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Comparison of somatic clones of the eye in the analysis of cell growth.

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Introduction

Cell proliferation and increases in cell size are the processes that contribute to cell growth. Organs or tissues have a tendency to develop within the constraints of a normal final size and vary from a large number of small cells to a small number of large cells (Day and Lawrence, 2000). One important pathway in the control of cellular growth is the insulin receptor signaling pathway, which is highly conserved among invertebrates and mammals (Brogiolo *et al.*, 2001). When manipulated, several components of this pathway can alter growth rates of *Drosophila melanogaster*. The *Drosophila* eye normally consists of 700 to 800 ommatidia that develop in a highly regulated manner (Baker, 2001) and is ideal for the study of cell growth and cell survival. Mosaic eye clones can be created via the yeast site-specific recombination FLP/FRT system (Theodosiou and Xu, 1998), which depends upon the presence of *FRT* sites and expression of the enzyme FLP directed in the developing eye tissue. In the presence of FLP, homologous chromosomes undergo mitotic recombination between the *FRT* sites located on chromosome pairs. Heterozygous parent cells produce homozygous tissue within a heterozygous organism. In this paper, two methods of generating somatic clones of the eye are compared in the study of cell growth.

Drosophila Strains and Culture

Experiments were carried out on standard media containing cornmeal, molasses, yeast, agar, and water at 25°C. The control line *w; +/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} akt1^{+}* (*w; FRT^{2A} FRT^{82B} akt1^{+}*) was obtained from Dr. Norbert Perrimon, Harvard University (Perrimon *et al.*, 1996). The P-element insertion line *akt1^{04226}* was obtained from the Bloomington *Drosophila* Stock Center. This line contains a P-element inserted within the 5' untranslated region of the *akt1* gene on the third chromosome (Perrimon *et al.*, 1996; Spradling *et al.*, 1999). The novel derivative of *akt1^{0422}*, *akt^{PR52}*, was generated by imprecise excision. Both *akt1^{04226}* and *akt1^{PR52}* were then recombined with the third chromosome *FRT*'s by standard means, and, therefore, have the genotype of *w; +/+; P[FRT ; w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} akt1^{04226}(or^{PR52})/TM6B* (*w; FRT^{2A} FRT^{82B} akt1^{04226} (or^{PR52})*). The stocks required for creation of somatic clones of the eye were received from the Bloomington *Drosophila* Stock Center. The full genotype of *Drosophila* stock containing *eyeless-FLP* is *y^{d2} w^{1118}; P{ry$^{+t7.2}$=ey-FLP.N}^{2}, P{GMR-lacZ.C(38.1)}^{TPN1}; P{ry$^{+t7.2}$=neoFRT}^{82B}, P{w^{+t*} ry^{+t*}=white-un1}^{90E}, l(3)cl-R3^{1}/TM6B, P{y$^{+t7.7}$ ry $^{+t7.2}$=Car20y}^{TPN1}, Tb^{1}* for method one (Newsome *et al.*, 2000), hereby termed the *ey-FLP* method. The genotype *y w; P{w^{+m}=GAL4-ey.H}$^{3-8}$, P{w^{+mC}=UAS-FLP1.D}^{JD1};*

P{ry$^{+t7.2}$=neoFRT}^{82B}, *P{w^{+mC}=GMR-hid}^{SS4}*, *l(3)CL-R^{1}/TM2* (Stowers and Schwarz, 1999) is the line used for method two, hereby termed the *ey-GAL4/UAS-FLP* method.

Assay Design

Males of *w; FRT^{2A} FRT^{82B} akt1^{+}*, *w; FRT^{2A} FRT^{82B} akt1^{04226}* and *w; FRT^{2A} FRT^{82B} akt1^{PR52}* lines are crossed to females possessing either *eyeless-FLP* or *eyeless-GAL4* and *UAS-FLP* plus the proximal 3R recombination site (*FRT^{82B}*) and a wild-type copy of *akt1*. To obtain the desired individuals, flies are collected upon eclosion based upon phenotypic markers (the absence of *Hu* or *Ubx*). For comparison, homozygous adult males have been analyzed. All collected flies are aged for three to five days, and then flash frozen at –70°C before preparation for Scanning Electron Microscopy (SEM). Preparation included mounting upon aluminum SEM studs, dessication, and sputter coating in gold. Three to five images of each genotype are taken by SEM (Hitachi S-570 SEM) at 150× magnification.

