# Supplemental Material: Data Analysis Code and Results

## December 19, 2024

This file reproduces the figures and statistics reported in the manuscript. As a guide, this table helps map variable names in the code to the biological meanings:

Code Category Name	Code Variable Name	Biological Variable Name	
new_gene	zip/+ sqh/+	$zip^1/+ sqh^{AX3}/+$	
dv5_base	+/+ dv5/+ dv5/RAR dv5/dv5	$Gli^+/Gli^+ \ Gli^{dv5}/+ \ Gli^{dv5}/Gli^{RAR77} \ Gli^{dv5}/Gli^{dv5}$	

# Load necessary libraries and do setup

```
library(tidyverse)
## -- Attaching core tidyverse packages ----- tidyverse 2.0.0 --
             1.1.3
## v dplyr
                       v readr
                                   2.1.4
## v forcats 1.0.0
                        v stringr
                                    1.5.0
## v ggplot2 3.4.4
                        v tibble
                                    3.2.1
## v lubridate 1.9.3
                        v tidyr
                                    1.3.0
## v purrr
              1.0.2
## -- Conflicts ----- tidyverse_conflicts() --
## x dplyr::filter() masks stats::filter()
## x dplyr::lag()
                   masks stats::lag()
## i Use the conflicted package (<a href="http://conflicted.r-lib.org/">http://conflicted.r-lib.org/</a>) to force all conflicts to become error
library(readxl)
library(ggplot2)
library(FSA)
## Warning: package 'FSA' was built under R version 4.3.3
## ## FSA v0.9.5. See citation('FSA') if used in publication.
## ## Run fishR() for related website and fishR('IFAR') for related book.
library(latex2exp)
```

## Warning: package 'latex2exp' was built under R version 4.3.3

```
# Set bootstrap samples
NUM_BOOTSTRAPS = 10000
```

# Get data ready

### Load and clean data

```
raw_data <- read_excel("WingScores_original.xlsx",</pre>
   col_types = c("skip", "numeric", "numeric",
                 "skip", "numeric", "numeric", "numeric",
                 "skip", "numeric", "numeric", "numeric",
                 "skip", "numeric", "numeric", "numeric"))
# Select the first 90 rows
WingScores by cols <- head(raw data, 90)
str(WingScores_by_cols)
## tibble [90 x 11] (S3: tbl_df/tbl/data.frame)
## $ +/+ & zip/+ : num [1:90] 0 0 0 0 1 0 1 1 1 0 ...
## $ +/+ & sqh/+ : num [1:90] 0 0 0 0 0 0 0 0 0 ...
## $ dv5/+ & +/+
                   : num [1:90] 0 0 0 0 1 0 2 0 1 1 ...
## $ dv5/+ & zip/+ : num [1:90] 0 0 1 0 1 1 2 0 0 2 ...
## $ dv5/+ & sqh/+ : num [1:90] 1 0 1 1 0 2 1 0 0 0 ...
## $ dv5/RAR & +/+ : num [1:90] 5 3 3 5 4 5 4 3 2 3 ...
## $ dv5/RAR & zip/+: num [1:90] 6 3 4 3 3 5 2 2 4 4 ...
## $ dv5/RAR & sqh/+: num [1:90] 2 5 2 4 6 6 5 5 5 6 ...
## $ dv5/dv5 & +/+ : num [1:90] 6 4 5 4 2 6 4 4 4 4 ...
## $ dv5/dv5 & zip/+: num [1:90] 5 5 5 6 4 4 4 7 6 5 ...
## $ dv5/dv5 & sqh/+: num [1:90] 4 4 7 6 6 5 3 4 6 4 ...
```

```
head(WingScores_by_cols)
```

```
## # A tibble: 6 x 11
     '+/+ & zip/+' '+/+ & sqh/+' 'dv5/+ & +/+' 'dv5/+ & zip/+' 'dv5/+ & sqh/+'
##
            <dbl>
                           <dbl>
                                        <dbl>
                                                          <dbl>
                                                                          <dbl>
## 1
                                              0
                                                                               1
## 2
                 0
                               0
                                              0
                                                              0
                                                                               0
                 0
                                              0
                                                              1
                                                                              1
## 4
                 0
                               0
                                              0
                                                              0
                                                                               1
## 5
                 1
                               0
                                              1
                                                              1
                                                                              0
                               0
## # i 6 more variables: 'dv5/RAR & +/+' <dbl>, 'dv5/RAR & zip/+' <dbl>,
     'dv5/RAR & sqh/+' <dbl>, 'dv5/dv5 & +/+' <dbl>, 'dv5/dv5 & zip/+' <dbl>,
## #
     'dv5/dv5 & sqh/+' <dbl>
## #
```

