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Shar-pei mediates cell proliferation arrest during imaginal disc growth in *Drosophila*

Madhuri Kango-Singh[1], Riitta Nolo[1], Chunyao Tao[1], Patrik Verstreken[3], P. Robin Hiesinger[4], Hugo J. Bellen[3,4,5] and Georg Halder[1,2,3,*]

[1]Department of Biochemistry and Molecular Biology, M. D. Anderson Cancer Center, Houston, TX 77030, USA
[2]Program in Genes and Development, M. D. Anderson Cancer Center, Houston, TX 77030, USA
[3]Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030, USA
[4]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA
[5]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA
*Author for correspondence (e-mail: ghalder@mdanderson.org)

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SUMMARY

During animal development, organ size is determined primarily by the amount of cell proliferation, which must be tightly regulated to ensure the generation of properly proportioned organs. However, little is known about the molecular pathways that direct cells to stop proliferating when an organ has attained its proper size. We have identified mutations in a novel gene, *shar-pei*, that is required for proper termination of cell proliferation during *Drosophila* imaginal disc development. Clones of *shar-pei* mutant cells in imaginal discs produce enlarged tissues containing more cells of normal size. We show that this phenotype is the result of both increased cell proliferation and reduced apoptosis. Hence, *shar-pei* restricts cell proliferation and promotes apoptosis. By contrast, *shar-pei* is not required for cell differentiation and pattern formation of adult tissue. Shar-pei is also not required for cell cycle exit during terminal differentiation, indicating that the mechanisms directing cell proliferation arrest during organ growth are distinct from those directing cell cycle exit during terminal differentiation. *shar-pei* encodes a WW-domain-containing protein that has homologs in worms, mice and humans, suggesting that mechanisms of organ growth control are evolutionarily conserved.

Key words: *Drosophila*, Imaginal discs, Cell proliferation, Apoptosis, WW domain-protein

INTRODUCTION

Cell proliferation must be tightly regulated to ensure the development of properly proportioned organs and tissues. During development, most organ primordia grow by increasing in cell number until the appropriate organ size is attained. Cells then exit from the cell cycle and differentiate into specific cell types. Defects in cell cycle exit result in excess cells and underlie tumor progression. Mechanisms that promote cell cycle exit are thus fundamental to development and understanding them should help us elucidate how tumors progress.

The *Drosophila* imaginal discs provide an excellent model system to study how cell proliferation is regulated during organ growth (Edgar and Lehner, 1996; Johnston and Gallant, 2002). Imaginal discs are epithelial sacs that differentiate into the external structures of head, thorax and genitalia of the adult fly (Cohen, 1993). Each disc develops from 10-30 precursor cells that proliferate extensively during the larval stages to give rise to approximately 50,000 cells in case of wing and eye discs, before differentiating into the corresponding adult structures during metamorphosis (Bryant, 1978). The growth of imaginal discs to specific sizes and shapes is directed by secreted signaling molecules including Decapentaplegic (Dpp), a TGFβ homolog, Wingless (Wg) and Hedgehog (Hh), which act as morphogens to induce patterning and growth (Day and Lawrence, 2000; Lawrence and Struhl, 1996; Serrano and O'Farrell, 1997). Although these factors may still be expressed, imaginal disc cells stop proliferating when discs reach their correct size (Bryant and Levinson, 1985). In addition, transplantation experiments revealed that developing discs transplanted into adult hosts grow until they reach their normal size and shape but do not grow larger than normal size even though they are not forced to differentiate (Bryant and Levinson, 1985; Garcia-Bellido, 1965). Therefore, mechanisms exist that terminate cell proliferation when discs have reached their correct size.

The generation of discs with stereotypical sizes and shapes is not the result of a predetermination of the number of cell divisions of progenitor cells. This is evident because the size of clones of imaginal cells is variable and thus not determined (Postlethwait, 1978), and cells can compensate for growth defects by extra proliferation. The flexibility of cell lineages is best illustrated by the observation that mitotic clones of cells that are induced to grow faster than their neighbors are significantly larger compared with wild-type clones (Morata

and Ripoll, 1975). Notably, after such manipulation of proliferation rates, the final pattern and size of the adult structures are normal. Moreover, discs can regenerate missing parts after surgical manipulation (Bryant, 1978; Bryant and Simpson, 1984) and when ~75% of the progenitor cells of imaginal discs are killed by X-rays, the remaining cells proliferate and compensate for the loss of cells (Haynie and Bryant, 1977). Hence, cell proliferation is plastic and cells in a developing tissue adjust their proliferation depending on whether more cells are needed to build a normal sized structure (Day and Lawrence, 2000; French et al., 1976; Garcia-Bellido and Garcia-Bellido, 1998). However, the molecular mechanisms that direct cells to stop proliferating once the primordium of a structure has reached the correct size are poorly understood.

In principle, defined organ size can be generated either by regulating the extent of cell proliferation or by eliminating superfluous cells through programmed cell death, or both. Only limited amounts of cell death are observed during imaginal disc growth (Milan et al., 1997; Wolff and Ready, 1991), indicating that disc size is primarily, albeit not exclusively, controlled at the level of cell division. Thus, factors must exist that regulate the decision of imaginal disc cells to re-enter or exit the cell cycle to mediate growth control.

The *Drosophila* eye is particularly well suited to identify factors that regulate cell proliferation. First, the various stages of cell division and differentiation can be accurately followed in eye imaginal discs. Second, defects in growth control and differentiation can be easily scored. In the early growth phase of the eye disc, cell cycles are not synchronized and proliferating cells are evenly distributed throughout the disc (Baker, 2001). Later, during the third larval stage, a wave of differentiation called the morphogenetic furrow sweeps across the eye disc from posterior to anterior (Wolff and Ready, 1993). Cells anterior to the furrow are developmentally uncommitted and divide asynchronously, whereas cells within the furrow arrest in the G1 phase of the cell cycle, synchronize and either start to differentiate into photoreceptor cells as they leave the furrow or undergo one additional round of cell division, referred to as the second mitotic wave before differentiating into the remaining photoreceptor, cone, pigment and bristle cells (Baker, 2001; Dickson and Hafen, 1993; Wolff and Ready, 1993). Thus, different modes of cell proliferation control can be studied with single cell resolution.

To gain insights into the mechanisms that regulate cell proliferation during organogenesis, we conducted a genetic screen in *Drosophila* to identify mutations that affect adult eye size. We describe the identification and phenotypic characterization of a novel gene, *shar-pei* (*shrp*), that is required for cells to terminate proliferation once imaginal discs have reached their correct size. Flies with *shrp* mutant tissues have enlarged structures that contain more cells of normal size. These overgrowths result from an extended period of cell proliferation, accompanied by a decrease in cell death. Based on these observations, we propose that *shrp* regulates organ growth by promoting both cell cycle exit and apoptosis. As *shrp* is not required for terminal cell cycle exit, we conclude that control of proliferation arrest during organ growth is separable from, and probably precedes, cell cycle exit during terminal differentiation. The presence of *shrp* homologs in other species suggests that the mechanisms that control tissue size are evolutionarily conserved.

