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A Genome Wide Search for Proteins that Control the Balance Between Proliferation and Differentiation in the *Drsophila* Ovary, an *in vivo* model System

-Avanti Jakatdar-

Avanti Jakatdar is currently a senior, pursuing a Bachelor of Science in Biology and a minor in German and Chemistry at Case. She has worked in an Orthopaedics lab and currently works in a Genetics lab at Case Western Reserve University's School of Medicine. This summer, Avanti participated in the Summer Program in Undergraduate Research, and she hopes to eventually pursue a degree in medicine. Besides research, she enjoys a capella music and is the director and president of Dhamakapella, Case Western Reserve's South Asian a capella group. She is also an avid classical dancer.

-Acknowledgements-

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ABSTRACT

Understanding how progenitor cells stop proliferating and enter a differentiation pathway is a fundamental question in cancer and stem cell research today. The long term goal of this study is to identify proteins required for the transition between proliferation and differentiation in the *Drosophila* ovary, which is a model system for studying stem cells and tumor formation. To meet this objective, gene trap technology was used, which involves the insertion of green fluorescence protein (GFP) via a P element transposon called P{GFP}. When P{GFP} is inserted into an intron, it 'traps' the protein and functions as an exon, fusing the endogenous protein with GFP. This GFP fusion protein allows for examination of the expression pattern of the trapped gene *in vivo*. Transgenic lines that contain single random P{GFP} insertions are identified using our genetic screening.

Live ovaries were screened to identify patterns of GFP expression in the transgenic lines. Thus far, over 300 lines were screened and two were identified that express GFP. By searching for genes that are highly expressed in tumor cells, the probability of trapping interesting genes was increased in proliferating or differentiating cells. High resolution microscopy was used to determine if GFP expression is occurring in stem cells or differentiating cells and also to compare expression in wild type ovaries to tumorous ovaries. Inverse PCR and sequencing was used to identify the endogenous gene in which the GFP insertion has occurred. Knowing the identity of the trapped protein will lay the foundation for understanding the role of the protein in the pathway that controls the choice between proliferation and differentiation.

INTRODUCTION

Stem cells are undifferentiated cells that undergo asymmetric division to produce a daughter cell that remains a stem cell and proliferates, and a progenitor cell, whose progeny differentiate into a defined cell type. Progenitor cells undergo limited cell division in the time between leaving the stem cell niche and beginning differentiation, and can sometimes 'de-differentiate' to regain the stem cell's self renewing properties. The regaining of stem cell-like self-renewing properties is thought to be a characteristic of tumorous cells.

The model used for the study of stem cells and tumor development is the *Drosophila melanogaster* ovary (Figure 1, provided by Johnie Chau). The ovary consists of 15-20 tubes called ovarioles. Two or three germline stem cells (GSCs) are located in the germarium, the anterior end of the ovariole. The daughter cell that remains in contact with the somatic cap cells at the tip of the germarium remains a stem cell and the other daughter cell, the progenitor or cystoblast (CB) cell, undergoes cell division.

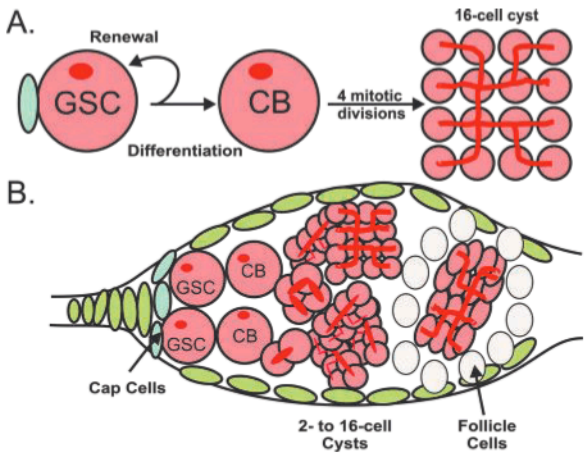

Figure 1. Our *in vivo* model system: the *Drosophila* ovary

The fly has proven to be a good model for gene identification and function studies. The simple anatomy of the fly germline allows for unambiguous identification of stem cells and their progeny *in vivo.* Also, results can be directly applied to human stem cell biology because the stem cell system appears to be conserved between flies and vertebrates.