## Pivot data into long format

```
WingScores_long <- WingScores_by_cols %>%
 pivot_longer(
   cols = everything(),
                               # Specify all columns to be pivoted
   names_to = "mutation", # Name of the new column for variable names
values_to = "score" # Name of the new column for scores
  ) %>%
  drop_na()
dv5 levels \leftarrow c("+/+", "dv5/+", "dv5/RAR", "dv5/dv5")
WingScores <- WingScores_long %>%
  separate(mutation, into = c("dv5_base", "new_gene"), sep = "&", convert = TRUE) %>%
  mutate(dv5_base = trimws(dv5_base),
         new_gene = trimws(new_gene),
         dv5_base = factor(dv5_base, levels = dv5_levels, ordered = TRUE),
         new_gene = as.factor(new_gene))
str(WingScores)
## tibble [656 x 3] (S3: tbl_df/tbl/data.frame)
## $ dv5_base: Ord.factor w/ 4 levels "+/+"<"dv5/+"<..: 1 1 2 2 2 3 3 3 4 4 ...
## $ new_gene: Factor w/ 3 levels "+/+", "sqh/+",..: 3 2 1 3 2 1 3 2 1 3 ...
## $ score : num [1:656] 0 0 0 0 1 5 6 2 6 5 ...
head(WingScores)
## # A tibble: 6 x 3
    dv5_base new_gene score
    <ord> <fct>
##
                     <dbl>
## 1 +/+
             zip/+
## 2 +/+
            sqh/+
                           0
## 3 dv5/+ +/+
## 4 dv5/+
              zip/+
                           0
## 5 dv5/+
              sqh/+
                           1
## 6 dv5/RAR +/+
                           5
summary(WingScores)
       dv5_base
                 new_gene
                                  score
##
## +/+ :180 +/+ :269
                              Min.
                                     :0.000
## dv5/+ :177
                 sqh/+:191
                              1st Qu.:0.000
## dv5/RAR:164
                  zip/+:196
                              Median :1.000
## dv5/dv5:135
                              Mean :1.805
##
                              3rd Qu.:4.000
                              Max. :7.000
##
```

# Explore

This section gives some summary statistics and initial visualizations of the dataset.

Counts of scores (how many samples for each crossed category)

```
count_table <- WingScores %>%
  count(dv5_base, new_gene)
# Convert the count table into a two-way table
counts_two_way_table <- count_table %>%
 pivot_wider(names_from = new_gene, values_from = n)
rownames(counts_two_way_table) <- NULL</pre>
counts_two_way_table <- counts_two_way_table %>% column_to_rownames(var = "dv5_base")
print(counts_two_way_table)
           sqh/+ zip/+ +/+
##
## +/+
              90
                    90 NA
## dv5/+
              43
                    44 90
## dv5/RAR
              40
                    34 90
## dv5/dv5
             18
                    28 89
```