MATERIALS AND METHODS

Fly stocks

shrp mutants were isolated from an *ey*FLP-mediated EMS mutagenesis screen using a strategy described earlier (Newsome et al., 2000). Adult heads were screened for phenotypes that displayed defects only in size/growth but retained wild-type pattern elements on the head cuticle. A total of six *shrp* alleles were recovered, balanced and tested for intra-allelic complementation. To generate mutant clones, *FRT82B shrp/TM6B* virgin females, carrying either *y w eyFLP* or *y w hsFLP* on the X chromosome, were crossed to either *y w; FRT82B P[w$^+$]ubi-GFP^{nls}/TM6B* (Datar et al., 2000) or *y w; FRT82B cell lethal P[w$^+$]/TM6B* (Newsome et al., 2000). Stocks used for meiotic mapping and male recombination were: *y w; P[w$^+$]how^{E7-3-4}* (Lo and Frasch, 1997), *y w; P[w$^+$] crb^{j1B5}/TM3, Sb* (Spradling et al., 1999), *y^1; ry^{506} P[SUP] KG05850, y^1; ry^{506} P[SUP] KG02176, y^1; ry^{506} P[SUP]CG444^{KG03827}* (http://flypush.imgen.bcm.tmc.edu/pscreen) and *ry^{506} P[ry$^+$]klg^{rN712}/TM3, Sb* (Spradling et al., 1999). Other stocks used are *Df(3R)hh* (Mohler and Vani, 1992), *Df(3R)M95A* (Reuter et al., 1986), *Elav-GAL4 UAS-mCD8-GFP* (Lee and Luo, 1999), *GMR-GAL4* (Freeman, 1996) and *UAS-p35* (Hay et al., 1994).

Meiotic mapping of *shrp* with *w$^+$* marked P elements

Flies carrying the *shrp^1* allele were crossed to flies carrying one of the five P elements in the 94A-96A region (see fly stocks above). Transheterozygous virgin females (*y w/y w; shrp ^{1} / P*[*w*$^+$]) were collected and crossed to males of the genotype *y w eyFLP/Y*; *st shrp^4 ca/TM3 P[w$^+$] Sb*. Recombination between *shrp^1* and a given P element would produce wild-type chromosomes that lack *shrp^1* and the P element. Half of these chromosomes were recovered over the *st shrp^4 ca* chromosome and the other half over *TM3 P[w$^+$]*. The reciprocal chromosomes carried both *shrp^1* and the P element. Recombinant flies of the genotype *y w / y w; + / st shrp^4 ca* were the only progeny that did not carry a *P[w$^+$]* and were identified by their white eyes among the rest of the red-eyed progeny. The frequency of white-eyed progeny is thus equal to half the meiotic distance in cM.

Scanning electron microscopy, immunohistochemistry and in-situ hybridization

Adult flies with heads in which over 90% of cells were mutant, were processed for SEM by using the hexamethyldisilazane (HMDS) method (Braet et al., 1997) with modifications. Flies were fixed for a day in 70% acetone, and washed twice in 100% acetone for 4 hours each. Acetone was then exchanged with HMDS through two washes in 1:1 acetone:HMDS and two washes in 100% HMDS over 2 days. Samples were air dried for 1 day prior to sputter coating with 25 nm platinum alloy. Antibody staining of imaginal discs carried out as described earlier (Halder et al., 1998). The following antibodies were used (dilutions in parenthesis): rat α-Elav (1:30) (O'Neill et al., 1994), rabbit α-Dlg (1:2000) (Cho et al., 2000), rabbit α-Drice (1:2000) (Yoo et al., 2002), guinea-pig α-Dlg (1:2000) (Woods and Bryant, 1991), guinea-pig α-Sens (1:1000) (Nolo et al., 2000), mouse α-BrdU (1:50, Becton-Dickinson) and mouse α-CycE (1:50) (Richardson et al., 1995). Secondary antibodies were donkey Fab fragments from Jackson Immuno Research. BrdU incorporation was carried out as described (de Nooij et al., 1996) by incorporating BrdU for 1 hour. For in situ hybridization, *Drosophila* cDNA clone RE52745 (ResGen Invitrogen Corp.) was used as template to generate DIG-labeled RNA probes (Roche), and in situ hybridization was performed as described (Nolo et al., 2000).

FACS analysis and cell counts

To analyze cell cycle and cell size distribution of *shrp* mutant and wild-type cell populations, wing imaginal discs containing mutant clones were dissected from transheterozygous larvae of the genotype *y w hsFLP; FRT82B shrp/FRT82B P(w$^+$) ubi-GFP^{nls}*. Clones were induced 24-48 hours after egg laying (AEL) by administering a heat-shock at 34°C for 30 minutes and discs were dissected 72 hours later. About 80-100 wing discs were dissected in PBS and transferred to 5 ml polystyrene tubes containing Trypsin-EDTA (Sigma, T-4174): PBS 9:1 v/v and 0.5 μg/ml Hoechst 33342 (Neufeld et al., 1998). Cells were dissociated for 4 hours by gentle shaking. The dissociated cells were analyzed on a Becton Dickinson Vantage Fluorescence activated cell sorter (FACS) and more than 50,000 mutant cells were scored for each sample. Data were analyzed with the Cell Quest program. For cell counts, wing discs from *y w hsFLP; FRT82B shrp / FRT82B P(w$^+$) ubi-GFP^{nls}* transheterozygous larvae were dissected 48 hours after clone induction, fixed in PEM (100 mM PIPES, 2 mM $MgSO_4$, 1 mM EGTA, 4% formaldehyde) on ice for 1 hour, washed briefly in PBT (PBS + 0.3% Triton X-100) and incubated with Hoechst (0.02 μg/ml) for 25 minutes. The discs were washed twice in PBT and mounted in Vectashield (Vector Labs). Cell numbers were determined by counting the nuclei (Hoechst-stained) of cells in mutant clones (GFP negative) and associated wild-type twin clone (GFP-positive) on a Zeiss axioplan fluorescence microscope.

RESULTS

To identify novel components of growth control pathways, we performed a genetic screen in adult *Drosophila* to isolate mutants in which tissue size but not tissue patterning is affected. Because genes involved in growth control may have ubiquitous functions, we anticipated that animals homozygous for mutations in these genes might die during embryogenesis. We therefore screened randomly mutagenized chromosome arms that were made homozygous only in the head using an *eyeless* enhancer driven Flipase transgene (*eyFLP*) (Newsome et al., 2000). Mutations in several genes were isolated that resulted in enlarged heads but did not affect patterning. These include mutations in the *Drosophila* homologs of *PTEN* and *TSC1/2* tumor suppressor genes, which act in cell growth control pathways that affect cell number as well as cell size (Potter and Xu, 2001; Stocker and Hafen, 2000; Tapon et al., 2001), mutations in *warts/lats*, a previously described tumor suppressor gene encoding a Ser/Thr kinase (Justice et al., 1995; Xu et al., 1995) that affect cell number but not cell size and mutations in a previously undescribed gene. We named this gene '*shar-pei*' because of its folded cuticle phenotype in the head, which resembles the folded skin of Shar-pei dogs.

Mutations in *shar-pei* cause overgrowth of adult structures

Mutations in *shrp* were isolated on chromosome arm 3R using chemical mutagenesis. Complementation tests showed that six mutations (*shrp$^{1-6}$*) that caused a head overgrowth phenotype fail to complement each other. All mutations showed a very similar phenotype and caused early larval lethality. Given the nature of the molecular lesions (see below) it is likely that they are either null alleles or very severe loss-of-function alleles. All experiments involving cell clones were performed with at least three independent alleles.

The heads of flies in which over 90% of cells are homozygous mutant for *shrp^1* are proportionally larger than other structures but have a normal overall pattern, including bristles, ocelli and ommatidia (Fig. 1A-F). All mutant fly heads have folded head cuticle and eye tissue (Fig. 1C-F) and over 15% of flies are severely affected (Fig. 1D). Smaller clones generated by heat-shock induced Flipase expression do not exhibit this folding phenotype. Folding may therefore be a secondary consequence of limited space within the pupal case, which does not allow overgrown tissue to fully expand. In addition to producing structures that are too big, *shrp* mutant cells appear to out-compete wild-type cells. The flies shown in Fig. 1A,B are *eyFLP* induced genetic mosaics in which clones are genotypically marked *white*, heterozygous portions are wild type and twin clones were eliminated by a cell lethal mutation to increase the amount of mutant cells in the eye (Newsome et al., 2000). When *white* cells proliferate at a normal rate, heterozygous red cells contribute about 20% of cells in this type of experiment. In contrast, eyes with white marked *shrp^1* mutant clones are predominantly white (Fig. 1B) and contain fewer wild-type ommatidia when compared with a control fly shown in Fig. 1A. Hence, the phenotype suggests that *shrp* mutant cells proliferate more rapidly than wild-type cells (Kirby and Bryant, 1982; Moberg et al., 2001; Simpson and Morata, 1981).