The genetic screen that was carried out is designed around two main points. First, control of germ cell fate decisions occurs at both the transcriptional and post translational level, and the study therefore uses 'protein trapping' rather than expression arrays. Second, it is known that the proliferation/differentiation decision is made by stem cell-like cells as they leave the stem cell niche, but it is impossible to screen for genes expressed specifically in these cells because there are only a few in each ovariole at any given time. We screen instead for genes expressed in the snf^{148} tumor cells, as studies conducted in Dr. Salz's lab indicate that snf^{148} tumors are rich in stem cell-like cells arrested in an intermediate stage of development. By comparing GFP expression in tumorous and wild types ovaries, lines that express GFP specifically in these arrested, intermediate cells but not differentiating cells can be identified. Proteins that are ectopically expressed in tumor cells may be coded by genes that must be silenced in order for differentiation to proceed. That is, these genes may be responsible for the continued proliferation in tumor cells, and further studies will show how exactly these genes function in the proliferation/differentiation decision.

To identify genes expressed in *snf* tumors, a collection of lines that contain single, random insertions of GFP were generated and screened. The screen is based

on other successful models that used the P-element based protein trap transposon called P{GFP}, which contains an artificial exon encoding GFP flanked by splice site acceptor and donator sequences (Morin, X., R. Daneman, M. Zavortink, and W. Chia, 2001). It functions as an exon when inserted into an intron fusing the endogenous protein with GFP. P{GFP} can also function as an enhancer/gene trap and in most cases, GFP expression accurately reproduces the developmental expression pattern of the trapped gene (Buszcak M, Paterno S, Lighthouse D, Bachman J, Planck J, et al., 2007).

The process of gene trapping is shown in Figure 2, provided by Dr. Salz. Other researchers in Dr. Salz's lab had already identified two lines that are expressed in the intermediate cells, and more lines expressing GFP are being trapped and identified using the screen.

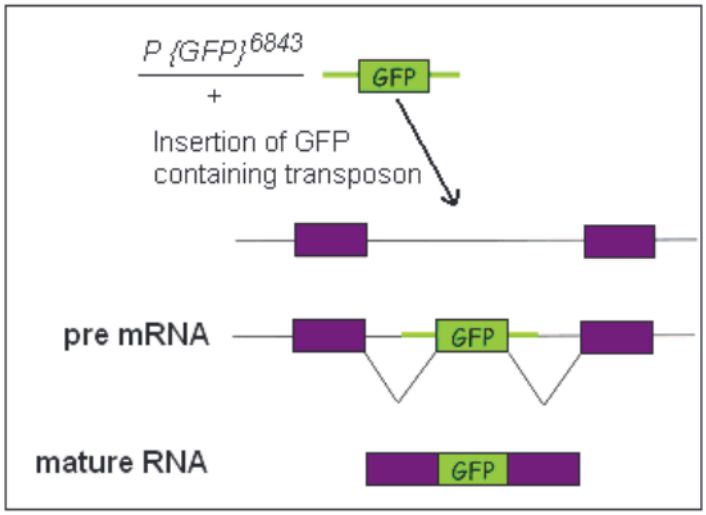

Figure 2. Trapping genes with a GFP exon

MATERIALS AND METHODS

Unfixed, dissected ovaries were screened directly for naturally fluorescent GFP expression. The ovaries were from females containing the P{GFP} insertion. Possible expression patterns that could have been detected are shown below (Fig. 3, provided by Johnie Chau).

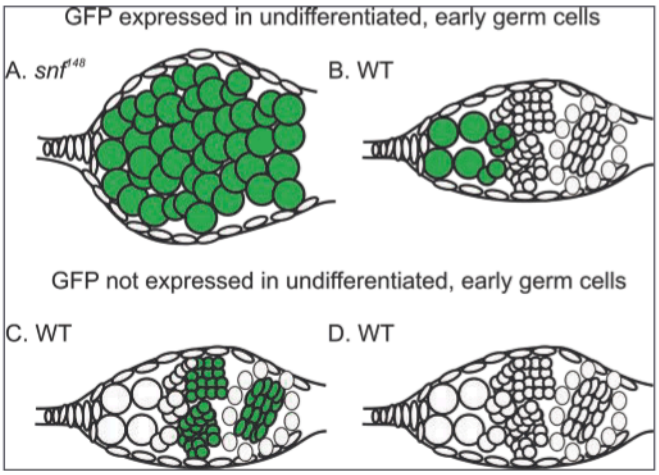

Figure 3. Possible GFP expression patterns. A) snf^{148} ovaries were screened for GFP expression throughout the germline. B-D) Expression patterns that could have been detected in wild type ovaries are B) expression in undifferentiated, early germ cells, C) expression in differentiated cells and D) no expression in the germline

