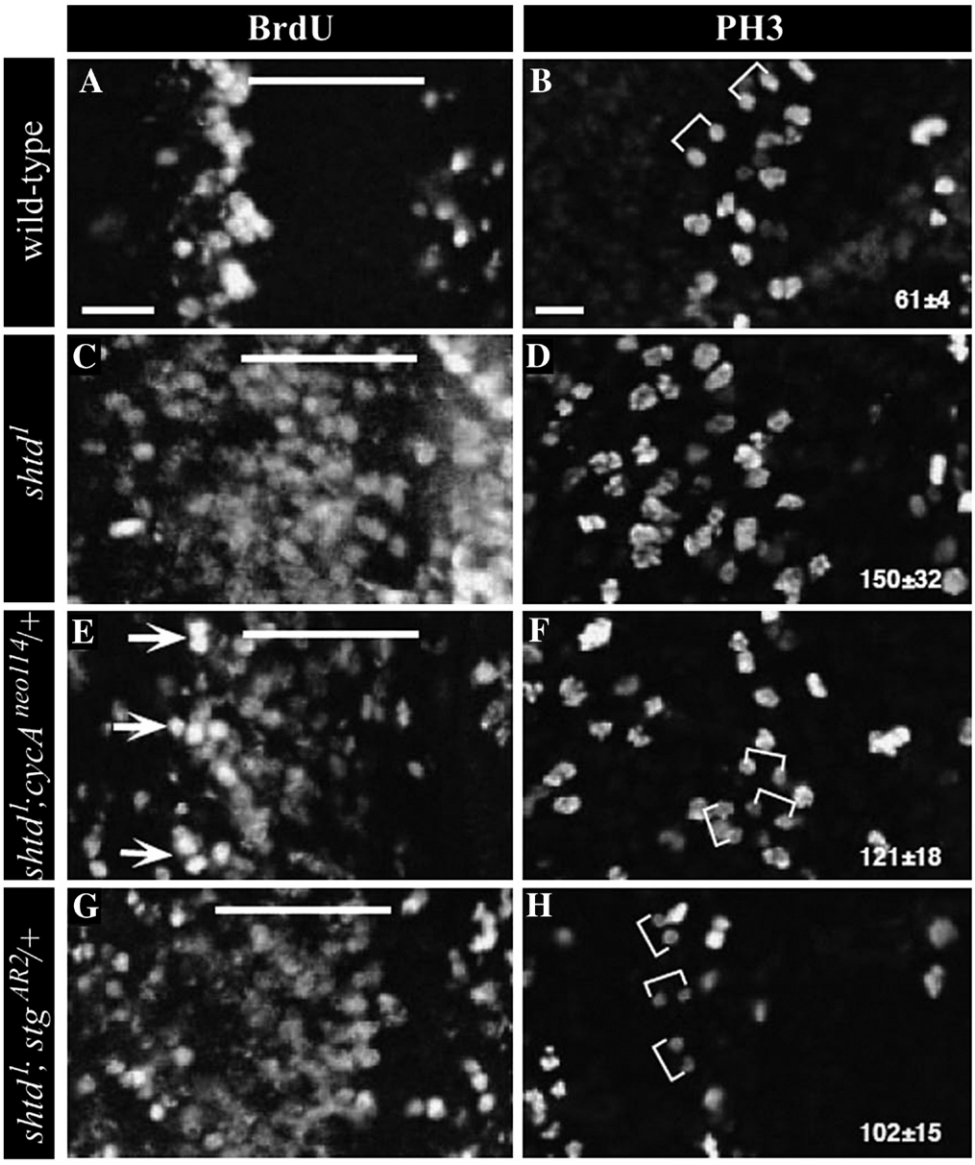


Fig. 4. Reducing *stg* dosage suppresses the mitotic delay phenotype of *shtd*$^{1}$. BrdU (A, C, E, G) and PH3 labeling (B, D, F, H) to show S phase and mitotic cells, respectively, in wild-type (A, B), *shtd*$^{1}$ (C, D), *shtd*$^{1}$*; CycA*$^{neo114}$/+ (E, F), and *shtd*$^{1}$*; stg*$^{RXT13}$/+ (G, H). The MF is indicated by a line and brackets show pairs of anaphase or telophase cells. Arrows indicate clusters of S phase cells with a more wild-type appearance that arise upon reducing *CycA* gene dosage in the mutant. Mitotic cells were counted in a unit area (wild-type, $n=3$; *shtd*$^{1}$, $n=7$; *shtd*$^{1}$*; CycA*$^{neo114}$/+, $n=4$; *shtd*$^{1}$*; stg*$^{RXT13}$/+, $n=4$) and the average number of PH3-positive cells is shown ±SD. Nuclei with visibly divided chromosome masses were counted as two units. Scale bars, 10 μm.

