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Role of a Specific P53 Binding Site in Limiting Tissue Overgrowth

Cheyenne Helflin, Haya Ghannouma, & Lei Zhou
University of Florida

Faculty mentor: Lei Zhou, Department of Molecular Genetics and Microbiology

Abstract

The p53 protein is an important transcription factor known for maintaining tissue homeostasis by activating genes that have antiproliferative function, such as pro-apoptotic and cytostatic genes. Transcriptional activation of proapoptotic genes has displayed a fundamental role in mediating apoptosis during *Drosophila* embryogenesis and tissue development. Using Chromatin Immunoprecipitation Sequencing (ChIP-Seq) and RNA sequencing (RNA-Seq) methods, in previous studies, we have identified p53 binding sites potentially responsible for p53-mediated induction of pro-apoptotic genes following DNA damage. We have since generated fly lines with the p53 binding site deleted by CRISPR-Cas9-mediated genome editing. To study the effects of the p53 binding site knockout (p53BSKO) on tissue homeostasis, wings of the knockout fly line were dissected, mounted, and then compared against wild type wings. Results show p53BSKO animals had an increase in wing size compared to that of the wild type. FijiWings 2.2 macros software was used to measure wing hair (trichome) densities, which is directly proportional to cell numbers. This analysis showed that p53BSKO led to hyperplasia of the wing as compared to the wild type. Our study indicated that this single p53 binding site is required for ensuring the right number of cells in a given tissue, likely through mediating over proliferation-induced apoptosis.

Introduction

The tumor suppressor p53 is known for its role in regulating cellular stress responses and is involved in a number of important functions related to cell cycle regulation and tumor suppression (Amundson, Myers, & Fornace, 1998), including its role in transactivating various proapoptotic genes such as *reaper* and *hid* (Zhang et. all, 2008). Dysfunctional p53 has been discovered in a wide variety of invasive cancers, where the mutant protein loses its tumor-suppressive function and mediates metabolic changes that lead to cancer development. (Duffy, Synnott, & Crown, 2017). Our recent work has also shown that modifications to the p53 binding site can lead to its suppression and result in hyperplasia, the overgrowth of cells within organs and tissues (Zhang et al., 2014). Hyperplasia is considered one of the first steps in cancer development, as the loss of cell cycle regulation is an important part of the transition of a healthy cell into a cancerous one. In *Drosophila*, the activation of proapoptotic genes is mediated by the p53 response element (p53RE) located within the Irradiation Responsive Enhancer Region

(IRER). The IRER is a ~33 kilobase region that has been shown to help suppress tumor growth by regulating three proapoptotic genes: *reaper*, *hid*, and *sickle*. The region is transcriptionally active during the "sensitive" stage (stages 9-11) of *Drosophila* embryonic development, or 4 to 7 hours after egg laying, and is highly responsive to irradiation (Zhang et. all, 2008). Epigenetic regulation of the IRER post stage 12 closes the region off from binding by p53, leading towards embryonic resistance to irradiation-induced apoptosis (Zhang et. all, 2008).

The IRER has also been linked to the regulation of hyperplasia in body tissues. Wings from animals deficient for the IRER have been compared to wild type wings in previous studies and have found to be significantly larger. Analyzing wing hair densities further confirmed this as IRER deficient (Df(IRER)) animals had a greater density than wild type, reflecting ~10-20% more number of cells (Zhang et. all, 2014). From this, it was concluded that the IRER is necessary to regulate the expression of proapoptotic genes, which in turn mitigates hyperplasia in tissue. (Zhang et. all, 2014)

Here, we seek to explore the role of the p53 binding site in limiting hyperplasia. By use of CRISPR-Cas9-mediated genome editing (DeCiaccio, Acuna, Ghannouma, et al unpublished data) we were able to generate fly lines with the p53 binding sequence (p53BS) deleted and studied the effects of the mutation on *Drosophila* tissue development. Females from these lines were crossed with Df(IRER) males and allowed to grow to adulthood, where their wings were dissected and compared to those of wild type animals. Our hypothesis is that the p53BS is required for mediating overproliferation-induced cell death and ensuring appropriate tissue size during development.

