Homework3

Name: Nuttapong Mekvipad, Ryan William Moreau, Rani Nielsen, Silvija Pupsaite, Liuqing Zheng

Group: 7

Question 1.1

```
wt1_quant <- read_tsv("./salmon_result_part1/WT1/quant.sf")
```

```
## Parsed with column specification:
## cols(
##   Name = col_character(),
##   Length = col_double(),
##   EffectiveLength = col_double(),
##   TPM = col_double(),
##   NumReads = col_double()
## )
```

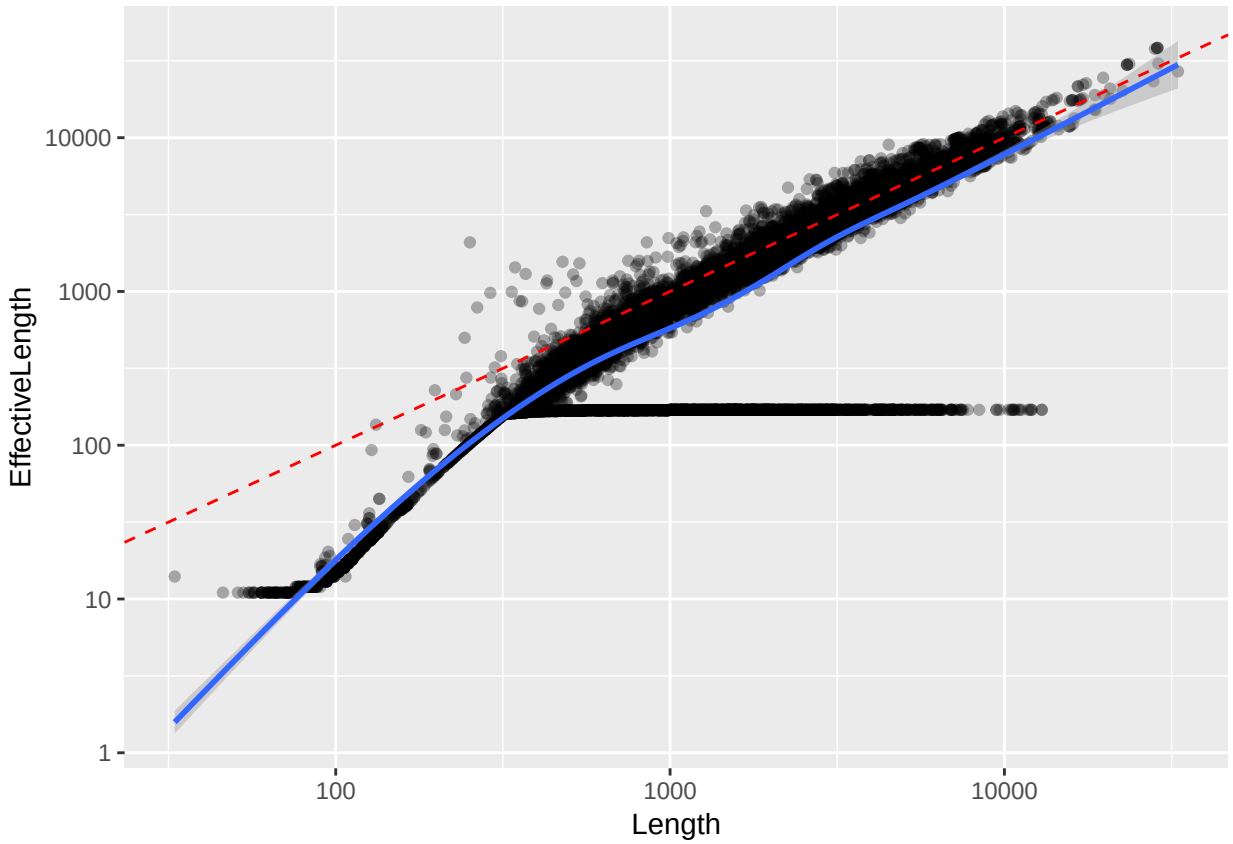
Here we plotted the isoform lengths versus effective lengths, and used geom smooth to show the trend line of the relationship between isoform lengths and effective lengths. The dash line shows the relationship where isoform lengths are equal to effective lengths.