To generate lines containing a single GFP insertion, different genotypes of flies were selected and mated based on eye color and shape until females with the insertion were obtained. First, males containing the P{GFP} transposon and transposase were mated to females homozygous for snf^{148}. Progeny in which the transposon had jumped to an autosome were then selected and mated to homozygous snf^{148} females. Finally, females with the insertion were selected and screened for GFP expression. The screen is shown to the right in a figure

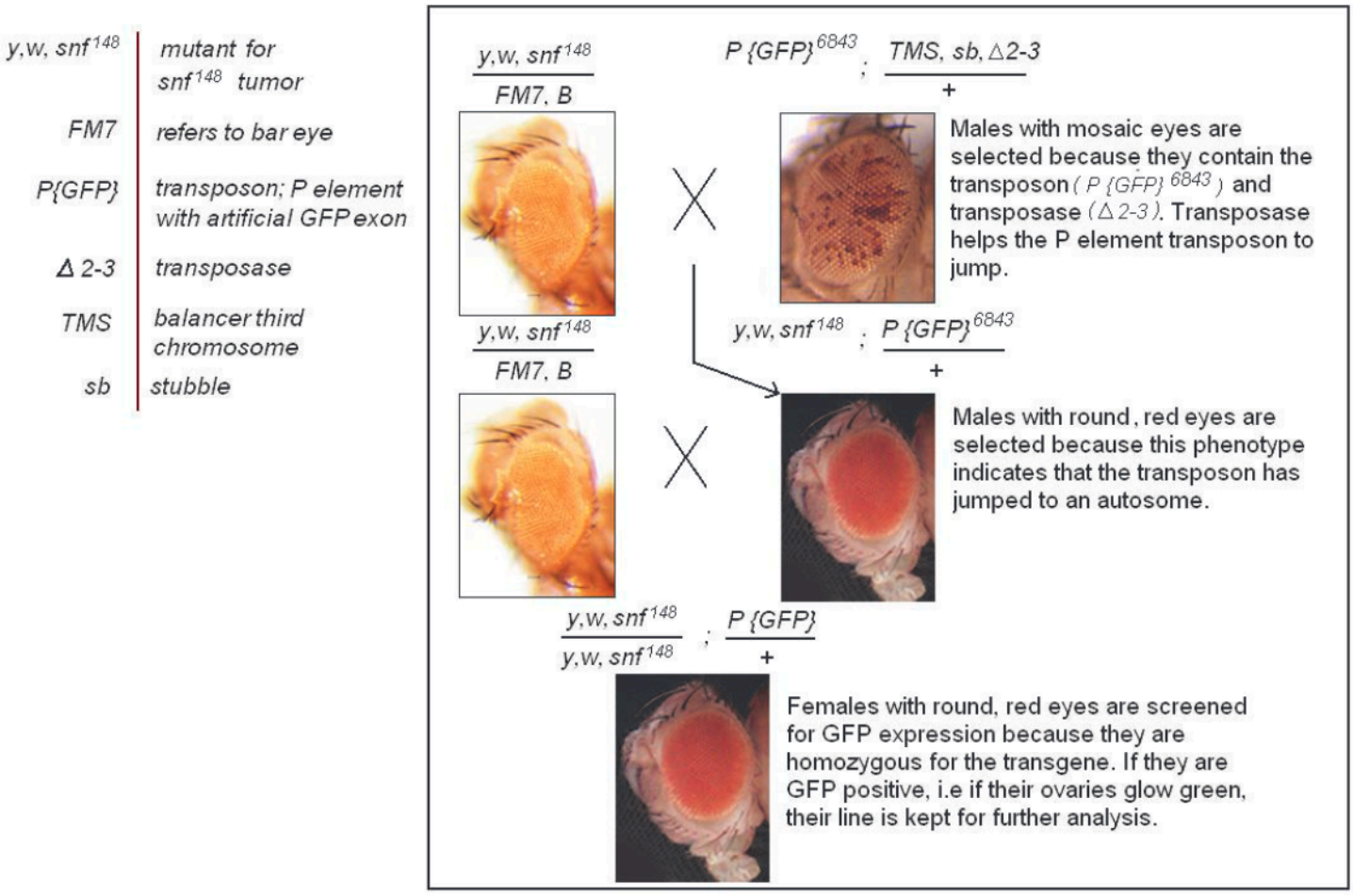

Figure 4. The GFP Screen

created by the author. Once a line expressing GFP was identified, the line was expanded to obtain a sufficient number of flies for confocal microscopy and inverse PCR.