Materials and Methods

CRISPR-Cas9 Generated P53BSKO Fly Lines

Fly lines used in the experiment were generated using CRISPIR-Cas9-mediated genome editing to delete the p53 binding site (P53BSKO). Fly lines were generated with a full deletion (R8-1) and a partial deletion (R28-5) of the p53 binding site motif. They were compared to R24-5 (with a 3 bp deletion outside of the motif) and wild type lines R15-1 and R42-5 (which underwent CRISPR-Cas9 genome editing). Figure 1 shows the p53 binding site motif, the R8-1 deletion and the R15-1 wild type line.

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                                                    RPR-gRNA
Reference                                                                    PAM
..TTCGGAATGGGTTTTCAGATTTCTTCACTCAGCGTGCATCGACGTGTTCGAAGGCCTATTTGGGTTCCTTTGTGTGTGTC..
R8-1
..TTCGGAATGGGTTTTCAGATTTC-----------------------------------------TTCCTTTGTGTGTGTC..
R15-1
..TTCGGAATGGGTTTTCAGATTTCTTCACTCAGCGTGCATCGACGTGTTCGAAGGCCTATTTGGGTTCCTTTGTGTGTGTC..
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Figure 1. Gene sequence of CRISPR-Cas9 Generated p53 Binding Site Knockout Fly Lines. R8-1 contains a 41 bp deletion eliminating the full p53 binding site (highlighted in yellow), gRNA sequence (in blue), and PAM site (in red). R15-1 has undergone CRISPR-Cas9 with no mutations in the region of interest.

Drosophila strains and culture

Flies were maintained in a standard corn-agar medium at 25°C. The strains used in this study are described in Table 1. Fly lines generated through CRISPR-Cas9 genome editing were used to create the experimental crosses. For each cross, 3-5 virgin females from each of the CRISPR-Cas9 lines were placed with 3 males from a stock heterozygous for the IRER deletion (M019), as shown in Figure 2, to generate fly lines that are transheterozygous for the P53BSKO and Df(IRER). In the first cross, R8-1 virgin females with a full p53 binding motif deletion (R8-1) were crossed with and M019 males. Flies heterozygous for both the p53 binding site deletion and Df(IRER) were selected for analysis. For the second cross, R15-1 virgin females—a wild type fly line that underwent CRISPR-Cas9 genome editing but have no p53 binding site deletion—were crossed with M019 males. Flies heterozygous for R15-1 chromosome and Df(IRER) were selected for analysis.

Table 1. Genotypes of Parent and Experimental Fly Lines (A Database of Drosophila Genes and Genomes, flybase.org)

Fly Line	Genotype	Notes
G085	w[1118]	
M069	w[*]; RPR BS KO (R8-1)/Tm3,Sb[1],Ser[1]	Mutation R8-1 for P53 BS near RPR. 18403346:TTCGGAATGGGTTTTCAGATTTC------------------------------TTCCTTTGTGTGTGTC:18403425 (41bp deletion)
R8-1	w[*]; RPR BS KO (R8-1)/Df(IRER)B11	

M192	w[*];RPR BS KO (R15-1)/Tm3, Sb[1], Ser[1]	Generated from R15-1 CRISPR CAS 918403346:TTCGGAATGGGTTTTCAGATTTCTTCACTCAGCGTGCATCGACGTGTTCGAAGGCCTATT (no mutation)
R15-1	w[*];RPR BS KO (R15-1)/Df(IRER)B11	
M019	w[*]; Df(IRER)B11/TM3 Sb Ser p{w+=?GFP}	Df(IRER) verified 06-10-2016 by Ben & Aaron; isogenic avoid water; PCR verfied deletion on 2012.