From the plot, we can see that the actual relationship of isoform lengths and effective lengths was not linear relationship where isoform lengths are equal to effective lengths, but the effective lengths were actually shorter than the isoform lengths. This is due to the fact that during the cDNA library preparation step we fragmented the cDNA and filtered out the fragments that were smaller than certain threshold. These short fragments were mostly from the both end of cDNA; therefore the effective lengths were shorter than actual isoform lengths.

Moreover, we can see that the deviation from identity line for the shorter isoform was much greater than the longer isoform. This is because the filtered out fragment made up to greater proportion of based pair in shorter isoform than longer isoform given the certain filtering threshold.

```
wt1_quant %>% ggplot(aes(x=Length, y=EffectiveLength)) +
  scale_x_continuous(trans='log10') + scale_y_continuous(trans='log10')  +
  geom_point(alpha=0.3) +
  geom_smooth() + geom_abline(color = "red", linetype=2)
```

```
## `geom_smooth()` using method = 'gam' and formula 'y ~ s(x, bs = "cs")'
```



Question 1.2

From the figure in question 1.1, we can see the the set of outliers that followed the sigmoidal-like curve. These outliers might correspond to the cDNA fragments that came from RNAs that we do not have their cDNA read in this sample. So, the Salmon program did not have any read information, and needed to use other method for effective length estimation.

If their actual lengths were shorter than certain threshold, their effective lengths might be estimated using fixed equation by the Salmon program. This is why there was no straight line in plot before a certain effective length was reached. For the RNAs with actual lengths exceeding a certain threshold, their effective lengths might be set to fixed value which here was 170 bp. This corresponds to the upper bound of sigmoidal curve that we see in the plot.

Question 1.3

Here we imported salmon data and transformed the abundance matrix by the function $log_2(x+1)$ when x was abundance value. We can see the first 4 transcripts in transformed abundance matrix below.

```
all_salmons <- importIsoformExpression(parentDir = "./salmon_result_part1/")
```

```
## Step 1 of 3: Identifying which algorithm was used...
##     The quantification algorithm used was: Salmon
##     Found 6 quantification file(s) of interest
## Step 2 of 3: Reading data...
## reading in files with read_tsv
## 1 2 3 4 5 6
```

```
## Step 3 of 3: Normalizing FPKM/TxPM values via edgeR...
## Done
```

```
salmon_matrix <- as.matrix(all_salmons$abundance[,2:ncol(all_salmons$abundance)])
rownames(salmon_matrix) <- all_salmons$abundance[,1]

transformed_salmon <- log2(salmon_matrix+1)

transformed_salmon[1:4,]
```

```
##                       WT1       WT2       WT3    WTTPA1    WTTPA2 WTTPA3
## TCONS_00000001 0.2973299 0.0000000 0.0000000 0.3822156 0.0000000      0
## TCONS_00000002 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000      0
## TCONS_00000003 0.0000000 0.2984888 0.2253968 1.0124265 0.0000000      0
## TCONS_00003946 0.0392366 0.0000000 0.1913649 0.0000000 0.0564598      0
```

The reason why we added pseudocount = 1 to the abundance before apply log_2 to the abundance value is that if the abundance of the transcripts in some condition were 0, when we applied log_2 to 0 the value would become infinity and would not be applicable for further down stream analysis. Adding pseudocount = 1 to the abundance before transform by log_2 will prevent this problem, and also log_2 of 1 is 0 which is make sense for further analysis as the starting value before transform is already 0.