gamma-ray-induced lethal alleles, *shtd*$^{2}$ and *shtd*$^{3}$, were generated that also failed to complement the *shtd*$^{1}$ eye phenotype. Inverse PCR analysis from *shtd*$^{EP\Delta}$ showed that this allele results from a 33 kb deletion (Fig. 5B). To determine whether the remaining alleles contained chromosomal rearrangements, we performed PCR mapping across a 519 kb region corresponding to cytological bands 13B-E from *shtd*$^{1}$, *shtd*$^{2}$ and *shtd*$^{3}$ (Materials and methods). The *shtd*$^{2}$ allele also results from a deletion minimally 56 kb in size that completely overlaps the 33 kb deletion in *shtd*$^{EP\Delta}$; no chromosomal rearrangements were detected in *shtd*$^{1}$ or *shtd*$^{3}$ by this analysis. We conclude that the 33 kb region uncovered by *shtd*$^{EP\Delta}$ contains *shtd*.

The 33 kb region contains ten annotated genes (Grumbling, 2006). The cell cycle defects of *shtd* mutants described above led us to focus on CG9198, which encodes the Anaphase promoting complex 1 (Apc1), the largest subunit of the APC/C. The *Drosophila* Apc1 shows 36% identity and 54% similarity to the human and mouse proteins over its entire length. To determine if *shtd* corresponds to *Apc1*, we performed genomic rescue experiments. A genomic fragment containing *Apc1* was isolated from a P1 clone (Fig. 5B and Materials and methods), and multiple transgenic flies were established. Both the *shtd*$^{1}$ eye phenotype and the *shtd*$^{3}$ lethality were rescued by the genomic fragment containing *Apc1*. Sequence analysis showed that both *shtd*$^{1}$ and *shtd*$^{3}$ contain point mutations in *Apc1* (Fig. 5C). The *shtd*$^{1}$ allele contains a single Glu-to-Lys substitution at position 1184, while *shtd*$^{3}$ contains a substitution of Ala-to-Pro at 1287 and