Experimental Cross:

Generating $\boldsymbol{w}[*];\dfrac{\boldsymbol{p53\ BSKO}\ (\boldsymbol{R}\#-\#)}{\boldsymbol{Df}(\boldsymbol{IRER})\boldsymbol{B11}}$

R8-1 Female Virgins **OR** M192 R15-1 Female Virgins M019 Males

$w[*];\dfrac{p53\ BSKO\ (R\#-\#)}{Tm3,Sb[1],Ser[1]}$ X $w[*];\dfrac{\mathrm{Df(IRER)B11}}{Tm3,Sb,Ser,\mathrm{p\{w+=?GFP\}}}$

Examine F1 $\boldsymbol{w}[*];\dfrac{\boldsymbol{p53\ BSKO}\ (\boldsymbol{R}\#-\#)}{\boldsymbol{Df}(\boldsymbol{IRER})\boldsymbol{B11}}$ **for hyperplasia phenotype in the wings.**

Figure 2. Experimental Crossing Plan. Outcome of the F1 generation results in offspring that are transheterozygous for the p53 binding site knockout (P53BSKO) on one allele and IRER deficiency (Df(IRER)) on the other allele.

Wing size measurement and trichome density

The left and right wings of each experimental vial were dissected and mounted on microscope slides using Permount Mounting Medium, (Fisher Scientific, Fair Lawn, New Jersey, United States, Catalog # SP15-100, Lot#005968-24). Images of each wing were captured with a high-power microscope and uploaded into a FijiWings 2.2 macros software (Dobens & Dobens, "FijiWings", 2013). Figure 3 illustrates a representative dissected wing. Five longitudinal veins were identified and labelled as L1-L5. The line segment tool within the software, was used to measure the width of L5 to determine differences in wing size, as shown in Figure 2. The "75px sq trichome density" tool within FijiWings 2.2 macros software was also used to determine wing hair (trichome) densities within a selected $75px^2$ between L1-L2, L2-L3, L4-L5, and L5. Figure 3 shows the division of the wing used to determine the various compartments for trichome

measurements. The average of each compartment for each genotype was then calculated and used for comparative analysis.

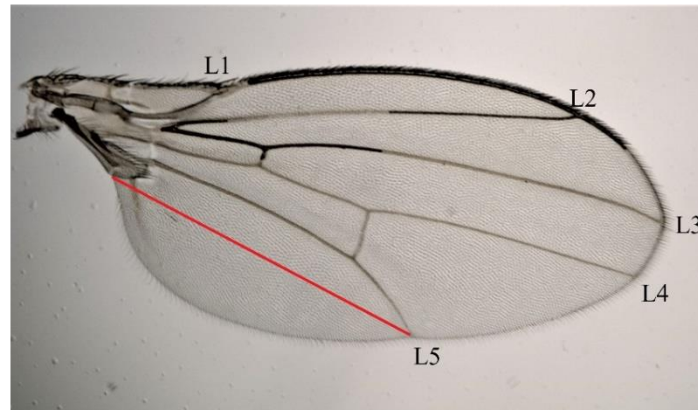

Figure 2. Representative image of a dissected right wing labelled with the longitudinal veins L1-L5. The red line indicates where the L5 measurement was extracted using the FijiWings' line segment tool.

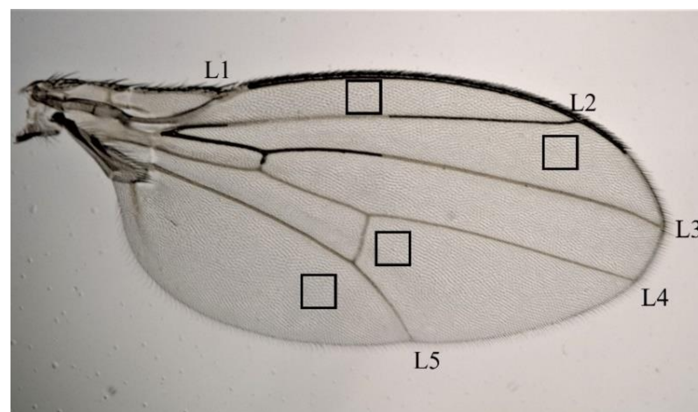

Figure 3. Representative image of a dissected right wing labelled with the longitudinal veins L1-L5. The squared indicate the areas where trichome densities were measured using the FijiWings 75px sq trichome density tool.