Question 1.4

We used tidyverse to extract top 100 most variable isoforms as shows by code below. We first converted abundance matrix to tibble. Then we used mutate() to apply the variance function in a rowise manner by called rowwise() function before mutate(). After that we sorted the tibble by variance and sliced out top 100 transcript with highest variance between samples.

```
salmon_tibble <- as_tibble(transformed_salmon, rownames=NA)

top100var <- salmon_tibble %>% rownames_to_column() %>% rowwise() %>%
  mutate(variance=var(c(WT1, WT2, WT3, WTTPA1, WTTPA2, WTTPA3))) %>%
  arrange(desc(variance)) %>% slice(1:100)

head(top100var, 5)
```

```
## Source: local data frame [5 x 8]
## Groups: <by row>
##
## # A tibble: 5 x 8
##   rowname          WT1   WT2   WT3 WTTPA1 WTTPA2 WTTPA3 variance
##   <chr>          <dbl> <dbl> <dbl>  <dbl>  <dbl>  <dbl>    <dbl>
## 1 TCONS_00010929  8.66  8.33  8.08   0      6.27   8.66    11.5
## 2 TCONS_00006168  5.98  0     0      0      3.88   5.44     8.27
## 3 TCONS_00006650  0     6.21  0      0      0      0        6.42
## 4 TCONS_00001502  8.07  2.44  4.50   3.44   7.26   8.10     6.20
## 5 TCONS_00003104  1.54  6.33  5.91   5.88   1.66   1.91     5.68
```