## Medians of score

```
# Summarize the data to get the median score for each combination of dv5 base and new gene
median_scores <- WingScores %>%
  group_by(dv5_base, new_gene) %>%
  summarize(median_score = median(score, na.rm = FALSE))
## 'summarise()' has grouped output by 'dv5_base'. You can override using the
## '.groups' argument.
med_summary_table <- xtabs(median_score ~ dv5_base + new_gene, data = median_scores)</pre>
med_summary_table['+/+', '+/+'] <- NA</pre>
print(med_summary_table)
##
            new_gene
## dv5_base +/+ sqh/+ zip/+
     +/+
##
                     0
##
     dv5/+
               0
                     0
                            0
##
     dv5/RAR
               3
                     5
                            4
     dv5/dv5
               4
                     5
                           5
##
```

### Means of score

```
# Summarize the data to get the mean score for each combination of dv5_base and new_gene
mean_scores <- WingScores %>%
  group_by(dv5_base, new_gene) %>%
  summarize(mean score = mean(score, na.rm = TRUE))
## 'summarise()' has grouped output by 'dv5_base'. You can override using the
## '.groups' argument.
mean_summary_table <- xtabs(mean_score ~ dv5_base + new_gene, data = mean_scores)
mean summary table['+/+', '+/+'] <- NA
print(mean_summary_table)
##
            new_gene
## dv5 base
                             sqh/+
     +/+
##
                        0.03333333 0.22222222
##
     dv5/+
             0.16666667 0.51162791 0.47727273
##
     dv5/RAR 2.61111111 4.40000000 3.97058824
     dv5/dv5 3.69662921 4.66666667 5.14285714
##
```

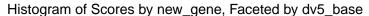
#### Plots of score distributions

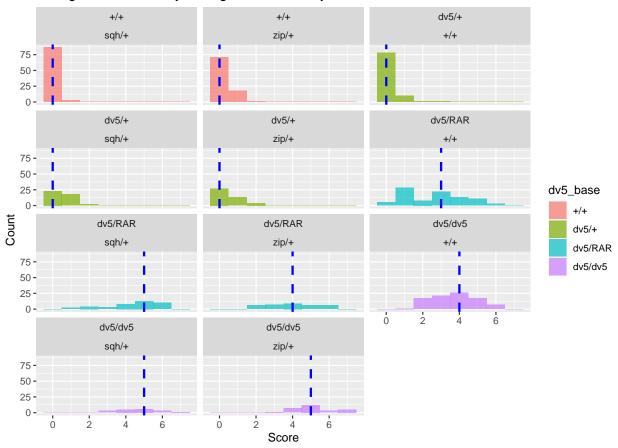
The histograms show the skewness of the data and indicate issues of normality of the distribution of scores. Since the data is measured on an ordinal scale with 7 possible values and does not satisfy the conditions of normality necessary to conduct ANOVA, we opt for focusing on the medians and using non-parametric test alternatives for assessing significance.

The dashed lines in the histograms are the medians for the respective distribution.

```
median_scores <- WingScores %>%
  group_by(dv5_base, new_gene) %>%
  summarize(median_score = median(score, na.rm = TRUE))
```