Confocal Microscopy

10 wild type flies and 10 mutants were selected from each GFP positive line in order to compare expression in wild type and tumorous ovaries. A three day antibody staining procedure with rabbit anti-GFP was applied to increase GFP expression for easier identification of expression patterns in the ovary. Slides were prepared by teasing apart the ovarioles and fixing them in *Vectashield Mounting Medium.* High resolution microscopy was then used to capture images of GFP expression in the ovarioles.

Inverse PCR

To locate the protein-trap insertion, the DNA flanking the insertion was recovered and sequenced using inverse PCR. The process of inverse PCR is shown in figure 5 (next page), provided by Johnie Chau. DNA was extracted from 30 mutants per line by treating them with buffer, a LiCl/KAc solution and TE. Then the DNA was digested with restriction enzymes HinP1 and Msp1. The transposon was ligated to the endogenous DNA using

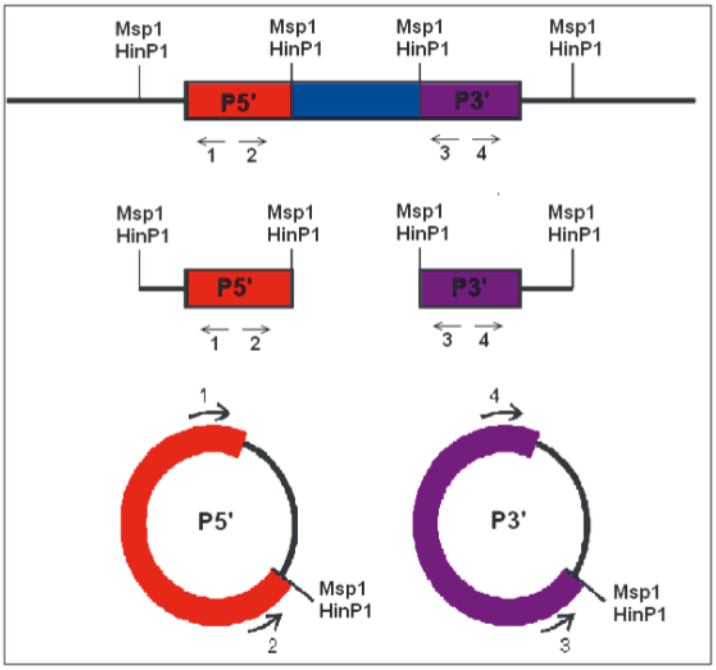

Figure 5. Inverse PCR.

1. Genomic DNA from a P{GFP} line is digested with restriction enzyme MspI or HinPI.

2. Fragments are made to self-ligate using T4 ligase.

3. 1-4 listed by the small arrows are the primers; red and purple bars represent ends of the transposon; black line represents endogenous DNA sequence.

ligation buffer and T4 Ligase. Finally, inverse PCR was performed with Pwht1 and Plac1 as 5' primers and Pry4 and Pry1 as 3' primers.

Gel Electrophoresis

This procedure was used to determine if the PCR products would be sufficient for obtaining good sequencing results. A 1.2% agarose gel was used to run the samples and the 100 bp marker.

BLAST

By analyzing the sequencing results, the endogenous gene was identified by its presence between the P{GFP} transposon and the restriction site. BLAST alignments were performed on the endogenous sequence and the *Drosophila* genome to identify the location of the insertion within the genome.

RESULTS

Confocal Microscopy

Shown to the right are high resolution pictures of lines that were used (Figure 6).

Inverse PCR and Gel electrophoresis

Shown to the right are results from the inverse PCR of lines LSK 1261 and 1335 (Figure 7). The products are different in size because they were digested with different restriction enzymes, either Msp1 or HinP1.

