Class 14: RNA-Seq analysis mini-project

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Background

The data for this hands-on session comes from GEO entry: GSE37704, which is associated with the following publication:

> Trapnell C, Hendrickson DG, Sauvageau M, Goff L et al. "Differential analysis of gene regulation at transcript resolution with RNA-seq". Nat Biotechnol 2013 Jan;31(1):46-53. PMID: 23222703

The authors report on differential analysis of lung fibroblasts in response to loss of the developmental transcription factor HOXA1. Their results and others indicate that HOXA1 is required for lung fibroblast and HeLa cell cycle progression. In particular their analysis show that "loss of HOXA1 results in significant expression level changes in thousands of individual transcripts, along with isoform switching events in key regulators of the cell cycle". For this session we have used their Sailfish gene-level estimated counts and hence are restricted to protein-coding genes only.

Data Import

```
counts <- read.csv("GSE37704_featurecounts.csv", row.names=1)
colData <- read.csv("GSE37704_metadata.csv")
```

Inspect and tidy data

Does the `counts` columns match the `colData` rows?

Ans. No, the `counts` columns are different compared to the`colData` rows.

```
head(counts)
```

```
                length SRR493366 SRR493367 SRR493368 SRR493369 SRR493370
ENSG00000186092    918         0         0         0         0         0
ENSG00000279928    718         0         0         0         0         0
ENSG00000279457   1982        23        28        29        29        28
ENSG00000278566    939         0         0         0         0         0
ENSG00000273547    939         0         0         0         0         0
ENSG00000187634   3214       124       123       205       207       212
                SRR493371
ENSG00000186092         0
ENSG00000279928         0
ENSG00000279457        46
ENSG00000278566         0
ENSG00000273547         0
ENSG00000187634       258
```

```
head(colData)
```

```
         id     condition
1 SRR493366 control_sirna
2 SRR493367 control_sirna
3 SRR493368 control_sirna
4 SRR493369      hoxa1_kd
5 SRR493370      hoxa1_kd
6 SRR493371      hoxa1_kd
```

```
colData$id
```

```
[1] "SRR493366" "SRR493367" "SRR493368" "SRR493369" "SRR493370" "SRR493371"
```

```
colnames(counts)
```

```
[1] "length"    "SRR493366" "SRR493367" "SRR493368" "SRR493369" "SRR493370"
[7] "SRR493371"
```

The fix here looks to be removing the first "length" column from counts:

```
countData <- counts[,-1]
head(countData)
```

```
                SRR493366 SRR493367 SRR493368 SRR493369 SRR493370 SRR493371
ENSG00000186092         0         0         0         0         0         0
ENSG00000279928         0         0         0         0         0         0
ENSG00000279457        23        28        29        29        28        46
ENSG00000278566         0         0         0         0         0         0
ENSG00000273547         0         0         0         0         0         0
ENSG00000187634       124       123       205       207       212       258
```

Check for matching countData and colData

```
colnames(countData) == colData$id
```

```
[1] TRUE TRUE TRUE TRUE TRUE TRUE
```

> Q. Complete the code below to remove the troublesome first column from countData

```
# Note we need to remove the odd first $length col
countData2 <- as.matrix(countData[,-1])
head(countData2)
```

```
                SRR493367 SRR493368 SRR493369 SRR493370 SRR493371
ENSG00000186092         0         0         0         0         0
ENSG00000279928         0         0         0         0         0
ENSG00000279457        28        29        29        28        46
ENSG00000278566         0         0         0         0         0
ENSG00000273547         0         0         0         0         0
ENSG00000187634       123       205       207       212       258
```

Q1. How many genes in total?

Ans. There are 19808 genes in total, but some of them have a count of zero.

```
nrow(countData)
```

```
[1] 19808
```

Q2. Filter to remove zero count genes (rows where there are zero counts in all columns). How many genes are left?