To test whether *shrp* affects cell proliferation in tissues other than the head, we induced random clones by heat-shock induced Flipase expression (Xu and Rubin, 1993). Such mutant clones resulted in overgrowths on thorax, wings, halteres and legs (Fig. 1G-J, not shown). As observed for the eye and head, these structures differentiated the correct tissue-specific cell types. We conclude that *shrp* is generally required to restrict the size of imaginal disc-derived adult structures, whereas tissue-specific cell-type specification and differentiation remain unaffected in *shrp* mutant cells.

shar-pei mutants produce extra interommatidial cells

To define the developmental basis for the enlarged tissue phenotypes, we focused on patterning and cell proliferation in the developing eye because the eye has a precise pattern of cell types and highly regulated cell proliferation (Baker, 2001; Kumar and Moses, 2000; Wolff and Ready, 1993). We first analyzed the pattern of differentiated photoreceptor cells in adult *shrp* mutant clones in 1 μm sections (Fig. 2A). We observed eight photoreceptors per ommatidium with a normal trapezoidal arrangement, indicating that this aspect of pattern formation is not affected. However, spacing between individual photoreceptor clusters was significantly increased in *shrp^5* clones when compared with wild-type areas (Fig. 2A arrowhead). To test whether the increased space was due to an excess of interommatidial cells, we stained wild-type and mutant midpupal retinas with an antibody against Discs-large (Dlg), a protein that localizes to apical junctions and hence reveals cell outlines (Fig. 2B,C) (Woods and Bryant, 1991). We found that *shrp^4* mutant clones exhibit a dramatic increase in the numbers of interommatidial cells (Fig. 2B) when compared with wild type (Fig. 2C). These extra interommatidial cells differentiated into pigment cells that produced normal pigmentation when clones were induced in a *w$^+$* background (not shown). These data indicate that Shrp regulates cell number but not differentiation in the retina.

The extra interommatidial cells could be due to excess cell

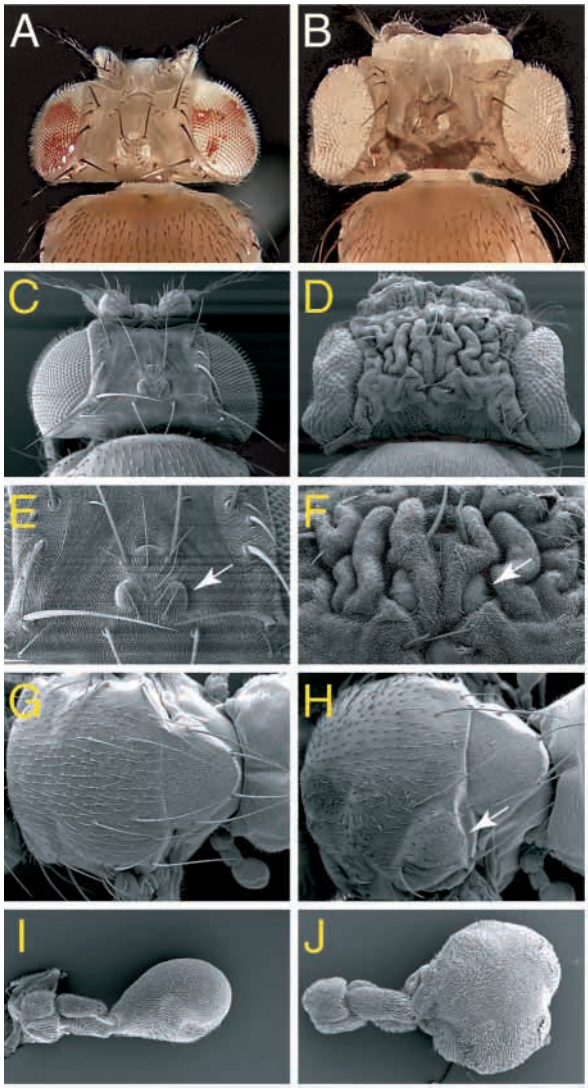

Fig. 1. *shar-pei* mutant clones result in outgrowths on head, thorax and halteres. Wild-type (left column) and mutant (right column) adult structures imaged by light and scanning electron microscopy (SEM). (A,B) Dorsal views of a normal sized fly (A) and a fly with a *shrp* mutant head (B). Both flies are genetic mosaics. We used the *eyFLP* transgene to induce recombination in most cells of the eye-antennal disc (Newsome et al., 2000). To increase the number of clone cells, a cell-lethal mutation on the homologous chromosome was used to eliminate homozygous twin clone cells (Newsome et al., 2000). In the normal sized fly, ~80% of cells are *white* but otherwise wild type. In the mutant fly, *white* cells are also homozygous mutant for *shrp*. These mutant cells make up virtually the entire eye. The body is wild type and serves as a reference for comparison of head sizes, because mitotic recombination was specifically induced in the developing head by using *eyFLP*. The genotypes are (A) *y w eyFLP; FRT82B/FRT82B cell lethal p[w$^+$]* and (B) *y w eyFLP; FRT82B shrp^1/FRT82B cell lethal p[w$^+$]*. (C,D) SEM images of a wild-type fly and a fly with a *shrp^3* mutant head produced by *eyFLP* induced mitotic recombination as for (B). (E,F) Higher magnifications of C,D. The mutant tissue is severely overgrown and folds up. Ocelli (arrows), bristles and hairs differentiated normally. (G,H) Wild-type thorax and a thorax with *shrp^3* mutant clones. The clones result in overgrown tissue (arrow). (I,J) Wild-type haltere (I) and haltere with *shrp^3* mutant clones (J). The mutant haltere is much larger than normal.

proliferation, increased spacing of photoreceptor clusters during patterning, lack of apoptosis or a combination thereof. In wild-type flies, interommatidial cells are initially produced in excess but the extra cells are later eliminated by apoptosis during pupal development in a process that requires cell-cell signaling (Rusconi et al., 2000). This system generates a very precise retinal lattice. To determine whether *shrp* mutant cells initiate the apoptotic program, we stained *shrp* mosaic pupal retinas with an antibody that detects the activated form of Drice, a caspase that triggers the apoptotic program and specifically marks cells undergoing apoptosis (Yoo et al., 2002). We detected many Drice-positive cells in wild-type retinal tissue, but none were found in *shrp^3* mutant territories (Fig. 2D,E). Importantly, all Drice-positive cells were wild type. This suggests that the apoptotic pathway is blocked in *shrp* mutant cells and that this block occurs upstream of Drice activation. We conclude that *shrp* mutant cells do not receive or are resistant to signals that induce apoptosis.

To test directly whether lack of apoptosis is sufficient to produce the *shrp* mutant phenotype, we compared the phenotype of *shrp* mutant retinas with that of wild-type retinas in which apoptosis was blocked by expressing the apoptosis inhibitor p35 (Hay et al., 1994). Ectopic expression of p35 eliminates most, if not all, normally occurring cell death in the retina (Hay et al., 1994) and results in extra interommatidial cells (Fig. 2F). However, the number of additional cells is significantly less than that observed in *shrp^4* mutant clones. (Fig. 2B). Therefore, while lack of apoptosis allows additional cells to survive, it is not sufficient to explain the amount of extra interommatidial cells generated in *shrp* mutants.