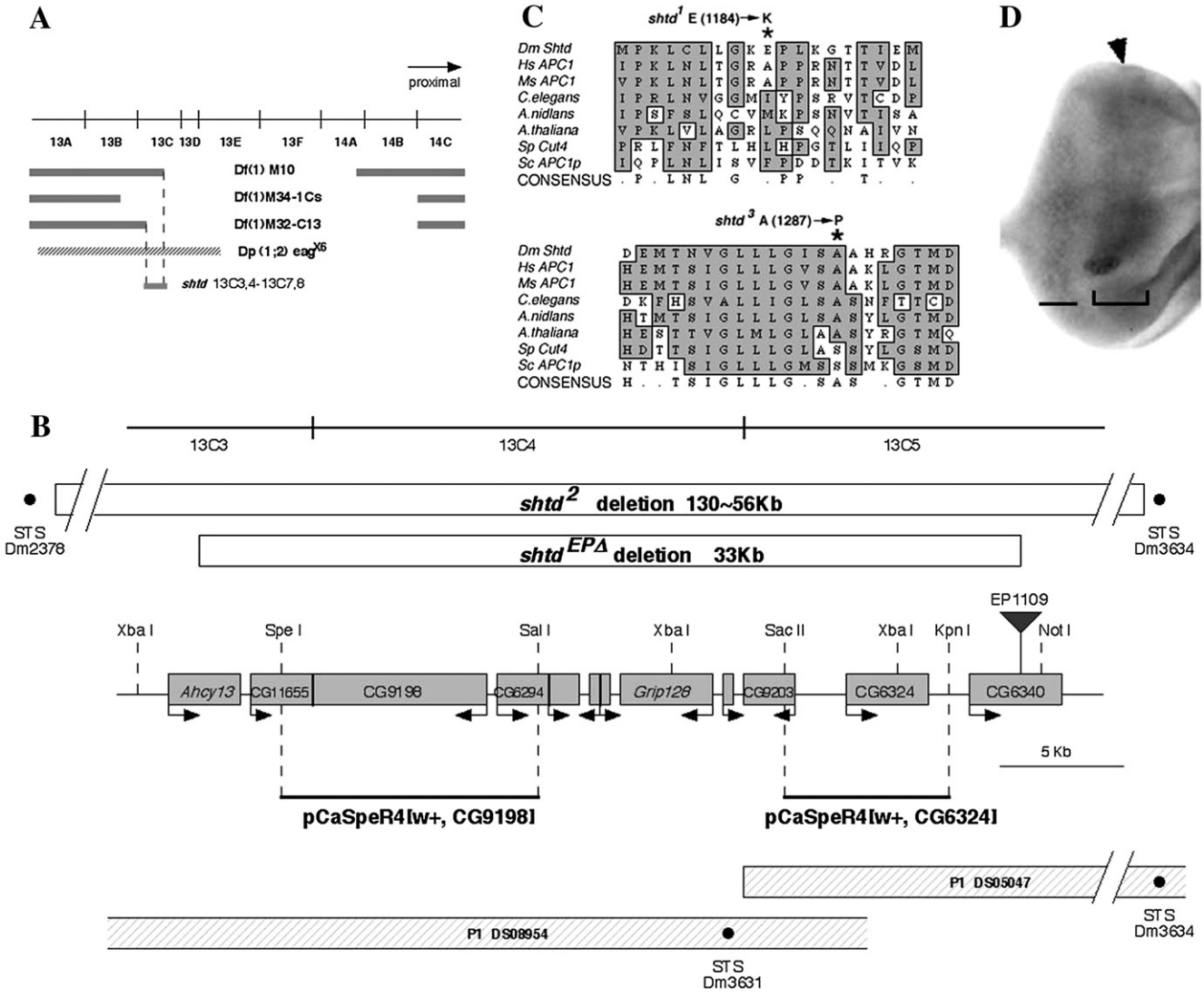


Fig. 5. *shtd* encodes the Apc1 homolog. (A) *shtd* deficiency mapping. Deficiencies uncovering the 13C region and the duplication chromosome *Dp(1;2) eag*$^{x6}$ are shown. The *shtd* gene maps by complementation to 13C3,4-13C7,8. (B) Schematic representation of the *shtd* locus. An approximately 35 kb region surrounding CG9198 is shown. The *shtd*$^{EP\Delta}$ and *shtd*$^{2}$ deletions are shown by open boxes; the breakpoints of *shtd*$^{2}$ fall outside of the range of this map, and the nearest intact STS markers are shown. The insertion site of EP1109, which was used for the imprecise excision, is shown by an inverted triangle. Predicted open reading frames are shown by shaded boxes, with the putative direction of transcription showed by the arrow. P1 clones and STS markers (black dots) are shown at the bottom. For the rescue experiment, genomic fragments were isolated from P1 clones (solid black lines). The genomic fragment that contains the CG9198 ORF rescued *shtd* mutant phenotype while the fragment that contains the CG6324 did not. The restriction enzyme sites used for subcloning are shown on the map. (C) The *shtd*$^{1}$ allele results from a substitution of lysine for glutamic acid at position 1184 (asterisk). The *shtd*$^{3}$ allele results from a substitution of proline for a conserved alanine at position 1287 (asterisk). (D) *shtd* mRNA expression pattern in third instar eye disc. Expression is higher in the domain surrounding the MF (bracketed region) and posterior portion of eye disc (bar). The arrowhead indicates the MF.

a 5 bp deletion in the promoter region of *Apc1*. Therefore, both *shtd*$^{1}$ and *shtd*$^{3}$ are loss-of-function alleles of *Apc1*.

Analysis of *shtd* mRNA expression pattern in embryos by *in situ* hybridization showed a large component of maternal mRNA during early stages of embryogenesis (Fig. S3A). Later expression is restricted to a particular patterned domain (Fig. S3C and S3D). In third instar eye discs, *shtd* expression is higher in the region covering the MF, and also in the posterior domain of eye discs (Fig. 5D). This mRNA expression pattern, particularly the region covering the MF, correlates with the region in which we observed the high level accumulation of Stg and mitotic cyclins in the *shtd*$^{1}$ mutants.