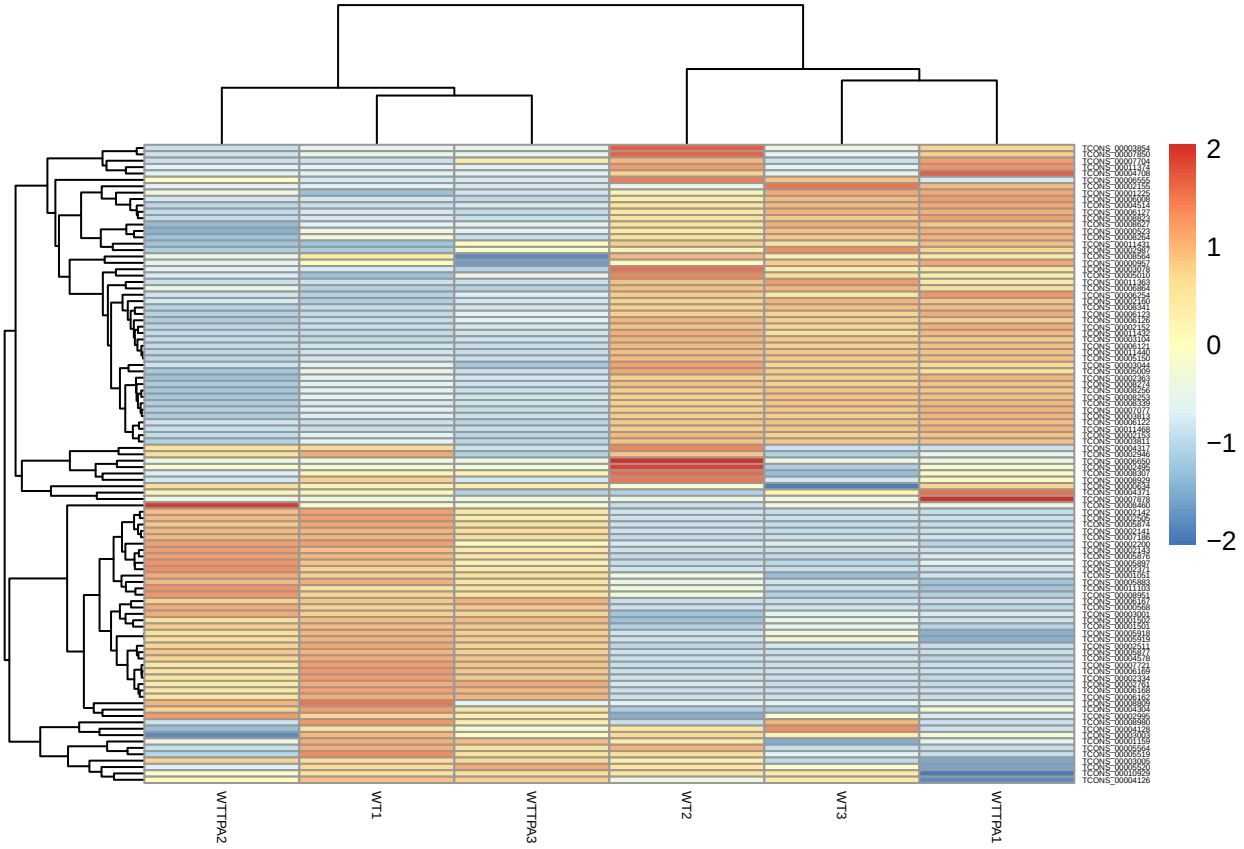
Question 1.5

We plotted the heatmap of matrix containing the transformed abundance value of top 100 transcripts with highest variance among samples using pheatmap function.

From the heatmap we can see that the samples could be clustered by the expression profile into 2 groups. The first group contained WT2, WT3 and WTTPA1, and the second group contained WT1, WTTPA2 and WTTPA3). We can also see that those 2 groups clearly had different expression profile. The genes in top

clusters of first group were expressed more than the bottom cluster, while the genes in top clusters of second group were expressed less than the bottom cluster.

```
top100var_mat <- as.matrix(top100var[,2:7])
rownames(top100var_mat) <- as.data.frame(top100var)[,1]
pheatmap(top100var_mat, show_rownames = TRUE, scale = "row", fontsize_row = 2.5,
         fontsize_col = 5)
```



Moreover, we can see that in our code the argument "scale" was changed form the default which is "none" to "row". This would tell the function to normalize the log_2 abundance value of all genes in each sample. The advantage of normalization is that it will put the log_2 abundance value on the same scale. This enabled us to compare the abundance value of the same gene between samples, and it also helped us to clearly see the different between high and low expression gene across all samples when plotting heatmap.

Question 2

```
all_salmons2 <- importIsoformExpression(parentDir = "./salmon_result_part2/")
```

```
## Step 1 of 3: Identifying which algorithm was used...
##     The quantification algorithm used was: Salmon
##     Found 6 quantification file(s) of interest
## Step 2 of 3: Reading data...
## reading in files with read_tsv
## 1 2 3 4 5 6
## Step 3 of 3: Normalizing FPKM/TxPM values via edgeR...
```

```
## Done
```

Question 2.1

First we loaded data into switchAnalyzeRList object using importRdata. The summary statistics of resulting switchAnalyzeRList were as below.

```
designMat <- data.frame(sampleID=colnames(all_salmons2$abundance)[-1],
                        condition=
                          str_replace(colnames(all_salmons2$abundance)[-1], "[0-9]", ""))

switchAnalyzeRList <- importRdata(isoformCountMatrix = all_salmons2$counts,
                                  isoformRepExpression = all_salmons2$abundance,
                                  designMatrix = designMat,
                                  isoformExonAnnoation =
                                    "./salmon_result_part2/subset.gtf",
                                  addAnnotatedORFs=FALSE)
```

```
## Step 1 of 6: Checking data...
## Step 2 of 6: Obtaining annotation...
##     importing GTF (this may take a while)
## Step 3 of 6: Calculating gene expression and isoform fraction...
##      2433 ( 24.33%) isoforms were removed since they were not expressed in any samples.
## Step 4 of 6: Merging gene and isoform expression...
##
  |
  |                                                                      |   0%
  |
  |================================                                      |  50%
  |
  |======================================================================| 100%
## Step 5 of 6: Making comparisons...
##
  |
  |                                                                      |   0%
  |
  |======================================================================| 100%
## Step 6 of 6: Making switchAnalyzeRlist object...
## Done
```

```
switchAnalyzeRList
```

```
## This switchAnalyzeRlist list contains:
##  7567 isoforms from 3304 genes
##  1 comparison from 2 conditions (in total 6 samples)
```

In total there were 7567 isoforms in the data set, and those isoforms came from 3304 genes. The samples came from two groups including the organism that splice factor X were knocked out and the wild type organism.

The options addAnnotatedORFs will be used only when we have the GTF file that specified the ORF regions of each isform in our data set, and want to add that annotation about the position of ORF on our transcript data. This ORF information can directly be used by IsoformSwitchAnalyzeR for down stream analysis such

as for analyzing the consequences of isoform switch instead of having to predict ORF from analyzeORF() function. However, we disabled this option here by setting it to FALSE as we did not have GTF file contain ORF information.