To investigate whether the extra interommatidial cells are due to abnormal ommatidial spacing during patterning, we stained developing mosaic eye imaginal discs for the neuronal marker Elav (Robinow and White, 1988) and the R8 marker Senseless (Sens) (Frankfort et al., 2001; Nolo et al., 2000). Elav is expressed in all differentiating photoreceptor cells and outlines differentiating photoreceptor clusters, while Sens is a marker for early pattern formation and ommatidial spacing, as well as R8 photoreceptors. Mutant ommatidial clusters have normal numbers of differentiating photoreceptor cells per ommatidium and are initially spaced correctly (Fig. 2G-I). However, at later stages in more posterior clones, spacing between photoreceptor clusters is increased (Fig. 2J-L). Thus, early retinal pattern formation is normal in *shrp* mutants.

shar-pei cell-autonomously restricts cell proliferation of uncommitted cells

To test directly whether *shrp* affects cell proliferation, we monitored the distribution of cell cycle progression in mutant third larval eye discs by bromodeoxyuridine (BrdU) incorporation (Fig. 3). In wild-type discs, BrdU-incorporating cells are randomly distributed in front of the morphogenetic furrow (Fig. 3A). In the furrow, cells synchronously arrest in G1 and do not incorporate BrdU (Fig. 3A, arrow). Posterior to the furrow, cells go through a synchronous S phase in the second mitotic wave, revealed as a band of cells incorporating BrdU (Fig. 3A, arrowhead). Few BrdU-positive cells are found posterior to the second mitotic wave (Fig. 3A). *shrp^1* mutant cells also synchronize their cell cycles in the furrow and progress normally through the second mitotic wave (Fig. 3B). However, in contrast to wild-type cells, *shrp^1* mutant cells still

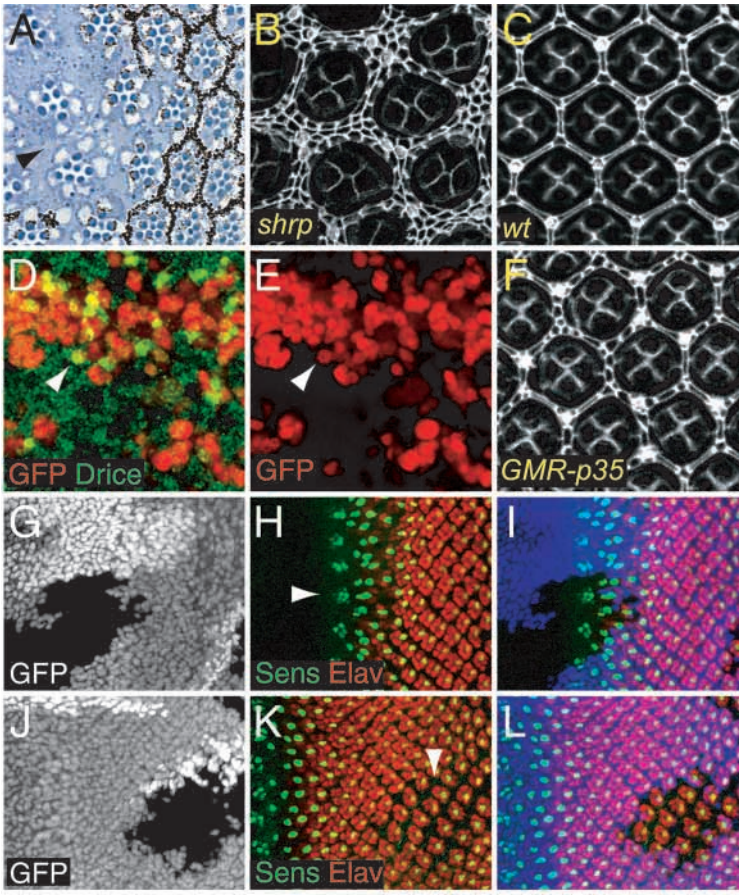

Fig. 2. *shar-pei* mutant clones in the eye show excess interommatidial cells, resistance to apoptosis and normal patterning. (A) Plastic thin section through an adult eye that is mosaic for *shrp*^{5} mutant cells. Mutant tissue lacks dark pigment granules in pigment and photoreceptor cells. Mutant ommatidial clusters have the normal complement of seven rhabdomeres in the correct trapezoidal arrangement. The spaces between the photoreceptor clusters, however, are significantly larger in mutant tissue than in wild-type tissue (arrowhead). (B,C) Mid pupal stage retinas with *shrp*^{4} mutant clones (B) and wild type (C) stained with anti-Dlg antibody to detect cell outlines. (D,E) Confocal section of the basal side of a 38 hours after puparium formation (APF) pupal retina mosaic for *shrp*^{3}. Mutant cells are marked by the absence of GFP expression (shown in red). The retina was stained with antibodies against activated Drice to detect apoptotic cells (green in D). All apoptotic cells are wild-type and express GFP (arrowheads). (F) Cell outlines in a retina expressing p35 under *GMR* control revealed by Dlg expression. (G-L) *shrp* mutant clones in third instar eye discs marked by the absence of GFP (grayscale in G,J and blue in I,L). Discs are stained for Sens (green) and Elav (red) expression. (G-I) A *shrp*^{6} mutant clone spanning the morphogenetic furrow (arrowhead) that shows normal patterns of Sens and Elav expression. (J-L) A *shrp*^{1} clone at the posterior edge shows normal patterning but increased spacing between ommatidial clusters (arrowhead). Anterior is towards the left in G-L.

display BrdU incorporation after the second mitotic wave (Fig. 3B, asterisk). The extra DNA synthesis is followed by cell division, as revealed by ectopic expression of phosphorylated histone H3 (PH3), which marks chromosomes during mitosis (not shown). This phenotype of *shrp* is cell autonomous, because only mutant cells undergo extra rounds of cell proliferation (Fig. 3C,D), and all territories of mutant cells show the excess interommatidial cell phenotype in pupal retinas, whereas non-mutant tissue appears wild type. Extra cell proliferation continues into the pupal stage but ceases by 24 hours after pupariation (not shown). Double labeling with BrdU and antibodies against Elav to detect differentiating photoreceptor cells revealed that only Elav-negative cells incorporated BrdU (Fig. 3E,F). Therefore, *shrp* is required to arrest cell proliferation in developmentally uncommitted cells after the second mitotic wave, but is not required for cell cycle arrest of differentiating photoreceptor cells. The ectopic proliferation produces extra interommatidial cells, which together with the lack of apoptosis, are sufficient to explain the overgrowth phenotypes observed in pupal and adult retinas.

shar-pei regulates Cyclin E levels

Cyclin E is limiting for S-phase initiation and progression during imaginal disc development and several tumor suppressor genes negatively regulate its activity or levels (de Nooij et al., 1996; Duman-Scheel et al., 2002; Lane et al., 1996; Moberg et al., 2001; Neufeld et al., 1998; Richardson et al., 1995). Cyclin E levels are upregulated in *shrp*^{1} mutant cells in the second mitotic wave and posterior to it (Fig. 4A-C arrows). Elevated levels were also observed just anterior to the second mitotic wave, although this effect was not as pronounced. The effect on Cyclin E is cell autonomous and observed in most or all mutant cells, even though only a fraction of them are actively progressing through S phase (Fig. 3C,D). Thus, the effect of Shrp on cell proliferation arrest may be mediated by regulating the levels of Cyclin E.

shar-pei mutant cells show accelerated cell proliferation during disc development

Our data show that although *shrp* mutant cells are able to exit the cell cycle during cell differentiation, they are delayed in arresting cell proliferation at the end of eye imaginal disc growth. To determine whether *shrp* has a function in uncommitted cells anterior to the morphogenetic furrow, we wanted to measure whether mutant eye discs were already larger than wild-type before ommatidial clusters are specified. Because initial spacing of photoreceptor clusters is normal in *shrp* mutant eye discs (Fig. 2G-I), the final number of ommatidia provides a measure of the number of cells present in mutant eye discs before R8 cells are specified in the morphogenetic furrow. We thus determined and compared the numbers of ommatidia in wild-type and mutant retinas (n=18 each) by counting clusters of photoreceptor cells revealed by Elav-Gal4 driven GFP expression (Lee and Luo, 1999). Mutant retinas contained an average of 913 ommatidial clusters (s.d.=40), whereas wild-type retinas had an average of 776