Statistical Analysis

Images of each genotype are analyzed using NIH Image J software. Ommatidia and bristles are counted from three images. Ommatidia area is determined by three independent measurements of the area of a cluster of seven ommatidia per picture, for three images. Results are graphed using GraphPad Prism (version 4.02). This program calculates the standard error of the mean to statistically compare the averages of ommatidia number, size, and bristle number between genotypes.

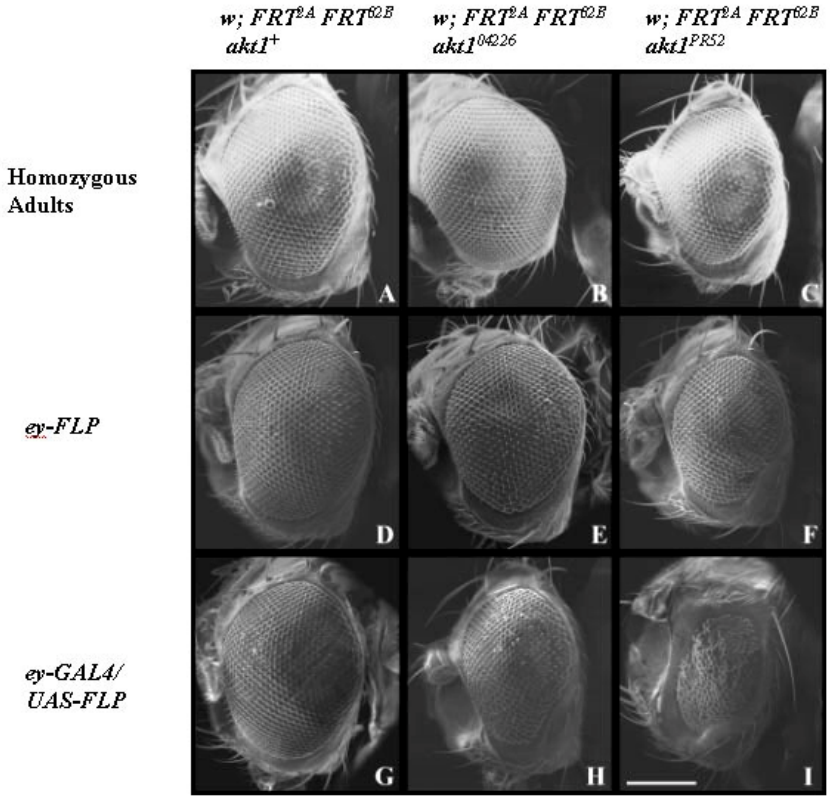

Figure 1. Eyes of *w; FRT^{2A} FRT^{82B} akt1^{+}*, *w; FRT^{2A} FRT^{82B} akt1^{04226}* and *w; FRT^{2A} FRT^{82B} akt1^{PR52}* homozygotes, *ey-FLP*-generated clones, and *ey-GAL4/UAS-FLP*-generated clones. Both methods of creating eye clones counteract the effects of developmental delay, as compared to the homozygous individuals. A-C are from homozygous adult males, D-F are somatic clones of the eye generated by the *ey-FLP* method, and G-I are somatic clones of the eye generated by the *ey-GAL4/UAS-FLP* method. Genotypes are: A, D, and G: *w; FRT^{2A} FRT^{82B} akt1^{+}*, B, E, and H: *w; FRT^{2A} FRT^{82B} akt1^{04226}*, C, F, and I: *w; FRT^{2A} FRT^{82B} akt1^{PR52}*. Scale bar represents 160 um.