Statistical Methods

Student t-tests were run on data in order to determine the significance of differences in compartment size and trichome density between the experimental lines and control lines. For comparisons between the wild type, G085, and the experimental lines (R8-1 and R15-1), a single-tailed t-test was used. When comparing the experimental lines to one another, a two-tailed t-test was used.

Results

To compare wing area, we took width measurements of the L5 compartments of both male and female flies for each genotype using FijiWings' line segment tool (as shown in Figure 4). We chose L5 because we have previously noted that the most significant size difference occurred

within this area of the wing. Measurements were recorded and the average was taken of left and right wings combined for each experimental genotype (R8-1 and R15-1), the parent genotype (M069 and M192), and G085 (negative control).

Females showed the most significant difference between experimental genotypes and G085, as shown in Figure 5. R8-1 females (heterozygous for both the full p53BSKO and Df(IRER)) showed a significant increase in size when compared to the G085 wild type control($p = 2.58E-07$). A similar increase in the L5 compartment width is noted when comparing R15-1 to G085 ($p = 0.003$). When comparing R8-1 to R15-1, it is important to note there is a significant difference in size between these experimental lines ($p = 0.003$), likely indicating that a homozygous deletion of the p53 binding site found in R8-1 results in greater hyperplasia than in R15-1, which is heterozygous for an unmutated p53 binding site. These increases in width could indicate hyperplasia resulting from an increase in cell number.

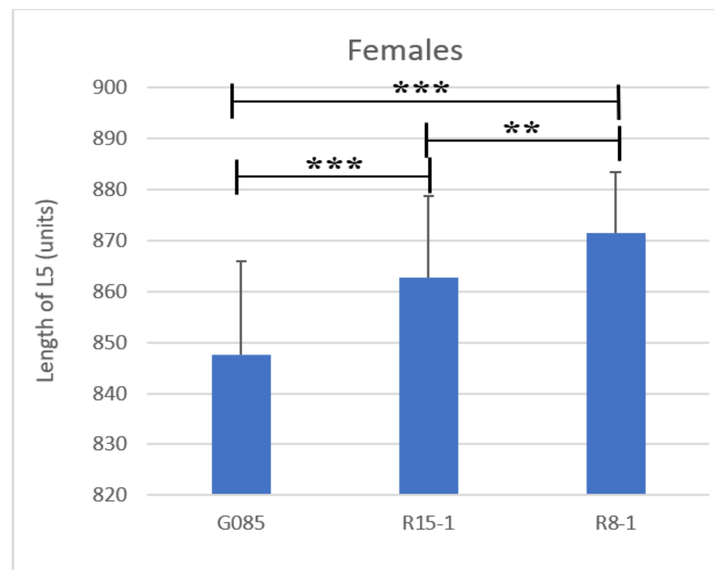

Figure 5. Average L5 Compartment Width for Females. Student t-test results indicate that there are significant differences in the L5 wing compartment size between the three genotypes, most notably when comparing R8-1 to the wild type, G085, and when comparing R8-1 to R15-1 (G085 vs. R8-1 $p = 2.58E-07$, G085 vs. R15-1 $p = 0.00035$, and R8-1 vs. R15-1 $p = 0.003$).

A small trend can be noted in male flies of each genotype, as shown in Figure 6, where R15-1 males displayed slightly wider L5 compartments than R8-1 males, which was an unexpected result. However, statistical analysis comparing each genotype showed no statistically significant

difference in wing size (G085 vs. R8-1 p = 0.249, G085 vs. R15-1 p = 0.244, and R8-1 vs. R15-1 p = 0.306).