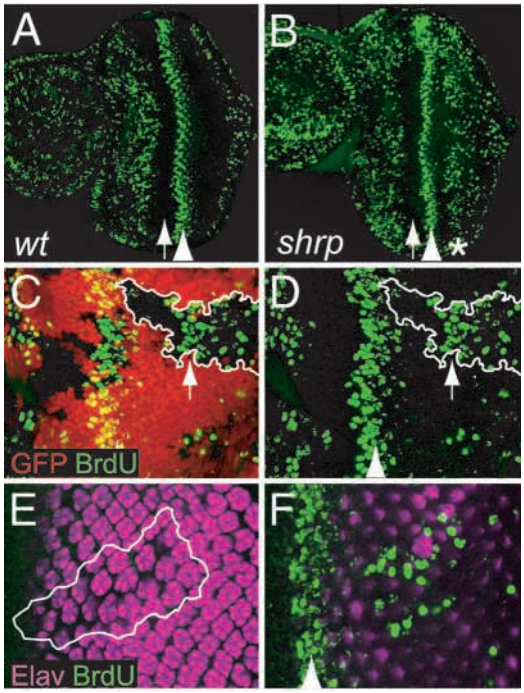

Fig. 3. *shar-pei* mutant cells display ectopic cell proliferation. All panels show imaginal discs stained to detect S phases by BrdU incorporation (green). (A) Wild-type and (B) *eyFLP* induced mosaic eye disc nearly entirely mutant for *shrp*^{1}. In wild-type, cells arrest in G1 phase in the morphogenetic furrow (arrow) and non-differentiating cells go through one synchronous S phase in the second mitotic wave (SMW, arrowhead). (B) *shrp*^{1} mutant cells also arrest in G1 and go through a synchronous SMW, but cells then continue to proliferate posterior to the SMW (asterisk). (C,D) BrdU incorporation (green) in *shrp*^{1} mutant clones marked by the absence of GFP (red). *shrp*^{1} mutant cells behind the SMW (arrowhead) continue to proliferate (arrows). This effect of *shrp* is cell autonomous. (E) Apical and (F) basal focal plane of an eye disc with a posterior *shrp*^{1} mutant clone stained for Elav (purple) and BrdU (green). Mutant cells were marked by the absence of GFP expression (not shown). The clone boundary is indicated by a white line in (E). BrdU-incorporating cells are located basally (F) and none of the Elav-positive cells incorporated BrdU. S phases in the SMW are marked by an arrowhead in F. Anterior is towards the left for all discs.

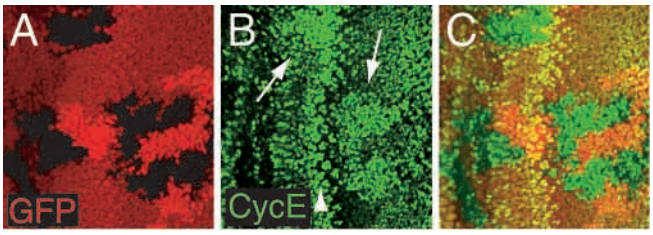

Fig. 4. *shar-pei* mutant cells upregulate Cyclin E levels. (A-C) *shrp*^{1} mutant clones in the eye disc marked by the absence of GFP expression (red) stained for Cyclin E (green). (C) merged channels. Cyclin E is upregulated in cells of *shrp* mutant clones (arrows), in particular posterior to the SMW (arrowhead).

(s.d.=45) photoreceptor clusters (Fig. 5). The two groups are significantly different by *t*-test (P<0.001). We conclude that *shrp* mutant eye discs are already larger than normal at the time when the positions of ommatidia are specified in the morphogenetic furrow. Shrp thus functions in uncommitted cells anterior to the morphogenetic furrow.

To test whether *shrp* affects the rate of cell proliferation during the growth phase of imaginal discs, we compared cell numbers in mutant clones and their associated twin clones in third instar wing discs (Fig. 6A). To reduce variability in the proliferation rate of wild-type twin clones, we used isogenized *FRT 82B ubi-GFP* chromosomes to generate mitotic clones. Cell numbers in *shrp*^{3} mutant clones were almost always larger than their twin clones, and the difference in cell numbers was significant when assessed using a *t*-test (P<0.001). The same experiment with a second allele, *shrp*^{4}, showed similar differences (not shown). By contrast, cell numbers in clones of the isogenized wild-type chromosome on which the *shrp* mutations were induced during the mutagenesis screen were similar and not significantly different from the corresponding *ubi*-GFP/*ubi*-GFP twin clones (Fig. 6B). Based on these cell counts and assuming exponential proliferation, the cell division rate of *shrp* mutant cells is 1.10 times faster than that of wild-type cells. Our data thus indicate that *shrp* mutant cells proliferated more. This phenotype is also manifest in mosaic adult eyes, where *shrp*^{1} mutant cells out-compete wild-type cells (Fig. 1A,B). Determination of the distribution of cell cycle phases in third instar wing discs using FACS analysis (Neufeld et al., 1998) showed that the population of *shrp*^{4} mutant cells has the same distribution of cell cycle phases as the wild-type cells (Fig. 6C). Thus, *shrp* mutant cells do not accelerate a particular step in the cell cycle. Rather, mutant cells show an even acceleration of cell cycle progression.

Manipulating the activity of cell growth regulators such as components of the insulin receptor signaling pathway results in larger organs because of more and larger cells (Johnston and Gallant, 2002; Potter and Xu, 2001; Prober and Edgar, 2001; Stocker and Hafen, 2000; Tapon et al., 2001; Weinkove and Leevers, 2000). To determine whether *shrp* also affects cell size, we stained mosaic wing discs with antibodies against Dlg to detect apical cell outlines. Cells in *shrp*^{3} mutant clones

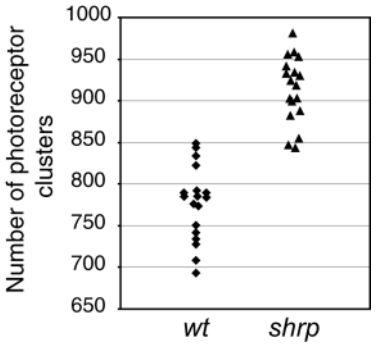

Fig. 5. *shar-pei* mutant retinas contain more photoreceptor clusters than wild type. The numbers of photoreceptor clusters in 18 wild-type (wt) and 18 *eyFLP* induced *shrp*^{1} mosaic retinas were counted in whole mid-pupal retinas. Photoreceptor clusters were visualized by Elav-GAL4 driven GFP expression. Each square/triangle represents one retina.

showed normal cell sizes, as judged by cell outlines (Fig. 6E-G) and had normal height as judged by the thickness of the wing disc epithelium in the mutant clones (not shown). In addition, rhabdomeres of mutant photoreceptor cells were of normal diameter (Fig. 2A), and *shrp* mutant cone and pigment cells are of normal size at the pupal stage (Fig. 2B,C). Furthermore, forward light scatter (FSC) data, a measurement of cell size collected by FACS analysis confirmed that mutant cells have normal size (Fig. 6D). Therefore, Shrp does not regulate cell size. Rather, extra proliferation of *shrp* mutant cells is induced by stimulation of cell growth and cell cycle progression, resulting in balanced growth and extra cells that are of normal size.