Ans. There are 15975 genes left.

```
to.keep.inds <- rowSums(countData) > 0
```

```
new.counts <- countData[to.keep.inds, ]
```

```
nrow(new.counts)
```

```
[1] 15975
```

Q. Complete the code below to filter countData to exclude genes (i.e. rows) where we have 0 read count across all samples (i.e. columns).

```
# Filter count data where you have 0 read count across all samples.
countData3 = countData[to.keep.inds, ]
head(countData3)
```

	SRR493366	SRR493367	SRR493368	SRR493369	SRR493370	SRR493371
ENSG00000279457	23	28	29	29	28	46
ENSG00000187634	124	123	205	207	212	258
ENSG00000188976	1637	1831	2383	1226	1326	1504
ENSG00000187961	120	153	180	236	255	357
ENSG00000187583	24	48	65	44	48	64
ENSG00000187642	4	9	16	14	16	16

Setup for DESeq

```
library(DESeq2)
```

Setup input object for DESeq

```
dds <- DESeqDataSetFromMatrix(countData = new.counts,
                              colData = colData,
                              design = ~condition)
```

```
Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
design formula are characters, converting to factors
```

Run DESeq

```
dds <- DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```
res <- results(dds)
```

```
head(res)
```

```
log2 fold change (MLE): condition hoxa1 kd vs control sirna
Wald test p-value: condition hoxa1 kd vs control sirna
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE       stat      pvalue
                <numeric>      <numeric> <numeric>  <numeric>   <numeric>
ENSG00000279457   29.9136      0.1792571 0.3248216   0.551863 5.81042e-01
ENSG00000187634  183.2296      0.4264571 0.1402658   3.040350 2.36304e-03
ENSG00000188976 1651.1881     -0.6927205 0.0548465 -12.630158 1.43990e-36
ENSG00000187961  209.6379      0.7297556 0.1318599   5.534326 3.12428e-08
ENSG00000187583   47.2551      0.0405765 0.2718928   0.149237 8.81366e-01
ENSG00000187642   11.9798      0.5428105 0.5215598   1.040744 2.97994e-01
                      padj
                 <numeric>
ENSG00000279457 6.86555e-01
ENSG00000187634 5.15718e-03
ENSG00000188976 1.76549e-35
ENSG00000187961 1.13413e-07
ENSG00000187583 9.19031e-01
ENSG00000187642 4.03379e-01
```

```
res2 = results(dds, contrast=c("condition", "hoxa1_kd", "control_sirna"))
res2_omitna <- res2[-which(is.na(res2$padj)),]
```

Q. Call the summary() function on your results to get a sense of how many genes are up or down-regulated at the default 0.1 p-value cutoff.

```
summary(res)
```