### *Genetic interactions identify stg, CycA, and apoptotic regulators as modifiers of shtd*$^{1}$

Dosage-sensitive enhancement or suppression of mutant phenotypes in *Drosophila* is a powerful method to identify genes that act in common genetic pathways. In particular, loci that dominantly suppress the *shtd*$^{1}$ mutant phenotype at reduced gene dosage are potential substrates for APC/C-mediated proteolysis or regulators of APC/C activity. We used a series of overlapping deficiencies of the second and third chromosomes to search for modifiers of the *shtd*$^{1}$ eye phenotype (Fig. 6B). Two deficiencies that uncovered the 99A region of the

third chromosome suppressed $shtd^{1}$; this region contains *stg*. Multiple alleles of *stg* strongly suppressed the $shtd^{1}$ eye phenotype (Fig. 6E, Table 1).

We also recovered genetic interactions between $shtd^{1}$ and genes previously shown to regulate CycA-dependent kinase activity (Table 1). Reducing the dosage of *cdc2*, *CycA*, or *rca1* all partially suppressed $shtd^{1}$ eye phenotype (Figs. 6D, F and data not shown). CycA was shown to be regulated by *rux* (Sprenger et al., 1997; Thomas et al., 1997). Increasing the gene dosage of *rux* or *fzr* partially reduced $shtd^{1}$ eye roughness (Table 1) whereas double-mutant combinations of the viable alleles $shtd^{1}$ and $rux^{3}$ or $fzr^{rape6}$ are synthetic lethal at pupal or early larval stages, respectively. Furthermore, the $rux^{3}$, $shtd^{1}$ double-mutant eye discs exhibited more severe defects of aberrant mitotic figures and abnormal ommatidial spacing than that seen in either $rux^{3}$ or $shtd^{1}$ alone (data not shown). These genetic