Results

Homozygous *akt1* mutant males have phenotypically smaller eyes than wild-type flies (Figure 1). With the *ey-FLP* method, control somatic eye clones developed 711 ± 15 ommatidia, with an area of 223 ± 5 um^2 and 540 ± 14 bristles (Figure 2). The *w; FRT^{2A} FRT^{82B} akt1^{04226}* eye was smaller in size compared to the *w; FRT^{2A} FRT^{82B} akt1^{+}* control, with 558 ± 14 ommatidia, with an area of 196 ± 4 um^2 and a bristle count of 452 ± 15. This weak allele produced a reduced number of ommatidia and exhibited a small amount of abnormal patterning, as well as misshaped ommatidia, not observed for the control clones. The P element derivative allele *w; FRT ^{2A} FRT^{82B} akt1^{PR52}* developed smaller and fewer ommatidia than either *w; FRT^{2A} FRT^{82B} akt1^{+}* or *w; FRT^{2A} FRT^{82B} akt1^{04226}*, with 482 ± 32 ommatidia of an area of 182 ± 3 um^2 and bristle count of 371 ± 33 that had irregular ommatidial pattern. In the *ey-GAL4/UAS-FLP* method, the *w; FRT^{2A} FRT^{82B} akt1^{+}* control eye had an average of 704 ± 3 ommatidia with an area of 212 ± 2 um^2 and 563 ± 8 bristles. The *w; FRT^{2A} FRT^{82B} akt1^{04226}* eye clones have 508 ± 21 ommatidia of an area of 179 ± 3 um^2 with 377 ± 11 bristles. The derivative *w; FRT^{2A} FRT^{82B} akt1^{PR52}* in this method produced 261 ± 8 ommatidia with an area of 149 ± 11 um^2 and 115 ± 11 bristles. All genotypes had some abnormal ommatidial patterning and shape in the *ey-GAL4/UAS-FLP* method. In both methods, the more severe allele, *w; FRT^{2A} FRT^{82B} akt1^{PR52}*, was found to be significantly reduced in ommatidia number and size, and in bristle number. However, it is only in the *ey-GAL4/UAS-FLP* method that a significant difference between the control and the weak allele, *w; FRT^{2A} FRT^{82B} akt1^{04226}*, was observed.

Discussion

The akt protein kinase is a central component in insulin receptor signaling, and acts through many downstream targets in cell survival, growth, proliferation, metabolism, and migration (recently reviewed in Manning and Cantley, 2007). Homozygous *akt1^{1}* mutant eyes are small, but co-expression of *akt* in these eyes was able to significantly suppress the reduction in cell size (Staveley *et al.*, 1998; Scanga *et al.,* 2000). Overexpression of *akt* in the developing eye was reported to produce enlarged eyes due to an increase in cell size but not in cell number (Verdu *et al.*, 1999). The akt kinase may exert its influence on cell growth through the activation of the target of rapamycin (TOR) complex, and downstream targets S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein (4EBP), which stimulate the initiation of protein synthesis (Manning and Cantley, 2007). In general, the akt kinase can alter cell number through its influence upon cell survival and proliferation, by blocking pro-apoptotic downstream targets BAD (and homologues) and the transcription factor foxo. The akt kinase can influence the overall size of tissues via cell proliferation through downstream targets, which function within cell cycle regulation. Regardless of the relative contributions of these molecular mechanisms, *akt* activity controls cell growth and survival in the developing *Drosophila* eye.

Comparison of the Two Methods of Creating Somatic Clones of the Eye

Both methods use *eyeless* enhancers to induce expression of the *FLP recombinase* gene. In the *ey-FLP* method, the *FLP* gene is under the control of four tandem repeats of a specific enhancer from the *eyeless* gene and a basal *hsp70* promoter. This allows for a direct expression of *FLP*. In the *ey-GAL4/UAS-FLP* method, the expression of *FLP* is indirect by the use of the UAS/GAL4 system. The *ey-GAL4* was constructed by cloning a 3.6 kb *Eco*RI fragment containing the eye-specific enhancer of the *eyeless* gene into a vector (Hazelett *et al.*, 1998). The expression of *eyeless* begins in the 6 to 23 cell-containing eye disc in embryogenesis and lasts until the last cell divisions required to

complete the ~15,000 cell-containing eye disc are carried out during the late third instar (Newsome *et al*., 2000). *FLP* is expressed during this time period and leads to the induction of recombination. There are roughly 10 to 12 rounds of post-embryonic cell divisions necessary to generate the number of cells in the eye disc (Newsome *et al.*, 2000). Heterozygous cells can give rise to homozygous cells during subsequent rounds of cell division, and this is increased with the sustained expression of *FLP* and mitotic recombination.