## 'summarise()' has grouped output by 'dv5\_base'. You can override using the
## '.groups' argument.



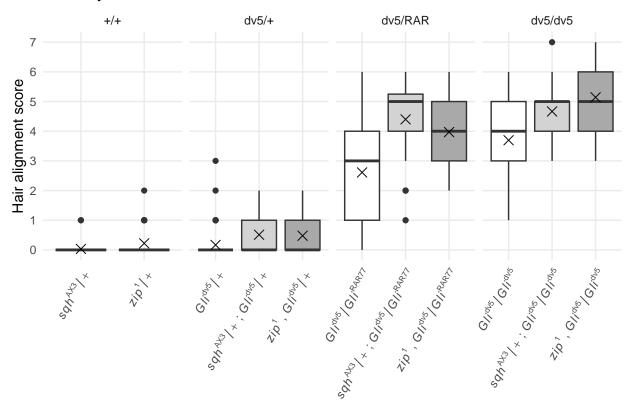


These boxplots clearly show differences in medians between levels within the same category. They also show a spread of scores in each category. The means on these plots are marked with an "X".

```
# Combine group and level into a single variable for x-axis
WingScores$x_combined <- paste(WingScores$dv5_base,</pre>
                                         WingScores$new gene, sep = ";")
# Make labels for the boxplot x-axis
custom labels <- c(</pre>
  "+/+;sqh/+"=TeX("$\\textit{sqh}^{AX3}/+$"),
  "+/+;zip/+"=TeX("$\\textit{zip}^1/+$"),
  "dv5/+;+/+"=TeX("$\\textit{Gli}^{dv5}/+$"),
  "dv5/+; sqh/+"=TeX("$\star {sqh}^{AX3}/+$; \star {gli}^{dv5}/+$"),
  "dv5/+;zip/+"=TeX("$ \\textit{zip}^1$, $\\textit{Gli}^{dv5}/+$"),
  "dv5/dv5; +/+ "= TeX("$\\text{Gli}^{dv5}/\\text{Gli}^{dv5}$"),
  "dv5/dv5; sqh/+"=TeX("$\\ tsqh}^{AX3}/+$ ; $\\ tstit{Gli}^{dv5}/\\ textit{Gli}^{dv5}$"),
  "dv5/dv5;zip/+"=TeX("$\\ textit{zip}^1$, $ \\ textit{Gli}^{dv5}/\\ textit{Gli}^{dv5}$"),
  "dv5/RAR;+/+"=TeX("$\\textit{Gli}^{dv5}/\\textit{Gli}^{RAR77}$"),
  "dv5/RAR; sqh/+"=TeX("$\\text{sqh}^{AX3}/+$ ; $\\text{Gli}^{dv5}/\\text{Gli}^{RAR77}$"),
  \label{lem:condition} $$ \dv5/RAR; zip/+"=TeX("$\times {zip}^1$ , $\times {Gli}^{dv5}/\textit{Gli}^{RAR77}$") $$
  )
# Boxplot
p <- ggplot(WingScores, aes(x = x_combined, y = score, fill = new_gene)) +</pre>
```

```
geom_boxplot(position = position_dodge(width = 1.0), width = 0.8) +
  stat_summary(fun = mean, geom = "point", shape = 4, size = 3, color = "black",
                 position = position_dodge(width = 1.0)) +
  facet_wrap(~ dv5_base, scales = "free_x", nrow=1) +
  scale_fill_manual(values = c("white","lightgrey","darkgrey")) +
  scale_x_discrete(labels = custom_labels) +
 labs(title = "Side-by-Side Box Plots", x = NULL,
      y = "Hair alignment score", fill = NULL) +
 theme minimal() +
  theme(axis.text.x = element_text(angle = 60, hjust = 1)) +
  guides(fill=FALSE) +
 scale_y_continuous(
  breaks = seq(0, 7, by = 1), # Setting breaks for every unit
 labels = seq(0, 7, by = 1), # Corresponding labels for the breaks
 minor_breaks = NULL
 )
## Warning: The '<scale>' argument of 'guides()' cannot be 'FALSE'. Use "none" instead as
## of ggplot2 3.3.4.
## This warning is displayed once every 8 hours.
## Call 'lifecycle::last_lifecycle_warnings()' to see where this warning was
## generated.
# Save the plot
# ggsave("figures/hair_scores_boxplots_scaled.tiff", plot = p, width = 10, height = 5, dpi = 300)
```

# Side-by-Side Box Plots



# Analysis

Through investigation, we found that trying to use two-way ANOVA with interaction leads to failure of the assumptions of normality of residuals and homogeneity of variance of residuals. Thus, we have to resort to non-parametric methods.