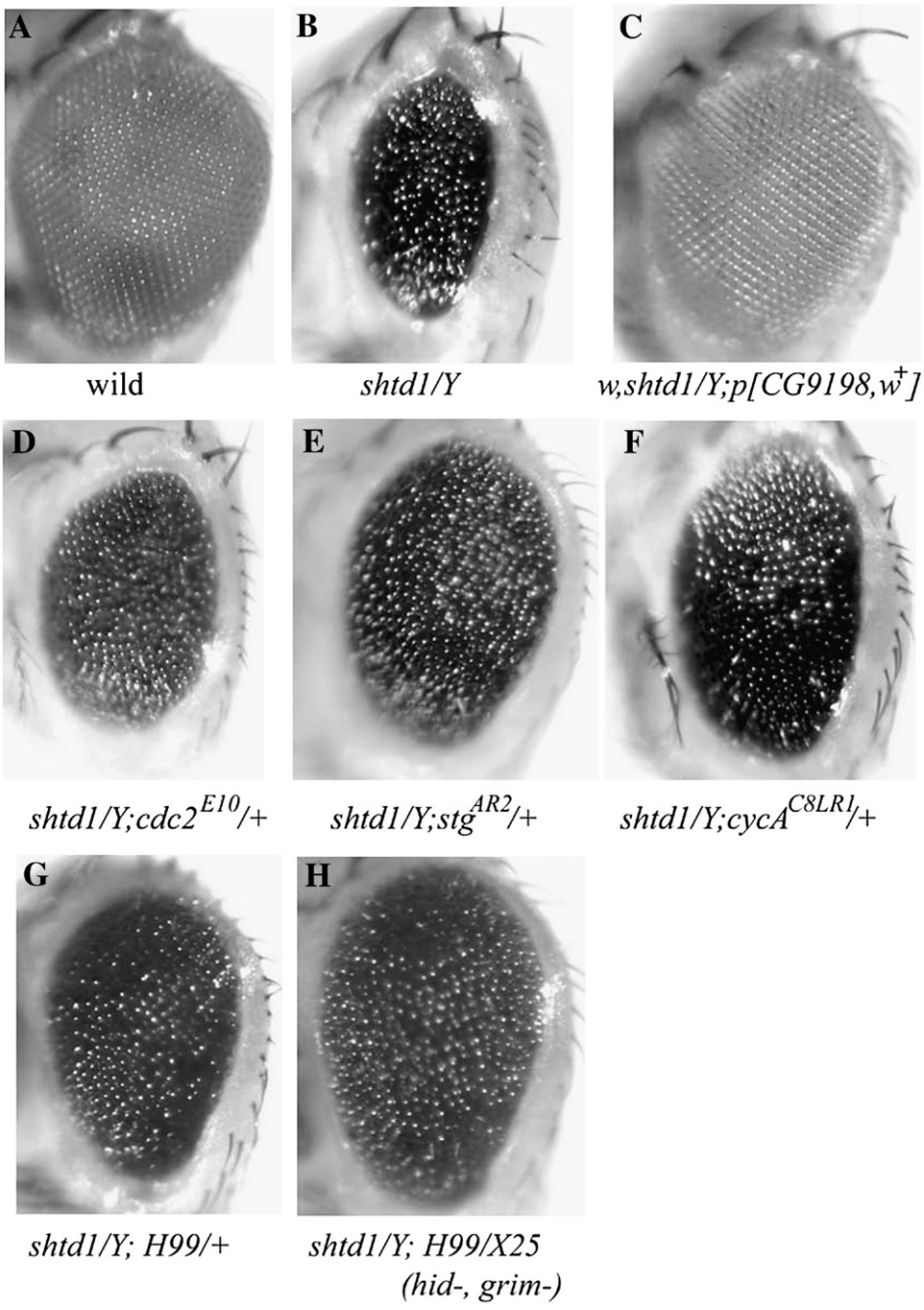


Fig. 6. Suppression of $shtd^{1}$ adult eye phenotypes. (A, B) A $shtd^{1}$ mutant male fly eye (B) displays a rough and reduced eye compared to wild-type (A). (C) The $shtd^{1}$ rough eye phenotype is completely rescued by introducing one copy of the *CG9198* gene. (D–H) $shtd^{1}$ modifiers. A one-copy reduction of *cdc2* (D), *stg* (E), or *CycA* (F) suppresses the $shtd^{1}$ eye phenotype. A one-copy reduction of the apoptosis regulators *rpr*, *hid* and *grim* (*Df(3L)H99*/+, G) or removal *hid* and *grim* (*Df(3L)H99/Df(3L)X25*, H) also shows suppression. The size of the compound eye is larger and the eye roughness is milder than in $shtd^{1}$ alone. Adult male fly eye images were taken under a Leica dissecting microscope.

Table 1
*shtd*$^{1}$ genetic interactions

| Gene | Allele/deficiency/transgene | Dosage | Effect [a] |
|---|---|---|---|
| *Cell cycle regulators* | | | |
| *stg/Cdc25* | *AR2, RXT13, 01215, 7B, 7M* | ↓ | Suppress |
| *CycA* | *neo114, C8LR1, 03946* | ↓ | Suppress |
| | *l(3)183, hari* | ↓ | None |
| *CycB* | *Df(2R)59AB* | ↓ | None |
| *CycB3* | *L6540* | ↓ | None |
| *CycE* | *AR95, 05206, P4, JP, l(2)35Dd* | ↓ | None |
| *CycH* | *EP3658* | ↓ | Enhance |
| *cdc2* | *B47, E10, E1-24, E51Q, Df(2L)J27* | ↓ | Suppress |
| *cdc2c* | *Df(3R)H81* | ↓ | None |
| *Cdk4* | *k06503, 05428, s4639* | ↓ | None |
| *rux* | *3, 8* | ↓ | Lethal [b] |
| | *pRux+6.0D1* | ↑ | Suppress [c] |
| *APC subunits and regulators* | | | |
| *mr/Apc2* | *1, 2* | ↓ | Suppress |
| *lmg/Apc11* | *03424* | ↓ | Suppress |
| *ida/Apc5* | *B4, D14* | ↓ | Suppress |
| *mks/Cdc27/Apc3* | *L7123* | ↓ | None |
| *fzr/rap/Cdh1* | *x-3, e6* | ↓ | Lethal [b] |
| | *Dp(1;2)rb±71 g* | ↑ | Suppress |
| *fzy/Cdc20* | *1, 4, 5, 6, Df(2L)r10* | ↓ | None |
| | *pFzy8+II.1, pFzy8+III.1* | ↑ | None [c] |
| *rca1/Emi1* | *IX KS2.30.1* | ↓ | Suppress |
| *Apoptosis regulators* | | | |
| *rpr, hid, grim* | *Df(3L)H99* | ↓ | Suppress |
| *hid, grim* | *Df(3L)X25* | ↓ | Suppress [d] |
| *rpr* | *Df(3L)XR38* | ↓ | Suppress [d] |
| *hid* | *05014* | ↓ | Suppress [d] |
| *th/diap1* | *4,5* | ↓ | Enhance |