Question 2.2

The reason why the annotation in the GTF file must be the exact annotation quantified with Salmon is because the IsoformSwitchAnalyzeR will annotate the isoform in isoformRepExpression data frame by matching the isoform_id in data frame with the isoform_id in GTF file. The isoform_id is not the fixed id for each transcript isoform unlike GenBank accession number/ENSEMBL id for transcripts. Therefore, the isoform_id can be varied between experiment. This is why we need to make sure that the annotation in GTF file is the exact annotation of the same data set from salmon in order to get correct annotation.

Question 2.3

The table of top 10 switching genes with consequences sorted by q-values could be make using below code. The top 10 genes were 5830418K08Rik, Ablim1, Tef, Xrcc6, Snx14, Slmap, Rac1, Fbxw7, Pld2 and Rrbp1, respectively.

```
switchList <- readRDS("./hw3switchList.Rdata")
top10switch <- extractTopSwitches(switchList, filterForConsequences = TRUE,
                                  sortByQvals=TRUE, n=10)
top10switch
```

```
##             gene_ref           gene_id     gene_name condition_1
## 1  geneComp_00100550       XLOC_047302 5830418K08Rik          WT
## 2  geneComp_00076087       XLOC_023295        Ablim1          WT
## 3  geneComp_00068215       XLOC_015573           Tef          WT
## 4  geneComp_00068223       XLOC_015581         Xrcc6          WT
## 5  geneComp_00101368 XLOC_048111:Snx14         Snx14          WT
## 6  geneComp_00066816       XLOC_014190         Slmap          WT
## 7  geneComp_00089842       XLOC_036766          Rac1          WT
## 8  geneComp_00081221       XLOC_028310         Fbxw7          WT
## 9  geneComp_00058485       XLOC_006025          Pld2          WT
## 10 geneComp_00080160       XLOC_027267         Rrbp1          WT
##    condition_2 gene_switch_q_value switchConsequencesGene Rank
## 1           KO        3.175544e-64                    TRUE    1
## 2           KO        1.155042e-15                    TRUE    2
## 3           KO        4.686282e-15                    TRUE    3
## 4           KO        9.951012e-13                    TRUE    4
## 5           KO        4.031854e-12                    TRUE    5
## 6           KO        6.992658e-11                    TRUE    6
## 7           KO        8.587909e-10                    TRUE    7
## 8           KO        7.331074e-09                    TRUE    8
## 9           KO        1.277562e-08                    TRUE    9
## 10          KO        1.922603e-08                    TRUE   10
```

Question 2.4

To made switch plot for top10 genes we used code below. The conditions were “KO” and “WT”.

```
top10genes_name <- top10switch[,"gene_name"]
switchCondition <- switchList$conditions[,1]

for(gene in top10genes_name){
  # best resolution pics that are not too big
```

```
  png(paste0(gene, ".png", collapse = ""), width = 1200, height = 1200, res=160)
  switchPlot(switchList, gene=gene, condition1 = switchCondition[1],
             condition2 = switchCondition[2])
  dev.off()
}
```

Question 2.5

We suspected that the knocked out gene coded for splicing factor, so we expected that the genes that were relevant to this knocking out should show clear splicing changes not other possible structural change such as transcription start site changes. This narrowed genes down from 10 genes to Rac1 where the swicth between isoform TCONS_00104606 and TCONS_00104607 clearly could happend only by splicing change. While in other genes the changes in isoform structure could be also caused by TSS change. Moreover, the isoform with significant increase in usage in knocked out mouse was non-coding isoform, while the isoform with significantly higher isoform usage in wild type mouse was the normal coding isoform that could be translated into functional protein. This means that this gene loss its function in knocked out mouse due to isoform switch. Therefore, we picked Rac1 as the most important gene.