### Kruskal-Wallis Test

A non-parametric test to see if there is a significant difference in the distributions of scores across the 3 levels of the new\_gene factor for each level of the dv5\_base factor. The following code and output shows that for each of the 4 levels of Gli, there is a significant difference in the distributions of the scores between the 3 new\_gene levels.

```
# Filter data for $Gli^+/Gli^+$
gli0_level_data <- WingScores %>% filter(dv5_base == '+/+')
kruskal_test <- kruskal.test(score ~ new_gene, data = gli0_level_data)
print(kruskal_test)

##
## Kruskal-Wallis rank sum test
##
## data: score by new gene</pre>
```

## Kruskal-Wallis chi-squared = 13.228, df = 1, p-value = 0.0002758

```
# Filter data for dv5/+
gli1_level_data <- WingScores %>% filter(dv5_base == 'dv5/+')
kruskal_test <- kruskal.test(score ~ new_gene, data = gli1_level_data)</pre>
print(kruskal_test)
##
##
    Kruskal-Wallis rank sum test
##
## data: score by new_gene
## Kruskal-Wallis chi-squared = 18.756, df = 2, p-value = 8.458e-05
# Filter data for dv5/RAR
gli2_level_data <- WingScores %>% filter(dv5_base == 'dv5/RAR')
kruskal_test <- kruskal.test(score ~ new_gene, data = gli2_level_data)</pre>
print(kruskal_test)
##
##
    Kruskal-Wallis rank sum test
##
## data: score by new_gene
## Kruskal-Wallis chi-squared = 35.569, df = 2, p-value = 1.889e-08
# Filter data for dv5/dv5
gli3_level_data <- WingScores %>% filter(dv5_base == 'dv5/dv5')
kruskal_test <- kruskal.test(score ~ new_gene, data = gli3_level_data)</pre>
print(kruskal test)
##
##
    Kruskal-Wallis rank sum test
##
## data: score by new_gene
## Kruskal-Wallis chi-squared = 26.71, df = 2, p-value = 1.585e-06
```

### Dunn's test for pairwise comparisons (post-hoc analyses)

The Dunn's test adjusted p-values are the ones we report in the manuscript (annotated on the boxplot). Below, we check within each dv5\_base category which pairs show significant differences in their medians using Dunn's test. The values we use are the ones in the column labeled P.adj.

```
When dv5_base = +/+
```

```
dunnTest(score ~ new_gene, data=gli0_level_data, method="bonferroni")

## Dunn (1964) Kruskal-Wallis multiple comparison

## p-values adjusted with the Bonferroni method.

## Comparison Z P.unadj P.adj

## 1 sqh/+ - zip/+ -3.637058 0.0002757703 0.0002757703
```

For  $Gli^+/Gli^+$ , there is no significant difference in the medians of the scores when the new\_gene is +/+ or when it is zip/+. However, the other two pairs show a significant difference in median scores.

#### When $dv5_base = dv5/+$

```
dunnTest(score ~ new_gene, data=gli1_level_data, method="bonferroni")

## Dunn (1964) Kruskal-Wallis multiple comparison

## p-values adjusted with the Bonferroni method.

## Comparison Z P.unadj P.adj

## 1 +/+ - sqh/+ -3.8536106 0.0001163887 0.000349166

## 2 +/+ - zip/+ -3.1239660 0.0017843108 0.005352932

## 3 sqh/+ - zip/+ 0.6516349 0.5146367289 1.0000000000
```

For the dv5/+ Gli level, there is no significant difference in the medians of the scores when the new\_gene is sqh/+ or when it is zip/+. However, the other two pairs show a significant difference in median scores.

### When $dv5_base = dv5/RAR$

```
dunnTest(score ~ new_gene, data=gli2_level_data, method="bonferroni")

## Dunn (1964) Kruskal-Wallis multiple comparison

## p-values adjusted with the Bonferroni method.

## Comparison Z P.unadj P.adj

## 1 +/+ - sqh/+ -5.490198 4.014842e-08 1.204453e-07

## 2 +/+ - zip/+ -3.823784 1.314191e-04 3.942574e-04

## 3 sqh/+ - zip/+ 1.172748 2.408968e-01 7.226903e-01
```

For the dv5/RAR Gli level, there is no significant difference in the medians of the scores when the new\_gene is sqh/+ or when it is zip/+. However, the other two pairs show a significant difference in median scores.