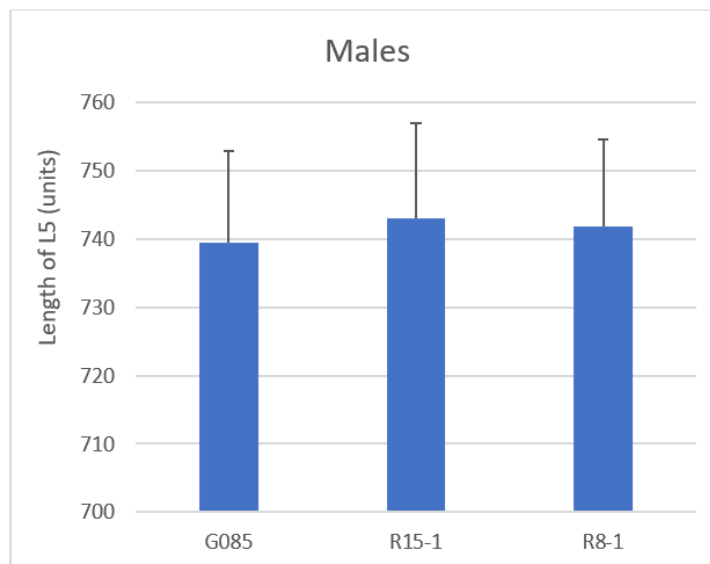

Figure 6. Average L5 Compartment Width for Males. Student t-test results indicate that there are no significant differences in the L5 wing compartment size between the three genotypes (G085 vs. R8-1 p = 0.249, G085 vs. R15-1 p = 0.244, and R8-1 vs. R15-1 p = 0.306).

Phenotypical differences between each genotype are more significant in female animals versus males, with the largest of these being between R8-1 and the negative control, G085. It is unclear why R15-1 showed a slightly increased width compared to R8-1 in males but not in females. We hypothesize there may be an off-target mutation during CRISPR-Cas9 genome editing when creating the R15-1 line.

Increased wing area alone cannot be used to determine whether the experimental fly lines experienced hyperplasia when compared to wild type flies. In order to support this data, we measured trichome densities of both experimental lines and G085, as described in Figure 3.

We first compared compartment data between both experimental lines as shown in Figure 7(a-b). Based on the data, there is a statistical difference in mean cell number between R8-1 and R15-1 within the posterior compartment L4-L5 (p = 0.003), showing R8-1 to have a greater mean cell number in females. These results agree with previous experiments that indicated an increase in cell number within the posterior compartment. For males, there was an increased mean cell number for R8-1 in compartments L2-L3 (p = 0.0044) and L5 (p = 0.0056). These results appear to indicate increased hyperplasia within R8-1 animals as compared to R15-1.