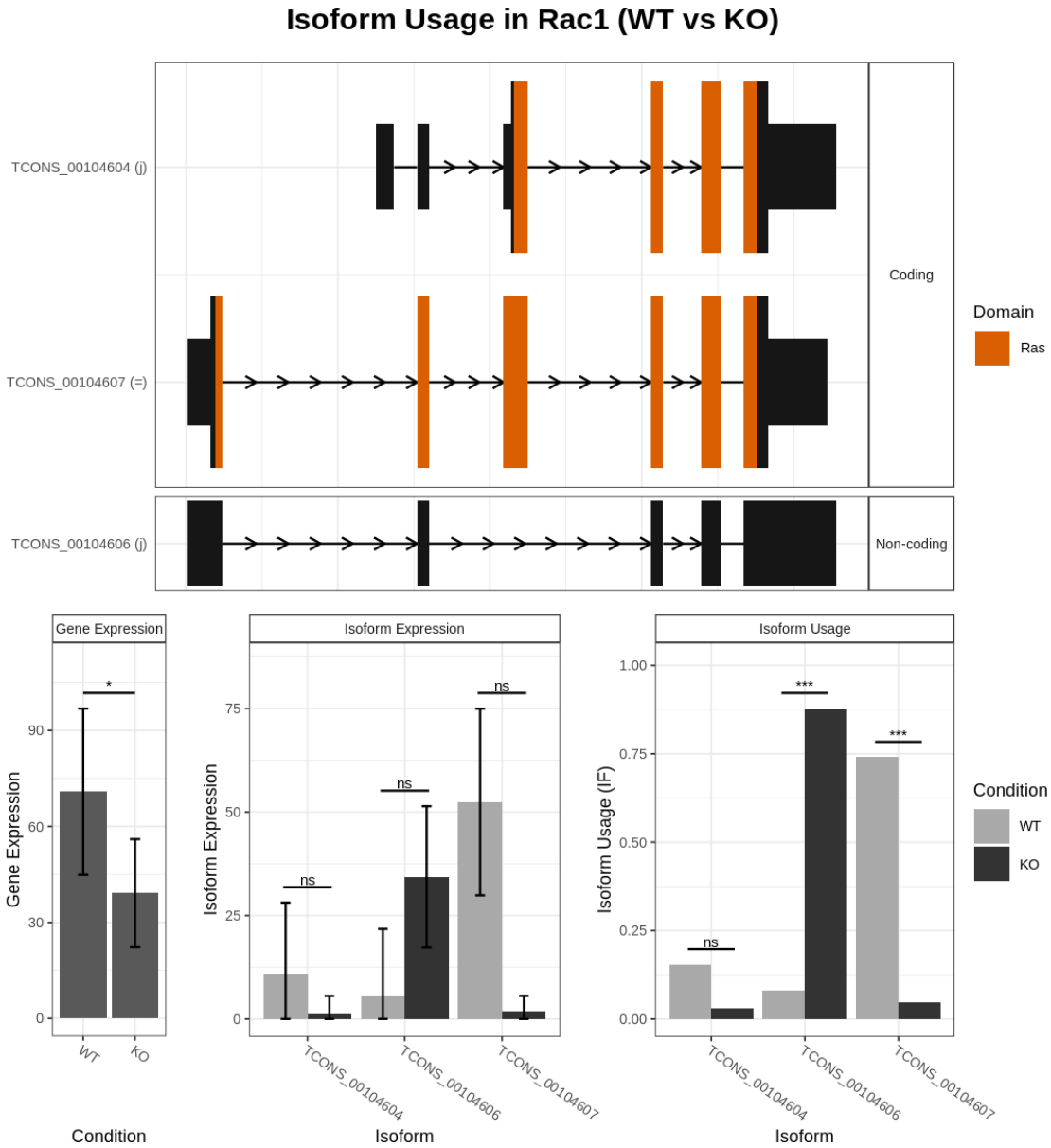


Figure 1: switchPlot of Rac1

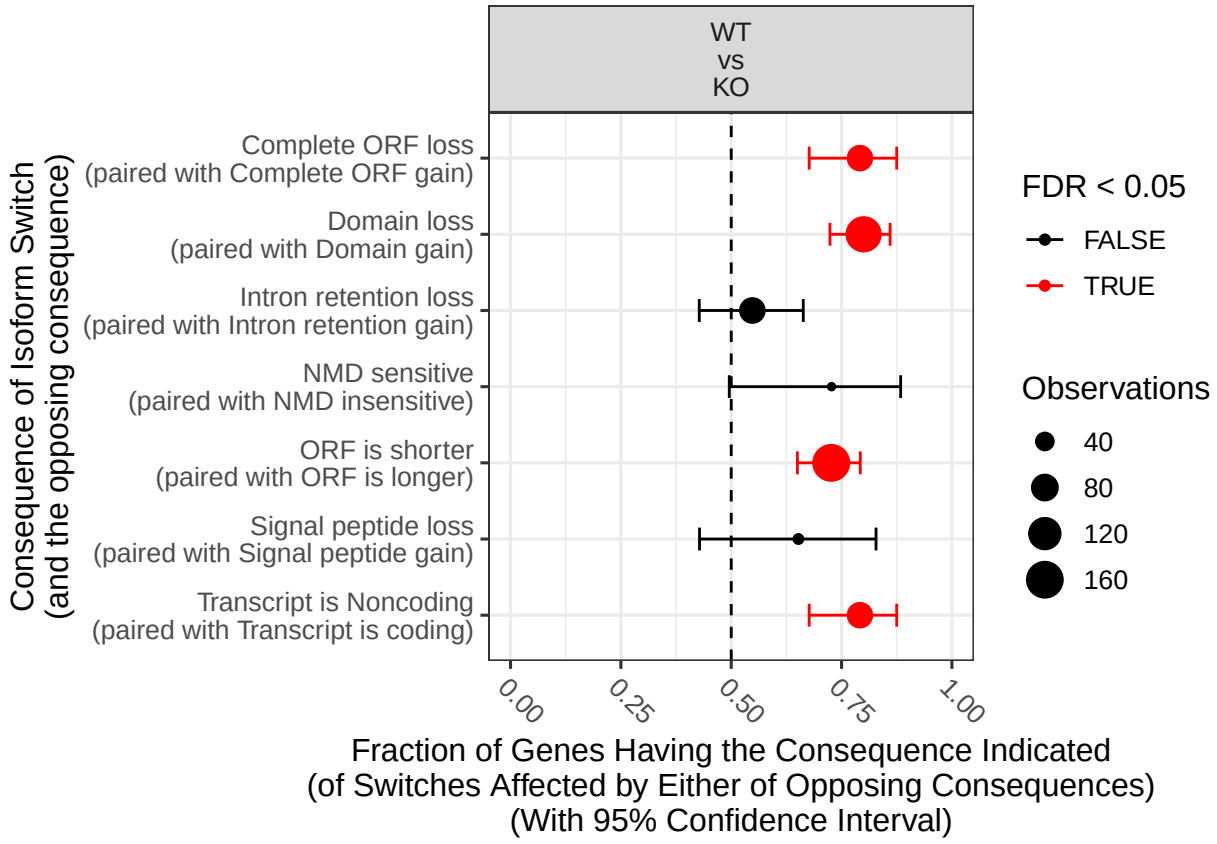
Question 2.6

From the global enrichment plot the general changes in isoform structure that were significant (FDR < 0.05) were ORF loss, domain loss, shortening of ORF, and conversion of transcript to non-coding transcript. All of these general changes can be introduced by both changes in splicing pattern or changes in trascription start site. So, we can not clearly say whether these patterns support our original hypothesis that factor X might be splicing factor.