### When dv5 base = dv5/dv5

```
dunnTest(score ~ new_gene, data=gli3_level_data, method="bonferroni")

## Dunn (1964) Kruskal-Wallis multiple comparison

## p-values adjusted with the Bonferroni method.

## Comparison Z P.unadj P.adj

## 1 +/+ - sqh/+ -2.770320 5.600117e-03 1.680035e-02

## 2 +/+ - zip/+ -4.830048 1.365001e-06 4.095003e-06

## 3 sqh/+ - zip/+ -1.094347 2.738030e-01 8.214091e-01
```

For the dv5/dv5 Gli level, there is no significant difference in the medians of the scores when the new\_gene is sqh/+ or when it is zip/+. However, the other two pairs show a significant difference in median scores.

## Bootstrapping for finding the signficance of the interactions

The strategy is described as follows:

- 1. Generate a stratified bootstrap sample for each of the dv5\_base levels with no new\_gene mutation.
- 2. Generate a stratified bootstrap sample for each of the new\_gene mutations (sqh/+ and zip/+) where dv5\_base = +/+.
- 3. Add the response values for each cross of the new\_gene and dv5\_base levels to get the expected additive value. Only compute as many added values as there were in the original dataset. So, for example, the original dataset had 18 values for dv5/dv5 with sqh/+ so only 18 rows will be computed in the bootstrap sample.
- 4. Find the median of each crossed-level.
- 5. Repeat this procedure 10,000 times to generate a null distribution.
- 6. Calculate a p-value as the number of bootstrap samples with median values greater than or equal to the observed value of the crossed levels of the original data. There will be 6 p-values to compute since there are 3 levels of interest of the dv5\_base and 2 levels of interest of the new\_gene.

The analysis in this section helps to establish how significant is the more-than-additive effect we see of combining, for example, sqh/+ with dv5/RAR (resulting in a score increase of 2 when 0 is expected).

### Function to calculate the expected medians of combining base levels from each factor

```
# Define the bootstrapping function
bootstrap_expected <- function(data, counts_two_way_table, n_bootstrap = NUM_BOOTSTRAPS) {
  set.seed(123) # For reproducibility
  boot_expected <- replicate(n_bootstrap, {</pre>
    # Resample the data stratified
    resample_data <- data %>%
      group_by(dv5_base, new_gene) %>%
      slice_sample(prop = 1, replace = TRUE) %>%
      filter((dv5_base == "+/+" & new_gene != "+/+") |
              (dv5_base != "+/+" & new_gene == "+/+")) %>%
      ungroup()
    # Calculate the expected additive effects
    # Loop over the vectors and filter data
    gli_vals <- c("dv5/+", "dv5/RAR", "dv5/dv5")
    new_gene_vals <- c("sqh/+", "zip/+")</pre>
    expected_data <- data.frame()</pre>
    for (i in gli_vals) {
      for (j in new_gene_vals) {
        # Filter the data by corresponding values in gli_vals and new_gene_vals
        gli_only <- resample_data %>%
          filter(dv5_base == i, new_gene == '+/+')
        new_gene_only <- resample_data %>%
          filter(dv5_base == '+/+', new_gene == j)
        # Only add as many values together as there were in the original dataset for the crossed levels
        num_to_compute <- counts_two_way_table[i, j]</pre>
```