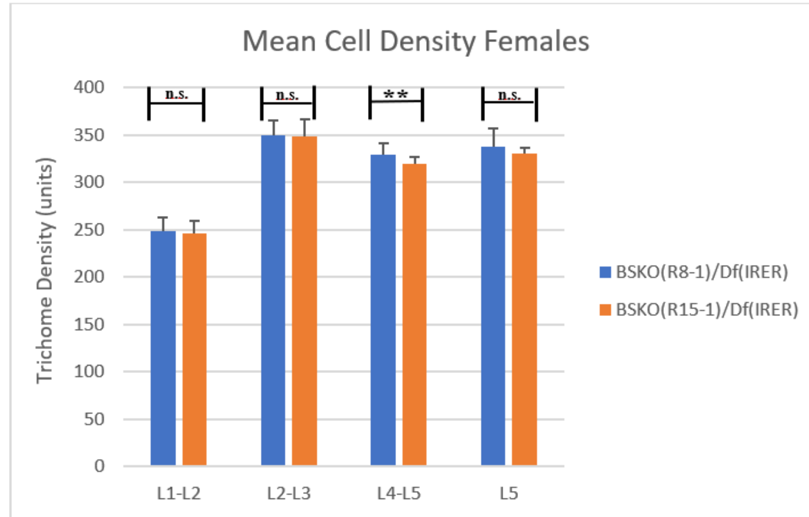

Figure 7a. Mean Cell Density (Females) of Experimental Lines. Student t-test results indicate significant differences in the mean cell density in compartment L4-L5 ($p = 0.003$), with no significance in compartments L1-L2 ($p = 0.662$), L2-L3 ($p = 0.882$), and L5 ($p = 0.081$).

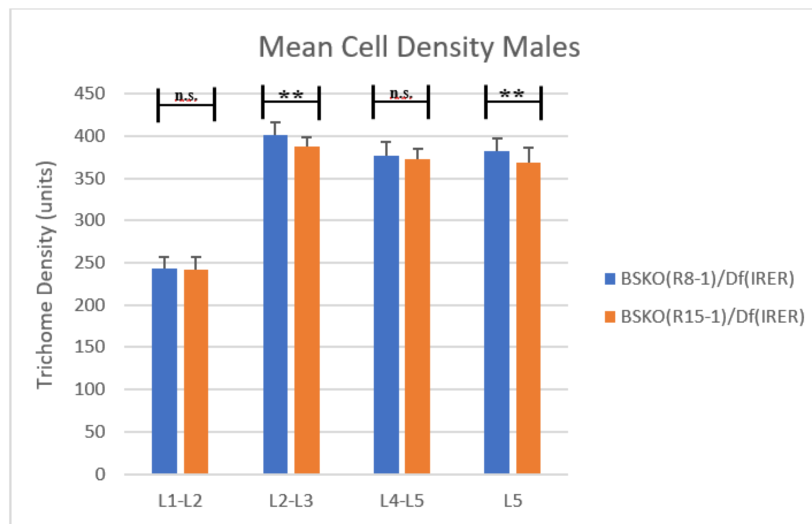

Figure 7b. Mean Cell Density (Males) of Experimental Lines. Student t-test results indicate significant differences in the mean cell density in compartments L2-L3 ($p = 0.0044$) and L5 ($p = 0.0056$), with no significance in compartments L1-L2 ($p = 0.796$) and L4-L5 ($p = 0.487$).

After comparing the experimental lines to one another, we then compared them to our wild type control (G085), as shown in Figure 8(a-b) and Figure 9(a-b). R8-1 showed the greatest statistical difference when compared to G085 for females (Figure 8a), particularly in compartments L1-L2 ($p = 2.9E-10$) and L5 ($p = 0.049$). It is worth noting that G085 appears to have a greater cell mean number for females in both the L2-L3 ($p = 0.00064$) and L4-L5 (p =

0.0028) compartments. Males (Figure 8b) showed an increased mean cell number for R8-1 in compartment L5 (p = 1.43E-06). There is no significant statistical difference in any of the other compartments of the wing (L1-L2 p = 0.321, L2-L3 p = 0.298, L4-L5 p = 0.167).