[a] Enhance or suppress refers to the *shtd*$^{1}$ eye phenotype. Unless otherwise indicated, genetic interactions were tested by reducing the dosage of the indicated gene in males (i.e., *shtd*$^{1}$*/Y; gene/+*).

[b] Interactions were assessed as double-mutant combinations.

[c] These crosses assessed an increase in gene dosage using genomic rescue constructs.

[d] Interactions were assessed as double mutant combinations (*shtd*$^{1}$*/Y; Df(3L)X25/Df(3L)H99, shtd*$^{1}$*/Y; Df(3L)XR38/Df(3L)H99* or *shtd*$^{1}$*/Y; hid*$^{05014}$*/Df(3L)H99*).

interactions are consistent with previous observations that *rux* mutants are defective in their ability to prevent the accumulation and/or activity of the mitotic cyclin, CycA (Avedisov et al., 2000; Foley et al., 1999; Foley and Sprenger, 2001; Thomas et al., 1997), and those showing a failure to degrade mitotic cyclins in *fzr* mutants (Sigrist and Lehner, 1997).

In addition, decreasing the dosage of apoptotic regulators such as *hid*, *rpr* or *grim* also significantly suppressed the *shtd*$^{1}$ eye phenotype (Figs. 6G and H). This is consistent with the observation that there is significantly increased apoptosis in *shtd*$^{1}$ mutants (Fig. 2F). Surprisingly, reducing the gene dosage of several APC/C subunits using available mutations suppressed *shtd*$^{1}$ (Table 1, Fig. S5). Examination of ectopic cell proliferation by BrdU incorporation and anti-PH3 staining showed that the observed suppression was correlated with a decreased ectopic BrdU incorporation in the MF and a corresponding increase in the number of anaphase cells (Fig. S6). Interestingly, the level of CycA was slightly reduced in *shtd*$^{1}$*; mr*$^{2}$*/+* and *shtd*$^{1}$*; ida*$^{B4}$*/+* eye discs as compared to that of *shtd*$^{1}$ (data not shown). It is possible that the partial APC/C complex without Apc1 may exert a dominant negative effect on the degradation of some key substrates.

The *shtd*$^{1}$ mutant phenotype is not limited to the eye. The wing margin bristles of the adult wing develop from the zone of non-proliferating cells (ZNC), which were shown to be arrested at G1 or G2 (Johnston and Edgar, 1998). The ordered array of wing margin bristles was disrupted in *shtd*$^{1}$ as compared to that in wild-type (Figs. S4A and S4B). This phenotype was suppressed by reduction of either *stg* or of apoptosis regulators (Figs. S4C–S4F), indicating that the observed genetic interactions between *shtd*$^{1}$ and *stg* or the apoptosis regulators are not limited to the eye.

### *Mutations in stg and CycA suppress shtd*$^{1}$ *through distinct mechanisms*

Genetic interaction experiments show that reducing the gene dosage of *CycA*, *cdc2* or *stg* suppresses the *shtd*$^{1}$ rough eye phenotype. In mammalian cells, a G1 form of the phosphatase, Cdc25A, functions in G1 to activate CycE- and CycA-Cdk2 complexes to promote S phase entry (Blomberg and Hoffmann, 1999). One model that would account for the observed genetic interactions is that Stg functions similarly in *Drosophila* to activate a CycA-dependent kinase complex in G1 cells which drives S phase entry. Alternatively, it is possible that *stg* suppresses the *shtd*$^{1}$ eye phenotype through a distinct mechanism. To distinguish between these two possibilities, we examined *shtd*$^{1}$ eye discs with reduced gene dosage of either *CycA* or *stg* for their ability to undergo G1 arrest and mitosis (Fig. 4).