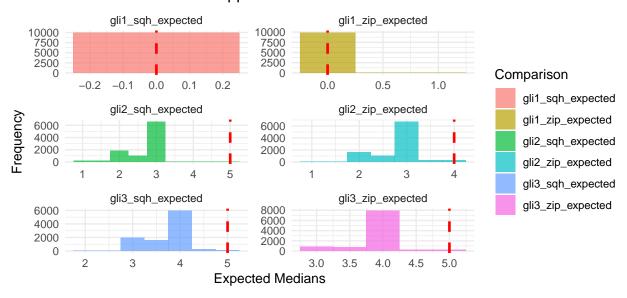
```
sum <- head(gli_only, num_to_compute)$score + head(new_gene_only, num_to_compute)$score</pre>
        expected <- data.frame(dv5_base = rep(NA, num_to_compute),
                               new_gene = rep(NA, num_to_compute),
                               score = rep(NA, num_to_compute))
        expected$score <- sum
        expected$dv5_base <- i</pre>
        expected$new_gene <- j
        expected data <- bind rows(expected data, expected)</pre>
      }
    }
    resample_data <- bind_rows(resample_data, expected_data)</pre>
    # Compute average for the resampled data
    resample_medians <- resample_data %>%
      group_by(dv5_base, new_gene) %>%
      summarize(average_response = median(score), .groups = 'drop') %>%
      filter((dv5_base != "+/+" & new_gene != "+/+"))
    # Combine expected into a vector
    boot_expected <- resample_medians$average_response</pre>
  })
  return(boot_expected)
# Perform the bootstrapping
boot_results <- bootstrap_expected(WingScores, counts_two_way_table, n_bootstrap = NUM_BOOTSTRAPS)
# Convert bootstrapped differences to a long format data frame for ggplot
boot_expected_long <- as.data.frame(t(boot_results)) %>%
  pivot_longer(cols = everything(), names_to = "Comparison", values_to = "Expected")
# Create labels for the comparisons
comparison_labels <- c("gli1_sqh_expected", "gli1_zip_expected",</pre>
                         "gli2_sqh_expected", "gli2_zip_expected",
                         "gli3_sqh_expected", "gli3_zip_expected"
 )
# Add labels to the data frame
boot_expected_long <- boot_expected_long %>%
  mutate(Comparison = factor(Comparison, labels = comparison_labels))
```

### Visualize

The plot below shows the distribution of the expected medians of the crossed levels of various bootstrap samples. The dashed red lines in each subplot indicate the observed median score for the respective combination of dv5\_base and new\_gene from the original data.

```
## Warning: Using 'size' aesthetic for lines was deprecated in ggplot2 3.4.0.
## i Please use 'linewidth' instead.
## This warning is displayed once every 8 hours.
## Call 'lifecycle::last_lifecycle_warnings()' to see where this warning was
## generated.
```

# Distribution of Bootstrapped Medians



To compute p-values for the interaction, we compute the proportion of interaction values that were greater than or equal to the true (observed) median score value for the respective combination of dv5\_base and new\_gene. The p-values can be interpreted in the standard sense: the probability of obtaining this evidence by chance against the null hypothesis. The null hypothesis is that the effect of the new\_gene (mutation) and dv5\_base (Gli) is additive. Thus, a low p-value suggests that our evidence is strong against the null hypothesis and the effects we see in the respective combination of Gli and mutation is more than additive.

## # A tibble: 6 x 3

##		Comparison	${\tt num\_extreme}$	<pre>prop_extreme</pre>
##		<chr></chr>	<int></int>	<dbl></dbl>
##	1	<pre>gli1_sqh_expected</pre>	10000	1
##	2	<pre>gli1_zip_expected</pre>	10000	1
##	3	<pre>gli2_sqh_expected</pre>	0	0
##	4	<pre>gli2_zip_expected</pre>	254	0.0254
##	5	gli3_sqh_expected	142	0.0142
##	6	gli3_zip_expected	212	0.0212

To read the table above, consider the row with "Comparison"=gli3\_zip\_expected. The num\_extreme =212, which tells how many of the bootstrap samples had a median score equal to or greater than the true value for that group from the original data.

The p-values for the significance of this result is given in the column titled prop\_extreme.