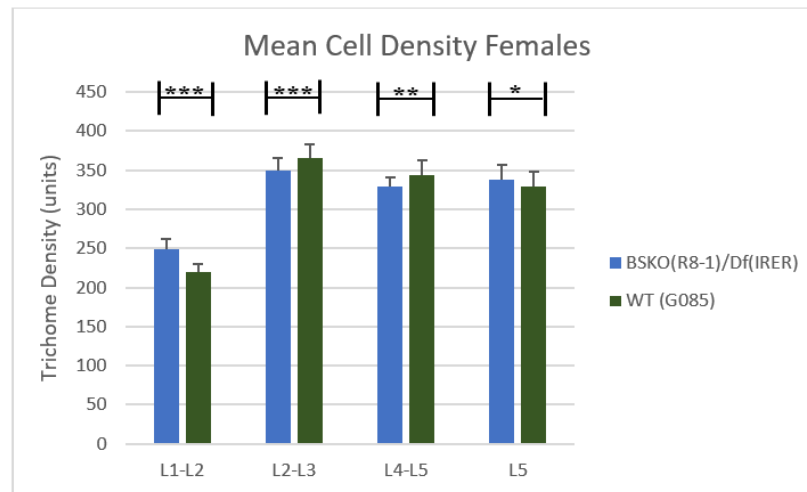

Figure 8a. Mean Cell Density (Females) for Experimental Line R8-1 as Compared to Wild Type Control. Student t-test results indicate significant differences in the mean cell density in compartments L1-L2 (p = 2.9E-10), L2-L3 (p = 0.0064), and L4-L5 (p = 0.0028), with minimal significance in compartment L5 (p = 0.049).

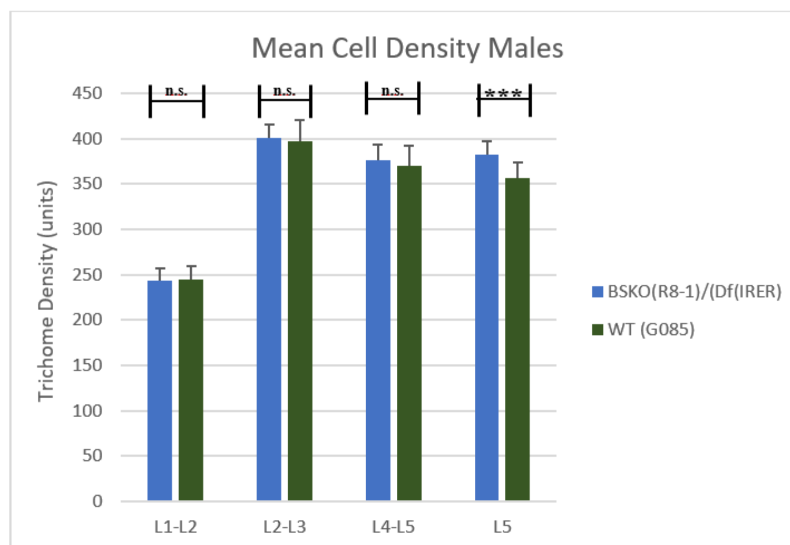

Figure 8b. Mean Cell Density (Males) for Experimental Line R8-1 as Compared to Wild Type Control. Student t-test results indicate significant differences in the mean cell density in compartment L5 (p = 1.43E-06), with no significance in compartments L1-L2 (p = 0.321), L2-L3 (p = 0.298), and L4-L5 (p = 0.167).

R15-1 females show significant differences when compared to the wild type G085 in compartments L1-L2 (p = 4.86E-07), L2-L3 (p = 0.004), and L4-L5 (p = 2.54E-06). Similarly,

R15-1 males showed significant differences in mean cell density in compartments L2-L3 (p = 0.0348) and L5 (p = 0.0137). This is important because we expected R15-1 animals to show a milder hyperplastic phenotype than R8-1, as they are only heterozygous for the p53 binding site.