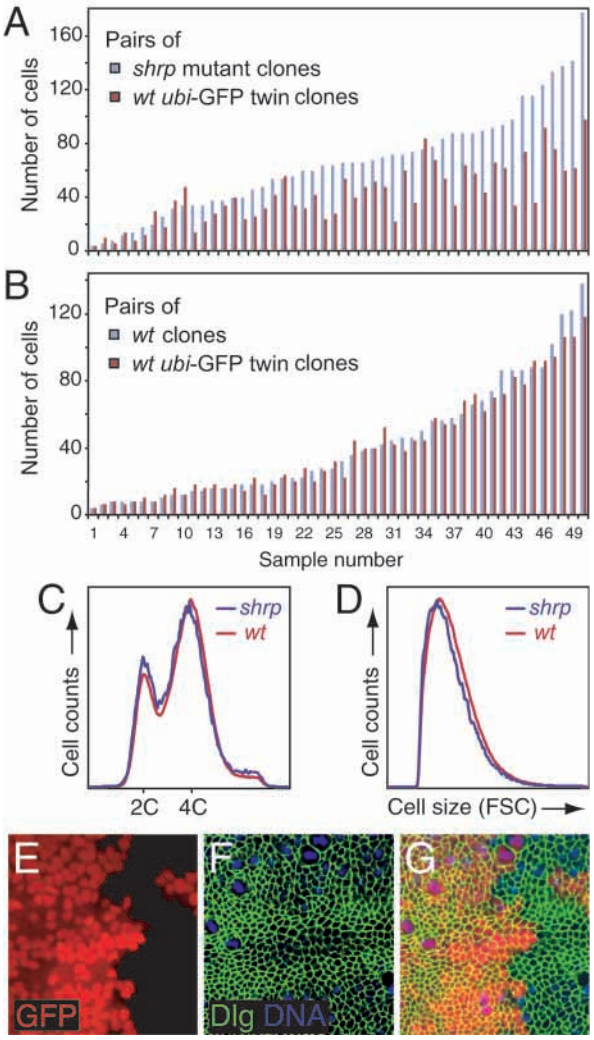

Fig. 6. *shar-pei* mutant cells proliferate faster, but show normal cell cycle profiles and cell size. (A) Cell numbers in 50 $shrp^3$ mutant clones (gray bars) and (B) 50 control clones of the isogenized wild-type FRT chromosome on which the *shrp* mutations were induced, compared with their twin clones (red bars). Twin clones were homozygous for an isogenized *FRT 82B ubi-GFP*^{NLS} chromosome. Cell numbers were counted in wandering third instar wing disc clones. (C) DNA profiles and (D) forward scatter distributions (FSC) of third instar wing disc cells measured by flow cytometry (FACS). $shrp^4$ mutant clones were induced at 24-48 hours after egg laying (AEL) and wing discs dissected 72 hours after clone induction. Blue trace represents *shrp* mutant cells, red trace wild-type cells. Mutant and wild-type cells were sorted by GFP expression. The mutant cell population had a similar distribution of cell cycle profiles and cell sizes when compared with the wild-type cells. (E-G) $shrp^3$ mutant clone in the presumptive wing pouch of a third instar wing disc marked by the absence of GFP expression (red). The disc is stained for Dlg to reveal cell outlines (green) and DNA to label nuclei (blue). Mutant cells have the same sized outlines as wild-type cells. Large cell outlines are from dividing cells showing apical mitotic figures (blue). (G) Merge of the three channels.

shar-pei encodes a conserved WW-domain containing protein

To identify the *shrp* gene, we first mapped the lethality associated with the mutations through deficiency mapping. We found that the *shrp* mutations failed to complement the deficiencies *Df(3R)hh* (93F;094D) and *Df(3R)M95A* (94D;095A), placing *shrp* within the 94D interval. Male recombination mapping further mapped *shrp* to a 150 kb interval between *klingon* (Butler et al., 1997) and *hedgehog* (Lee et al., 1992). Meiotic recombination mapping with several P elements as dominant markers in the region (http://flypush.imgen.bcm.tmc.edu/pscreen) (Fig. 7A), further placed *shrp* within a 90 kb interval (Fig. 7B). Based on the annotation of the *Drosophila* genome (Adams et al., 2000), we amplified and sequenced six predicted and conserved open reading frames in that region (Fig. 7B) and found mutations in CG13831 in all *shrp* alleles (Fig. 7C,D). The full-length *shrp* cDNA encodes a protein of 607 amino acids with two WW domains (Fig. 7C,D). WW domains are protein-protein interaction domains that bind to short proline-rich motifs, functionally resembling SH3 domains (Macias et al., 2002).

Database searches revealed that parts of Shrp are conserved in humans, mice (WW45) (Valverde, 2000), and *C. elegans* (T10H10.3) (Fig. 7C,D). In addition to the highly conserved WW domains, these proteins share a C-terminal domain that is specific for Shrp and not found in other proteins (Fig. 7C,D). Phylogenic analysis by neighbor joining of 202 WW domains (http://www.Bork.EMBL-Heidelberg.DE/Modules/ww/) revealed that the two WW domains of *Drosophila* Shrp and vertebrate WW45 are more closely related to each other than to other WW domains. Furthermore, only single copies of this gene were detected in all four species. We conclude that Shrp, WW45 and T10H10.3 are orthologous.

The alleles $shrp^{1-5}$ have point mutations that result in STOP codons, which truncate the proteins N-terminal to the WW domains (Fig. 7C,D). The sixth allele ($shrp^6$) has a 20 bp deletion that results in a frameshift between the WW domains and the Shrp-specific domain, resulting in the addition of 76 unique residues thereby effectively removing the Shrp specific domain. All six alleles are purely recessive and homozygous lethal at the first/second instar stage, and show the same lethal phase when heterozygous over *Df(3R)hh*. In addition, all alleles show similar phenotypes in mitotic clones in discs and adults. Thus, they all appear to be null alleles for *shrp* function. Because the frameshift allele $shrp^6$ is recessive and behaves as a null allele, the position of its lesion suggests that the conserved C-terminal domain is essential for Shrp function. In situ hybridization of imaginal discs revealed that *shrp* is ubiquitously expressed in the eye, wing and leg discs (Fig. 7E,F). This is consistent with our findings that *shrp* is required