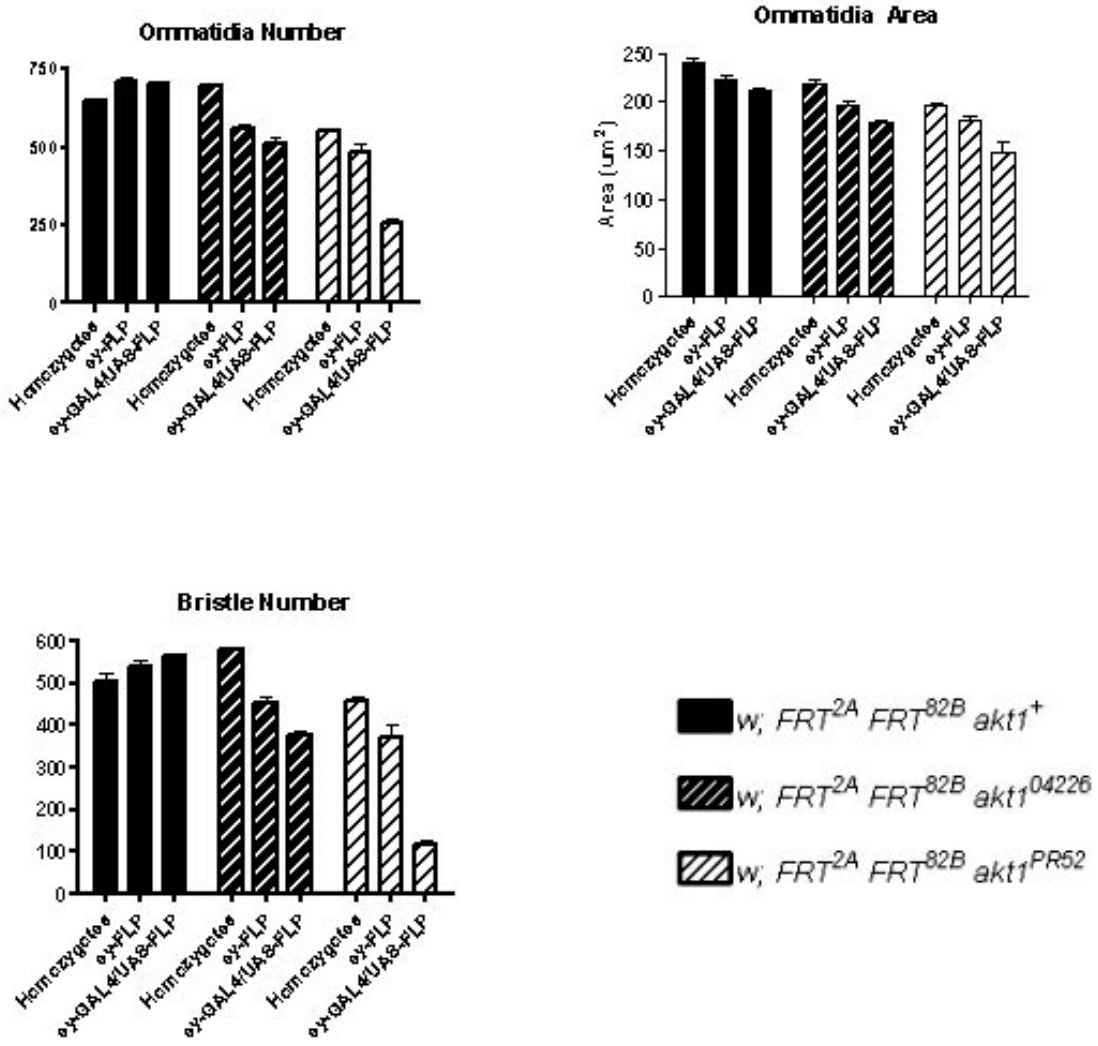

Figure 2. Comparison of ommatidia number, ommatidia size, and bristle number of *akt1* mutant homozygotes and somatic eye clones. The control line exhibits a small increase in both ommatidia and bristle number, and a slight decrease in ommatidia area in somatic clones when compared to homozygous individuals. The P-element insertion line *w; FRT^{2A} FRT^{82B} $akt1^{04226}$* has a larger number of smaller ommatidia and bristles compared to the control as a homozygote, but exhibits a decrease in all three phenotypic categories as a clone. The derivative *w; FRT^{2A} FRT^{82B} $akt1^{PR52}$* has fewer ommatidia and bristles, as well as smaller ommatidia, compared to both the control and *w; FRT^{2A} FRT^{82B} $akt1^{04226}$* in all three analyses. This decrease in size and number of cells is the most severe with the *ey-GAL4/UAS-FLP* method.

The *ey-FLP* method has been used both with and without the presence of a cell lethal. Without a cell lethal, the *ey-FLP* method results in 20-30% homozygous cells for the mutant allele of interest, and 70-80% of either homozygous or heterozygous wild-type cells (Newsome *et al.*, 2000). In the presence of a cell lethal, eye clones produce eyes that consist of 90-100% homozygous cells, although up to 10% of the eye may arise from heterozygous cells (Newsome *et al.*, 2000). The cell lethal is, therefore, able to enhance the generation of clone tissue and, therefore, allow for a more complete analysis of homozygous mutant tissue in an otherwise heterozygous animal.