Reducing the dosage of either *CycA* or *cdc2* in *shtd*$^{1}$ partially rescued the ectopic S phase phenotype seen in the mutants; the numbers of ectopic S phase cells within the MF were reduced relative to *shtd*$^{1}$ alone (Fig. 4E, data not shown), and groups of cells more strongly labeled with BrdU were observed just behind the MF in a position equivalent to the synchronous band of S phase cells seen in wild-type discs (Fig. 4E, arrows). In addition, reducing the gene dosage of *CycA* in *shtd*$^{1}$ also led to a moderate increase in the number of anaphase or telophase cells and a slight reduction in the total number of PH3-positive cells (from 150±32 to 121±18, Figs. 4D and F). It is possible that the increase in anaphase or telophase cells results from the restoration of normal S phase progression in a subset of cells behind the MF. By contrast, reducing the dosage of *stg* had no significant effect on the ectopic S phase phenotype of *shtd*$^{1}$ (Fig. 4G), but instead showed a significant suppression of the mitotic delay phenotype seen in and behind the MF than what was observed with the reduction of *CycA* (Fig. 4H). These discs exhibited fewer total number of PH3-labeled cells than the *shtd*$^{1}$ and *shtd*$^{1}$; *CycA/+* discs and a corresponding increase in telophase cells. These results suggest that the reduction of *stg* suppressed the *shtd*$^{1}$ mutant phenotype by partially restoring mitotic progression. This conclusion is further supported by the observation that we were unable to drive ectopic S phase

entry in the MF by overexpression of Stg (data not shown). Taken together, these observations suggest that partial loss of Apc1 function leads to hyper-accumulation of CycA and Stg, which results in ectopic S phase entry in MF, mitotic delay, and increased apoptosis. These phenotypes can be suppressed by reducing the dosage of *CycA*, *stg*, and components of the apoptosis pathway, respectively.

## Discussion

Our analysis of the *Drosophila* Apc1 homologue mutant *shtd*$^{1}$ revealed a failure in establishing G1 cell cycle arrest in the MF and a delay in progression through mitosis. The question is: how do the observed cell cycle defects lead to the dramatic developmental defects observed in *shtd*$^{1}$ mutants? As we did not observe BrdU incorporation of Sens-positive cells, it is likely that by the time Sens is induced in the future R8 cells, these cells have mostly completed S phase. Since initial Sens expression in the developing eye disc of *shtd*$^{1}$ mutants appears relatively normal, we propose that the initial specification of the first R8 cells proceeds relatively normally even in the absence of normal G1 arrest in the MF. However, these R8 photoreceptor cells that have completed S phase will proceed into mitosis, leading to highly defective arrangement of R8 photoreceptor cells. Since the rest of the photoreceptor cells and accessory cells are recruited after R8 specification, defects in the arrangement of R8 photoreceptors will cause severe defects in eye development. Of course, additional S phase and mitosis of other R cells types will also contribute to the *shtd*$^{1}$ mutant phenotypes. Consistent with this idea, reducing the dosage of *CycA* suppressed both the ectopic S phase entry in MF and the *shtd*$^{1}$ adult eye phenotype.

In addition to a failure to establish and maintain G1 arrest in MF, *shtd*$^{1}$ mutants also exhibited delayed progression through mitosis. While delayed progression through mitosis in the proliferating cells such as the anterior of the eye discs will not likely cause dramatic defects, it is possible that delayed progression through mitosis in the posterior may result in defects in photoreceptor cell recruitment and contribute to the significantly decreased R3 and R4 cells in *shtd*$^{1}$ mutants. It was shown previously that cell shape changes required for ventral furrow formation during *Drosophila* embryogenesis were incompatible with mitosis and that precocious mitosis in this region blocked ventral furrow formation (Grosshans and Wieschaus, 2000). Similarly, photoreceptor differentiation is preceded by significant cell shape rearrangements, forming clusters with differential adhesive properties (Brown et al., 2006). Therefore, it is possible that the cell shape changes required for the proper differentiation of photoreceptor cells are not compatible with mitosis. As proper differentiation of photoreceptor cells and the other accessory cells involve stepwise recruitment, it is conceivable that cells that remain in mitosis for an extended period of time can contribute to eye development defects. Consistent with this idea, reducing the gene dosage of *stg* significantly suppressed the mitotic delay posterior to the MF and significantly suppressed the *shtd*$^{1}$ adult eye phenotypes.