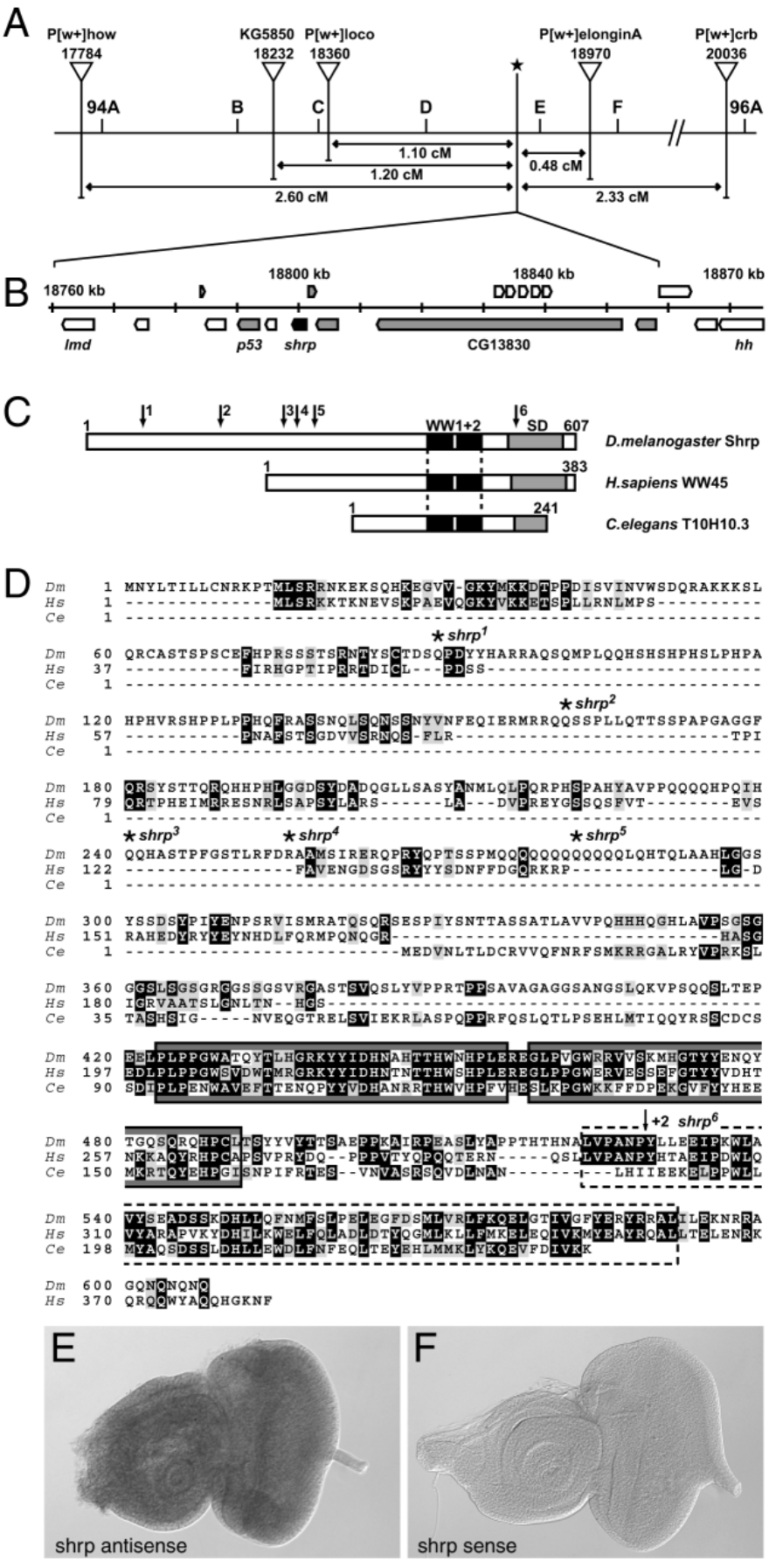

Fig. 7. Identification and sequence analysis of *shar-pei*. (A) Mapping of *shrp* relative to five P elements inserted in the 94A-96A region on 3R (horizontal line). Triangles show P elements with their names and genomic position in kb. Recombination distances between *shrp* (vertical line with star) and each P element are given in centiMorgan (cM, double arrows). (B) The genomic region of *shrp* determined by recombination mapping. Known and predicted ORFs are shown by arrowed boxes and genomic positions are given in kb above the DNA. The five gray boxed genes and *shrp* (black box) were sequenced. (C) Schematic representation of the protein structures of the fly, human and nematode Shrp homologs. Numbered arrows indicate the positions of the mutations in the six *shrp* alleles. The mutations in alleles 1-5 result in premature STOP codons, allele six has a +2 frameshift that results in the addition of 76 amino acids not related to any other protein in GenBank. (D) Sequence alignment of *Drosophila* Shrp (*Dm*), human WW45 (*Hs*) and *C. elegans* T10H10.3 (*Ce*). Identical residues are on black background, similar residues are shaded. The two WW domains are outlined by dark boxes and the *shrp*-specific domain is boxed by a broken line. Asterisks indicate the residues, the codons of which are mutated to STOP codons in the respective alleles, the position of the frame-shift in *shrp*^{6} is identified by an arrow. (E) Expression of *shrp* RNA in a wild-type eye-antennal disc. (F) The sense control shows no staining.

in all imaginal disc-derived tissues for proper cell proliferation arrest.

DISCUSSION

shar-pei promotes growth arrest and apoptosis but does not affect pattern formation or cell differentiation

Adult flies with *shrp* mutant cell clones have enlarged structures, which contain more cells of normal size. This phenotype results because *shrp* mutant cells proliferate faster than wild-type cells and because they do not terminate proliferation when imaginal tissues have reached their normal size. For example, *shrp* mutant cells in the eye disc undergo extra rounds of cell proliferation after the second mitotic wave, when wild-type cells do not normally proliferate anymore, resulting in extra interommatidial cells. In addition, Shrp effects cell survival. In wild-type, extra interommatidial cells are removed by apoptosis, while extra *shrp* mutant cells are not. Thus, Shrp has a dual function in promoting cell proliferation arrest and apoptosis. The effects of Shrp on apoptosis and cell proliferation may be

separate functions, because inhibition of apoptosis and stimulation of cell cycle progression are not necessarily linked (Asano et al., 1996; Du et al., 1996b; Moberg et al., 2001; Richardson et al., 1995).

Pattern formation appears to progress normally in *shrp* mutant clones. In the eye, clones show the normal complement and morphology of photoreceptor and cone cells. Clones on head, thorax, halteres and wings show normal patterning of bristles and other tissue-specific structures such as ocelli, wing margin bristles, haltere specific sense organs and tissue-specific cell type differentiation. In addition, mutant cell clones in wing discs respect both the AP and DV compartment boundaries and clone borders within compartments are jagged, indicating that cell affinities are not affected by the loss of *shrp* function. In summary, Shrp specifically regulates cell number by promoting cell proliferation arrest and apoptosis, but is not required for pattern formation or cell type differentiation.

Regulation of cell proliferation arrest during organ growth versus terminal differentiation

Shrp function is specifically required for the timely proliferation arrest of developmentally uncommitted cells, but not for terminal cell cycle exit during cell differentiation. In *shrp* mutant eye discs, cell cycle arrest in differentiating photoreceptor cells still occurs normally, and ectopic BrdU incorporation is confined to developmentally uncommitted cells. Consistent with this observation, we did not observe duplicated photoreceptors or cone cells in the eye or duplicated bristles on the head thorax or along the wing margin. Thus, *shrp* is not required for cells to exit the cell cycle during terminal differentiation. Rather, *shrp* is required for proliferation arrest before cells are induced to differentiate into specific cell types. Therefore, more precursor cells are generated in *shrp* mutants, which then differentiate normally but produce adult organs that are too big. For example, *shrp* mutant eyes have more ommatidia, indicating that mutant eye discs contain more cells than normal when ommatidial cell clusters are specified. The observation that *shrp* mutant clones produce overgrowths on disc-derived structures, including eyes, heads, wings, halteres, legs and thoraxes suggests that *shrp* function is ubiquitously required. The ubiquitous expression pattern of *shrp* in imaginal discs supports this conclusion. In the eye, we also observe an increase in the number of pigment cells per ommatidium, while the other cell types are present in normal numbers per ommatidium. This is because cell types are specified sequentially during eye development. First, some cells are specified as photoreceptors, while the other cells remain uncommitted. These uncommitted cells ectopically proliferate in *shrp* mutants and produce more interommatidial cells. Successively, the remaining cell types are recruited in normal numbers, but at the end, too many cells are leftover which differentiate into excessive numbers of pigment cells, the last cell type to differentiate.

The requirements of Shrp are distinct from those of genes required for cell cycle exit during terminal differentiation. *Dacapo* (*dap*), a *Drosophila* homolog of the Kip family of cyclin-dependent kinase inhibitors, is induced when cells exit the cell cycle prior to terminal differentiation (de Nooij et al., 1996; Lane et al., 1996). In *dap* mutant embryos, cells go through one extra round of cell division just prior to terminal differentiation. In the developing eye, *dap* expression is induced in differentiating photoreceptor cells, but not in developmentally uncommitted cells (de Nooij et al., 1996; Lane et al., 1996). Adult *dap* mutant eye clones do not show gross abnormalities or extra cells, but rare escapers show duplications of bristles on notum and wing margin (Lane et al., 1996). Downregulation of positive cell cycle regulators such as Cyclin E (Crack et al., 2002; de Nooij et al., 1996; Du and Dyson, 1999; Knoblich et al., 1994; Richardson et al., 1995; Richardson et al., 1993) and other negative regulators of cell proliferation such as Rbf (de Nooij et al., 1996; Du and Dyson, 1999; Du et al., 1996a) may act redundantly with Dap to promote cell cycle arrest. Nonetheless, while Dap is upregulated specifically in cells that withdraw from the cell cycle prior to terminal cell differentiation, Shrp is required in developmentally uncommitted cells during the growth phase of organs before terminal differentiation. The requirements for Dap and Shrp functions are thus spatially and temporally distinct.