BLAST

Sequencing results from PCR products showed the location of the transposon and the restriction site, whose sequences are known. In between the two was the sequence of the endogenous gene. Aligning the endogenous gene with the *Drosophila* genome showed us the approximate location of the gene within the genome. The 5' PCR product of LSK1093 digested with Msp1

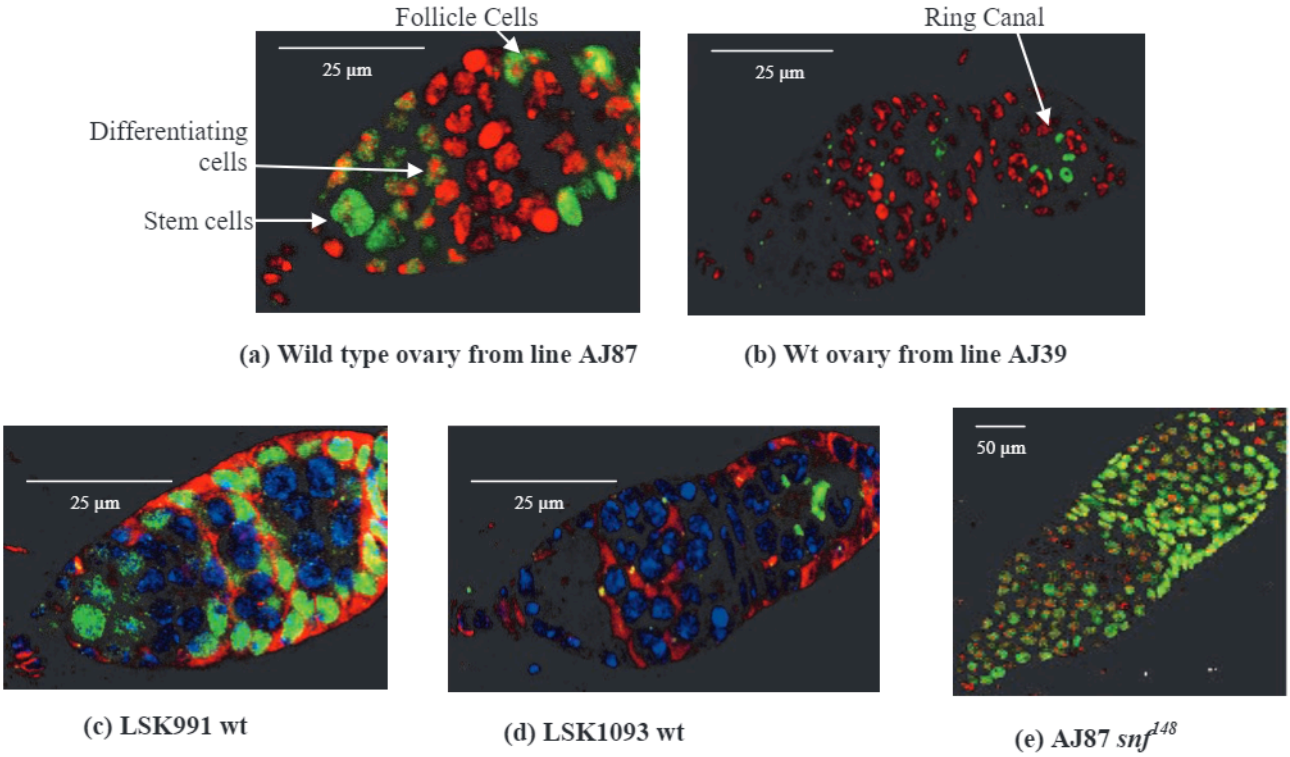

Figure 6. Confocal Imaging **a) Wild type ovary from line AJ87.** GFP expression is seen in the stem cells in the germarium, and in the follicle cells, **b) Wt ovary from line AJ39.** GFP expression is seen in ring canals, the region where interconnected germ cells connect, **c) Wt ovary from LSK991.** GFP expression is seen in the stem cells and follicle cells, **d) Wt ovary from LSK1093.** GFP expression is seen in the ring canals, **e) Tumorous ovary from AJ87.** Strong GFP expression is seen throughout the ovary. In (a), (b) and (e), DNA is colored red and GFP is green. In (c) and (d), DNA is colored blue and GFP is green, and 1B1 antibody causes the red color.