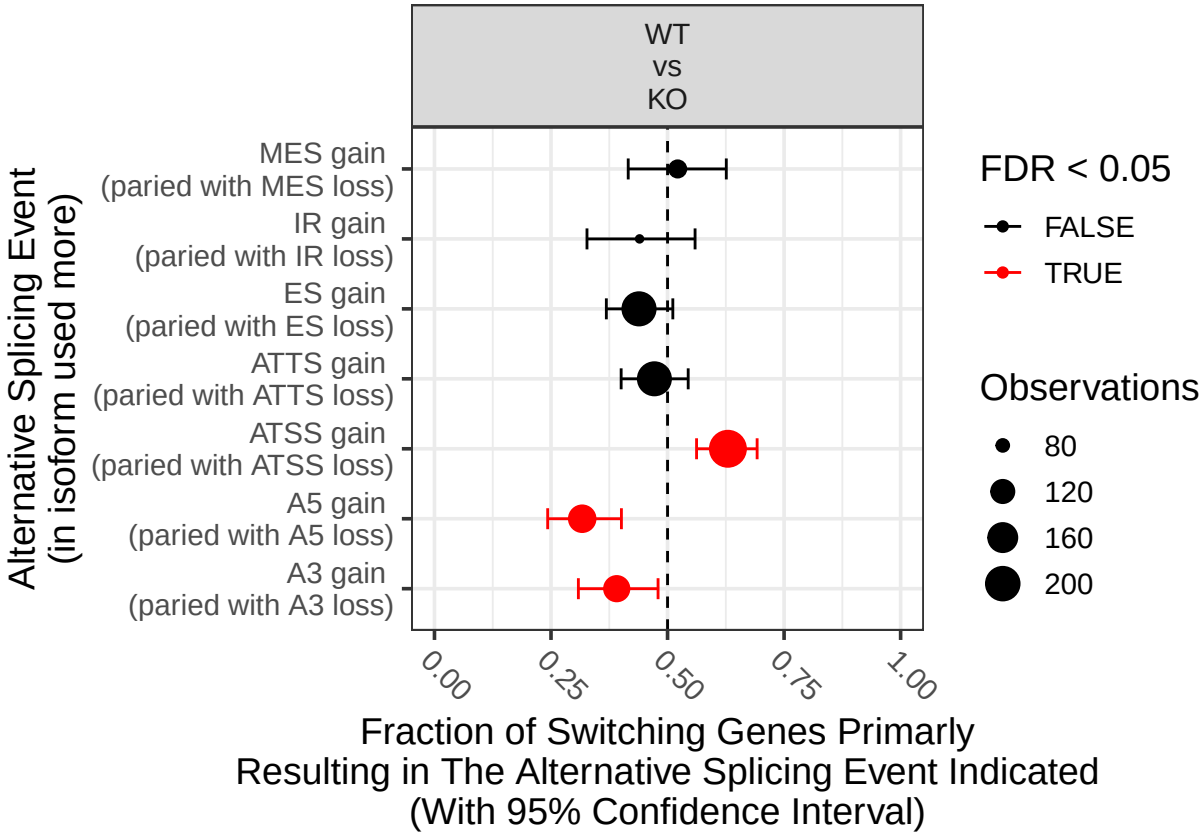
If factor X was actually splicing factor, we expected to observe that majority of switching would show alternative splicing. However, from alternative splicing analysis, we can see that less than half of switching showed the sign of MES loss, IR gain, ES loss and ATTs gain also with FDR higher than 0.05. Only ATSS was found in more than half of switching events with FRD < 0.05.

So, in related to our original hypothesis, we could not say yet that factor X is splicing factor as the results here not firmly supported this hypothesis.

```
extractConsequenceEnrichment(
    switchList,
    consequencesToAnalyze='all',
    analysisOppositeConsequence = TRUE,
    returnResult = FALSE
)
```




```
extractSplicingEnrichment(
    switchList,
    returnResult = FALSE
)
```


WT
vs
KO
Alternative Splicing Event
(in isoform used more)
MES gain
(paried with MES loss)
IR gain
(paried with IR loss)
ES gain
(paried with ES loss)
ATTS gain
(paried with ATTS loss)
ATSS gain
(paried with ATSS loss)
A5 gain
(paried with A5 loss)
A3 gain
(paried with A3 loss)
0.00
0.25
0.50
0.75
1.00
Fraction of Switching Genes Primarly
Resulting in The Alternative Splicing Event Indicated
(With 95% Confidence Interval)
FDR < 0.05
FALSE
TRUE
Observations
80
120
160
200