We propose that the arrest of cell proliferation during imaginal disc development is controlled by several genetically separable mechanisms. First, cells stop proliferating when imaginal discs have reached their correct sizes (Bryant and Levinson, 1985; Garcia-Bellido, 1965). This process requires Shrp function. Second, cells permanently exit the cell cycle during terminal differentiation. Because terminal cell cycle exit is part of cell differentiation, it is directly regulated by patterning mechanisms that determine the identity and position of each individual cell. This regulation is governed by tissue- and cell-type specific enhancers of cell cycle regulators such as *dacapo*, *cyclin E* and *string*, which all have complex *cis*-regulatory regions (Duman-Scheel et al., 2002; Jones et al., 2000; Lehman et al., 1999; Liu et al., 2002; Meyer et al., 2002). Similarly, patterned regulation of cell cycle progression may occur before terminal differentiation, as is observed in the second mitotic wave in the eye and in the zone of non-proliferation along the presumptive wing margin in the wing disc (O'Brochta and Bryant, 1985). None of these processes are affected in *shrp* mutants. Thus, the direct control of cell cycle progression by patterning mechanisms acts epistatically to the control of cell proliferation observed during organ growth and can impose cell cycle arrest on cells that otherwise may continue to proliferate. Therefore, *shrp* mutations do not deregulate cell proliferation of terminally arrested cells, and cells differentiate normally. In summary, Shrp identifies a molecular mechanism that is required to arrest cell proliferation during organ growth and that appears to be distinct from the ones used to arrest cells during terminal cell differentiation.

The function of *shar-pei* during proliferation arrest

What are the downstream effectors of Shrp that are deregulated in *shrp* mutants and induce cell proliferation? *shrp* mutant clones behind the second mitotic wave in eye discs show elevated levels of Cyclin E. Notably, Cyclin E was elevated in all developmentally uncommitted cells of the clones, apparently irrespective of the phase of the cell cycle. The effect on Cyclin E levels may thus be direct and not just a reflection of the ectopic cell proliferation observed in mutant clones. Precise regulation of Cyclin E expression and activity is crucial as ectopic expression of Cyclin E induces entry into S phase and limited cell proliferation in imaginal discs and embryos

(Knoblich et al., 1994; Neufeld et al., 1998; Richardson et al., 1995). Several other negative regulators of cell proliferation directly regulate the levels of Cyclin E activity. Dap directly inhibits Cyclin E/Cdk2 complexes (de Nooij et al., 1996; Lane et al., 1996), and Archipelago is required for degradation of Cyclin E (Moberg et al., 2001). The regulation of Cyclin E is thus likely to be an important downstream effect of Shrp function.

Ectopic expression of Cyclin E alone, however, is not sufficient to generate the number of extra cells observed in *shrp* mutant tissues (Neufeld et al., 1998; Richardson et al., 1995). Artificial acceleration of the cell cycle by ectopic expression of specific cell cycle regulators such as E2F accelerates cell division, but does not stimulate cell growth rates, and cells divide when they are smaller (Neufeld et al., 1998). This results in an increase in cell number and a concomitant decrease in cell size, yet does not affect the overall tissue size. Thus, cell cycle progression is not sufficient to drive cell and organ growth. Conversely, stimulating cell growth alone produces larger organs, but also affects cell size (Johnston and Gallant, 2002; Potter and Xu, 2001; Prober and Edgar, 2001; Stocker and Hafen, 2000; Tapon et al., 2001; Weinkove and Leevers, 2000). For example, artificially stimulating the activities of Ras, Myc or insulin receptor signaling produces more and bigger cells and thus bigger but otherwise normal flies. Thus, cell proliferation during organ growth requires coordinate stimulation of cell cycle progression and cell growth to produce normal sized cells. Because *shrp* mutant cells maintain normal size, Shrp appears to be required to regulate cell growth and cell cycle progression coordinately. Thus, Shrp probably regulates other targets driving cell cycle and cell growth in addition to Cyclin E.

Several other mutations have been described that fail to arrest imaginal disc growth and were thus classified as tumor suppressor genes (Gateff, 1994; Turenchalk et al., 1999; Watson et al., 1994). These include *discs-large* (*dlg*) (Woods and Bryant, 1991), *lethal giant larva* (*lgl*) (Gateff, 1978) and *scribble* (*scrib*) (Bilder and Perrimon, 2000), encoding proteins which form an architectural complex localized to septate junctions. Mutations in these genes disrupt septate junctions and apical-basal polarity of epithelial cells and result in neoplastic overgrowth of imaginal discs (Bilder et al., 2000; De Lorenzo et al., 1999; Johnston and Gallant, 2002). Mutations in a second group of *Drosophila* tumor suppressor genes cause hyperplastic overgrowth of imaginal discs that retain their single layered epithelial structure. These include *warts/lats*, which encode a kinase that regulates the activity of Cdc2 (Justice et al., 1995; Tao et al., 1999; Xu et al., 1995); *fat*, a large Cadherin (Mahoney et al., 1991); *hyperplastic discs*, a E3 ubiquitin ligase (Mansfield et al., 1994); and *discs overgrown*, a *Drosophila* homolog of Casein kinase Iδ/ε (Zilian et al., 1999). The imaginal disc overgrowth in mutants of both groups occurs during an extended larval period, and embryonic requirements for these genes appear to be provided by maternal contributions (Bilder et al., 2000; Bryant et al., 1988; Mansfield et al., 1994). By contrast, homozygous *shrp* mutant animals die as first/second instar larvae, which do not show gross morphological defects. Thus, zygotic expression of *shrp* is required for early larval viability, whereas that of other tumor suppressor genes is not. Cells homozygous mutant for neoplastic or hyperplastic tumor suppressor genes generally differentiate abnormally and show defects in cell morphology and/or pattern formation (Agrawal et al., 1995; Bilder and Perrimon, 2000; Bryant et al., 1988; Justice et al., 1995; Martin et al., 1977; Woods and Bryant, 1991; Xu et al., 1995; Zilian et al., 1999). These phenotypes are thus different from those of *shrp* mutant cell clones, which overproliferate but differentiate with normal cell morphology and patterning. In addition to these differences, clones of cells homozygous mutant for *shrp* proliferate more rapidly and have reduced apoptosis, while cells mutant for most other tumor suppressor genes have reduced viability and a decreased rate of cell proliferation (Bryant, 1987; Mansfield et al., 1994; Woods and Bryant, 1989). Only *fat* and *warts/lats* mutant cell clones proliferate faster (Garoia et al., 2000) (M. K. S., unpublished), similar to *shrp* mutant cells. However, the phenotypes of *shrp*, *fat* and *warts/lats* differ, as *fat* and *warts/lats* affect the morphology and pattern of adult tissues in addition to cell number. Therefore, *shrp* affects cell number more specifically than these other mutants, and future work will have to establish whether and how Shrp interacts with other tumor suppressor gene products to control tissue size.

In summary, our studies provide evidence that Shrp functions in the decision of imaginal disc cells to terminate proliferation and to exit the cell cycle once the correct disc size is attained. The determination of the effectors of Shrp action should reveal mechanisms by which cell growth and cell cycle progression are coordinately regulated during organ growth and how cells arrest proliferation once organs have reached their correct size. The presence of Shrp homologs in mouse and human suggest the existence of a conserved organ size control mechanism in mammals. The characterization of Shrp function should therefore provide valuable insights into the mechanisms that underlie tissue size regulation and cause disproportionate growth and tumorigenesis when defective.

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NOTE ADDED IN PROOF

While this manuscript was under review, Tapon et al. (Tapon et al., 2002) also reported the characterization of this gene.

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