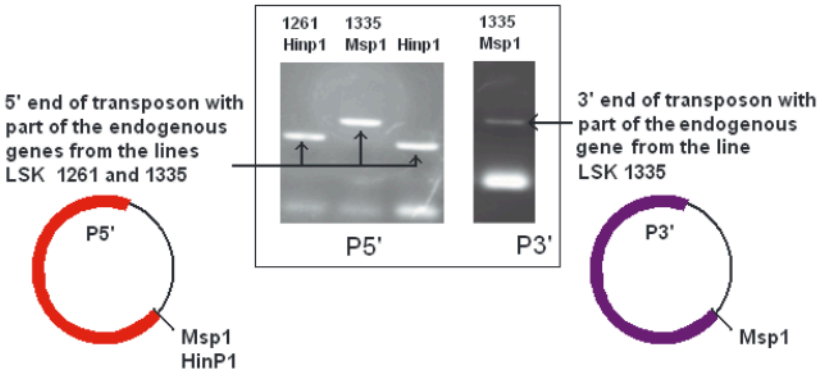

Figure 7. Inverse PCR. On the left are results obtained from 5' PCR primers Wht1 and Plac1. On the right are results from 3' PCR primers Pry4 and Pry1. The bright bands at the bottom of the gel are most likely primer dimers.

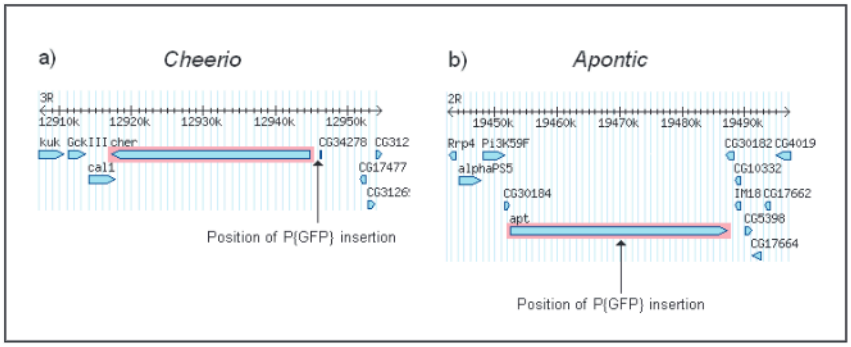

Figure 8. Location of the endogenous gene

A) from line LSK1093, digested with Msp1

B) from line LSK991, digested with HinP1

aligned with a region outside the 5' region of the 'Cheerio' gene. The 5' product of LSK991 digested with HinP1 aligned with part of the sequence of the 'Apontic' gene. The approximate locations of the P element insertions are shown above (Figure 8).

In the mutant AJ87, GFP expression was seen throughout the ovary (Figure 6), in the stem cell-like cells arrested in an intermediate stage of development. This may show that the 'trapped' protein is involved in arresting differentiation and restarting proliferation. GFP expression was seen in proliferating germ cells in the AJ87 and LSK991 wild types, which may show that the trapped protein has a role in early germ cell proliferation. Although the intention of the screening was to identify expression in proliferating cells, expression was unexpectedly found in ring canals in AJ39 and LSK 1093. Ring canals are cytoplasmic bridges that allow the flow of nutrients from nurse cells to oocytes. They are necessary for proper oocyte and egg chamber formation (Robinson D.N, Cant, K and Cooley, L., 1994). Therefore, expression in the ring canals may show that the trapped protein is involved in differentiation and proper cell development.

In LSK991, the GFP insertion occurred in Apontic, which is thought to be involved in transcriptional activity, mRNA binding and DNA binding. This gene may not be directly involved in stem cell proliferation. Cheerio is known to be involved in ring canal formation, but ring canals may only be required for proper differentiation and not for making the transition between proliferation and differentiation. Therefore, Cheerio may not have a direct role in controlling the balance between proliferation and differentiation.

Sequencing of lines AJ87 and AJ 39 is currently in progress, and it will be interesting to see if the insertions occurred in Cheerio and Apontic, as the expression patterns in AJ87 and AJ39 are consistent with LSK991 and LSK1093 respectively. The goal was to find genes that are highly expressed in tumor cells and may play a role in the stem cell's decision to proliferate or differentiate. In all four lines, GFP was highly expressed in tumor cells, and looking at expression patterns in the wild type offered a clue as to what type of role the trapped protein may have. Further analysis to determine the identity of the trapped proteins will lay the groundwork for understanding their role in the pathway that controls the choice between proliferation and differentiation.

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