One of the obvious phenotypes of *shtd*$^{1}$ mutants is the dramatically increased apoptosis in the larval eye disc and the small size of the adult eye. Although we do not know the exact cause for the extensive apoptosis in the eye disc, it is likely a consequence of the defects in cell cycle control or defects in differentiation. Consistent with this, dosage reduction of either *stg* or *CycA* partially restored cell cycle defects of *shtd*$^{1}$ mutant eye discs and partially suppressed adult eye size defect as well.

Although the primary developmental phenotypes observed in *shtd*$^{1}$ mutants are likely caused by defects in cell cycle control, this does not exclude the possibility that the APC/C may also function directly in regulating the properties of the postmitotic differentiating cells. Recently, a few studies identified crucial roles of APC/C-Cdh1 in controlling axon growth and patterning in developing brain and synapse size/function through proteolysis of key proteins (Juo and Kaplan, 2004; Konishi et al., 2004; Lasorella et al., 2006; van Roessel et al., 2004). Interestingly, the APC/C regulatory subunit Cdh1 but not Cdc20 is required to exert this postmitotic APC/C function.

The *shtd*$^{1}$ mutant phenotype has both similarities and differences with the *rux* mutant phenotypes (Thomas et al., 1994). Both the *shtd*$^{1}$ and the *rux* mutants showed accumulation of CycA and ectopic S phase in MF. In contrast, only *shtd*$^{1}$ but not the *rux* mutants displayed accumulation of Stg and delayed progression through mitosis. These observations are consistent with the observed suppression of *shtd*$^{1}$ and *rux* mutants by reducing the gene dosage of *CycA*: while *rux* mutants were fully suppressed by reducing *CycA* gene dosage (Avedisov et al., 2000; Foley et al., 1999; Foley and Sprenger, 2001; Thomas et al., 1997), the *shtd*$^{1}$ rough eye phenotype was only partially suppressed (Fig. 6F). These observations suggest that while the *rux* mutant phenotype results primarily from ectopic S phase entry due to CycA accumulation, the *shtd*$^{1}$ mutant phenotype results from both ectopic S phase entry and mitotic delay as a consequence of CycA and Stg accumulation, respectively. Currently, it is not clear how accumulation of Stg protein delays progression through mitosis. It is possible that in *shtd*$^{1}$, reduced APC/C activity is unable to quickly inactivate mitotic cyclin/Cdk activities, leading to delayed mitotic exit. Reducing the dosage of *stg* may help the inactivation of mitotic cyclin/Cdk activity during mitotic exit and promote mitotic progression. Alternatively, several APC/C subunits require phosphorylation on multiple sites for activity and dephosphorylation of the APC/C is sufficient for its inactivation (Herzog et al., 2005; King et al., 1995; Peters et al., 1996; Yamada et al., 1997). It is possible that Stg may directly or indirectly regulate APC/C activity to promote progression through mitosis.

In *Drosophila*, Stg protein levels are precisely controlled to promote the G2-M transition. Previous studies indicated that Stg is rapidly degraded upon exit from mitosis, although the mechanisms controlling its stability were not known (Edgar et al., 1994). More recently, studies in mammalian cells showed that degradation of Cdc25 upon exit from mitosis is dependent on APC/C-Cdh1 and a KEN-box motif at the N-terminus of Cdc25 (Donzelli et al., 2002). We found that the *Drosophila*

Cdc25 homologue Stg accumulates in a region that displays high levels of *stg* mRNA expression in *shtd*$^{1}$ mutants, suggesting that proteolysis of *Drosophila* Stg in the developing eye requires the Shtd function. Since we observed strong genetic interactions between *shtd* and *fzr*, which encodes the *Drosophila* Cdh1 subunit of APC/C complex, and since *fzr* mutants also accumulate Stg protein in the MF, we suggest that Stg degradation is regulated by APC/C-Cdh1.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.07.007.

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