The *ey-GAL4/UAS-FLP* method expresses *FLP* indirectly, so that an amplification of the recombinase is possible. This works through the *eyeless* control sequences enhancing the expression of *GAL4*, and then many copies of the GAL4 protein binding to *UAS* sites to lead to an excess of *FLP* expression. In addition, there is a *GMR-hid* transgene insertion distal to the *FRT* and wild-type copy of the gene of interest (Stowers and Schwarz, 1999). This gene will lead to the death of cells that carry it, including heterozygous cells. The *GMR* promoter leads to the expression of *hid* in late development, during metamorphosis (Stowers and Schwarz, 1999). While this is sufficient to remove heterozygous and homozygous wild-type cells, it does not leave sufficient time for the developing eye to compensate. The presence of both *GMR-hid* and a cell lethal allows this method to be effective in eliminating the non-mutant cells, and to produce an almost completely homozygous eye. Calculations determine that the percentage of heterozygous cells will decrease by 0.75 fold to result in the formation of 3 to 5% of the eye (Stowers and Schwarz, 1999). This represents a considerably smaller proportion of the eye when compared to the *ey-FLP* method.

Evaluation of Assay

The creation of homozygous clones of the eyes is effective in the study of a mutant phenotype in a heterozygous animal. When the homozygote is developmentally delayed, as is the case with *akt1* mutants (Slade and Staveley unpublished), clones are very informative. In homozygous individuals, a delay in development may allow for compensatory growth that could suppress the homozygous phenotype. The two methods for creating somatic clones of the eye, evaluated here, are more effective in revealing the mutant phenotype when compared to the analysis of the homozygous mutant adults. In the *ey-FLP* method, the *FLP recombinase* gene is expressed directly via the *eyeless* enhancer, and surviving heterozygous cells make up part of the eye. The *ey-GAL4/UAS-FLP* method has the *FLP recombinase* gene expressed in an indirect manner through the UAS-GAL4 system. This allows the amount of the FLP recombinase enzyme to be amplified that may lead to a greater effectiveness in the generation of clones and thus be more sensitive to alterations in growth as observed with *akt1* mutants. In addition, the presence of *GMR-hid* allows for heterozygous cells to be removed from the eye, to produce more clone tissue. These elements suggest that the *ey-GAL4/UAS-FLP* method is a sensitive method for analyzing the growth-dependent mutant phenotype in developmentally delayed adult organisms.

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Amplification of DNA from 30-year-old aceto-orcein stained salivary gland squash slides.

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Salivary gland squash slides have been used since the early days of genetics to visualize the fruit fly's polytene chromosomes. These slides represent an immense archive of genetic material for *Drosophila* and other genera. Aceto-orcein is commonly used to stain salivary gland preparation, as well as other cytological specimens, especially when trying to visualize chromosomal material. However, aceto-orcein stained tissue proves recalcitrant to yielding DNA for amplification. I describe here a simple procedural modification of a basic DNA extraction protocol that yields PCR-amplifiable DNA from aceto-orcein stained salivary glands.

The slides in question were made over 30 years ago from *Drosophila melanogaster* strains with presumptive tandem duplications of the maroon-like (*mal*) locus. These were apparently the result of spontaneous non-homologous unequal crossing over (Johnson and Smith, 1976; Johnson, 1977). These slides were prepared using a standard protocol involving 1) coating one slide with a gelatin subbing solution, 2) squashing salivary glands in 45% acetic acid between two slides using a bench vice, 3) freezing the slides on dry ice then popping them apart, 4) staining with aceto-orcein, 5) dehydration in alcohol, serial toluene treatment, and mounting a coverslip with CoverBond (Harelco). Various protocols to recover DNA were attempted repeatedly, including using the Pinpoint Slide DNA Isolation System™ (Zymo Research), all yielding no detectable DNA after PCR.

The key that made DNA isolation possible was a brief acidification step, presumably to remove the DNA-bound aceto-orcein. The successful protocol below is a modification of the Wizard® Genomic DNA Purification Kit procedure (Promega). The use of toluene here is to remove CoverBond which is toluene soluble.

Protocol

1) Carefully scrape salivary gland tissue off the slide with a new razor blade then soak the tissue in toluene for 5 minutes.
2) Add an excess of 95% ethanol and mix gently.
3) Centrifuge briefly to pellet the tissue flake then discard the supernatant.
4) Wash three more times with 95% ethanol (repeat steps 2 and 3).
5) Remove remaining ethanol in a vacuum centrifuge.
6) Dissolve the flake (as much as possible) in 600 µL Wizard Nuclei Lysis Solution.
7) Incubate at 65ºC for 30 minutes.