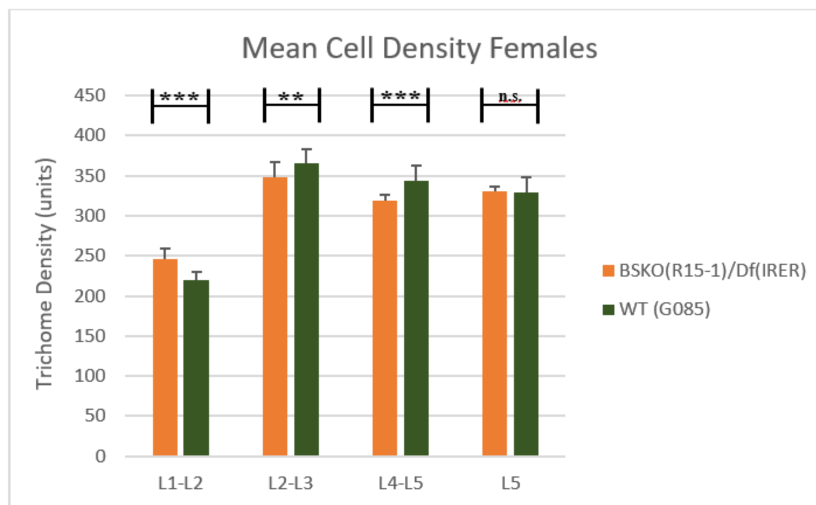

Figure 9a. Mean Cell Density (Males) for Experimental Line R15-1 as Compared to Wild Type Control. Student t-test results indicate significant differences in the mean cell density in compartments L1-L2 (p = 4.86E-07), L2-L3 (p = 0.004), and L4-L5 (p = 2.54E-06), with no significance in compartment L5 (p = 0.353)
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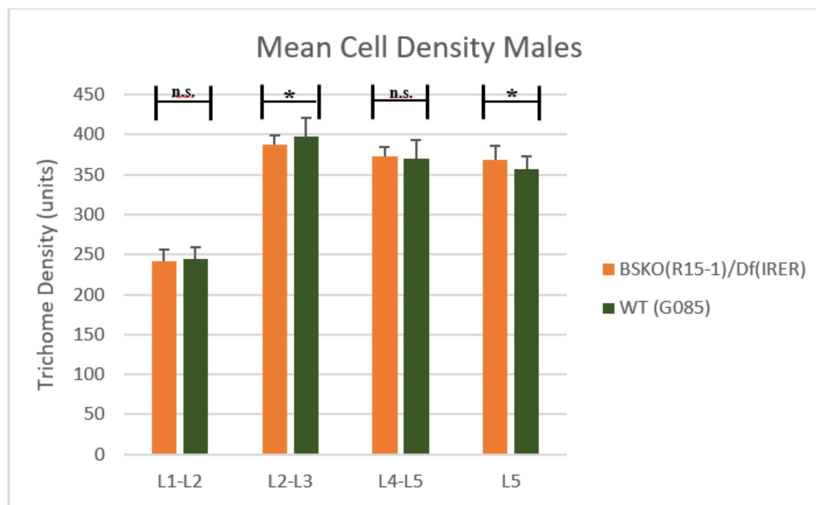

Figure 9b. Mean Cell Density (Males) for Experimental Line R15-1 as Compared to Wild Type Control. Student t-test results indicate significant differences in the mean cell density in compartments L2-L3 (p = 0.0348) and L5 (p = 0.0137), with no significance in compartments L1-L2 (p = 0.225) and L4-L5 (p = 0.307).

Discussion and Conclusion

Our experiment seeks to determine the relationship between p53-induced apoptosis and suppression of hyperplasia, which is considered one of the first steps towards cancer development. The results of our experiment indicated that there is a difference between p53BSKO(R8-1)/Df(IRER) flies1 and the wild type (control). Furthermore, the increase in cell density and L5 compartment width for p53BSKO(R15-1)/Df(IRER is unexpected as this fly line, unlike p53BSKO(R8-1)/Df(IRER), is heterozygous for the deletion to the p53 binding site motif. It is possible that there was some off-target mutation generated in the CRISPR-Cas9 genome editing process.

In order to verify our findings, further experiments need to be done in order to understand the significance of the difference between R8-1 and G085. The results of our experimental crosses will need to be compared to those of homozygous and heterozygous Df(IRER) animals as they will serve as a positive control to determine the extent to which the R8-1 deletion exacerbates hyperplasia. Our data set would be further improved by collecting more samples in order to have a larger and more even population sample, which would make comparing data sets much more reliable.

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