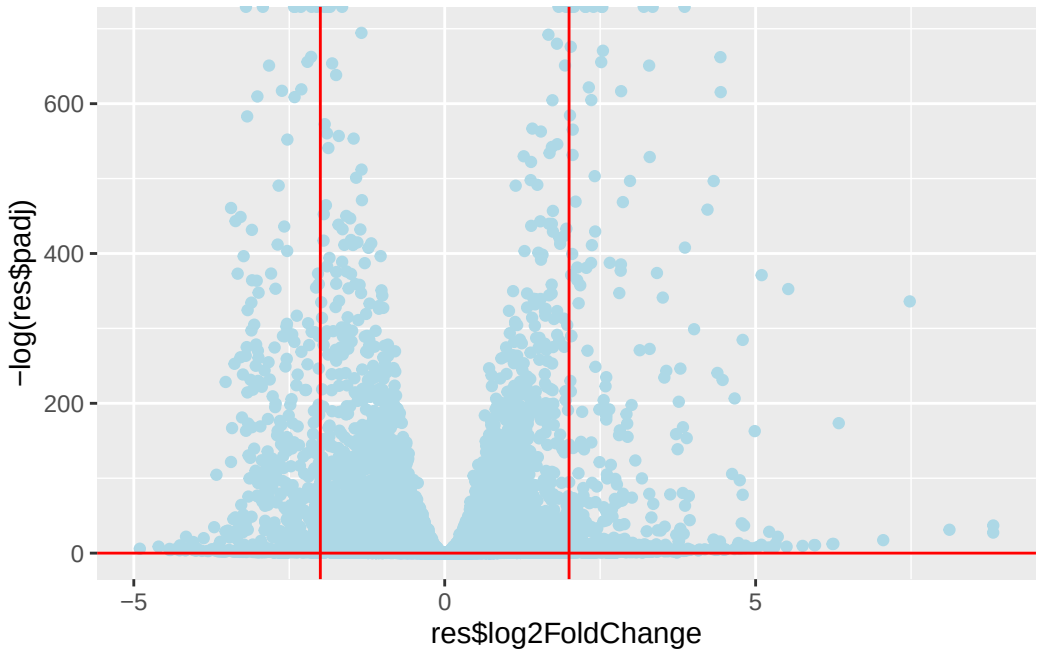
```
out of 15975 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 4349, 27%
LFC < 0 (down)     : 4396, 28%
outliers [1]       : 0, 0%
low counts [2]     : 1237, 7.7%
(mean count < 0)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

Volcano plot of results

```
library(ggplot2)
```

```
ggplot(res) +
  aes(res$log2FoldChange, -log(res$padj)) +
  geom_point(col="lightblue") +
  geom_vline(xintercept = c(-2,2), col="red") +
  geom_hline(yintercept = 0, col ="red")
```

```
Warning: Removed 1237 rows containing missing values or values outside the scale range
(`geom_point()`).
```



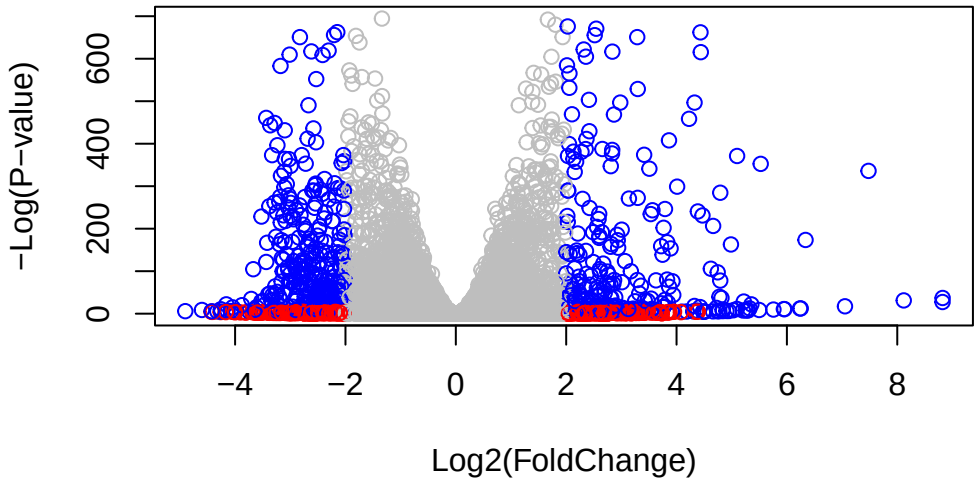
Q. Improve this plot by completing the below code, which adds color and axis labels

```
# Make a color vector for all genes
mycols <- rep("gray", nrow(res2_omitna) )

# Color red the genes with absolute fold change above 2
mycols[ abs(res2_omitna$log2FoldChange) > 2 ] <- "red"
```

```
# Color blue those with adjusted p-value less than 0.01
#  and absolute fold change more than 2
inds <- (res2_omitna$padj<0.01) & (abs(res2_omitna$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"

plot( res2_omitna$log2FoldChange, -log(res2_omitna$padj), col= mycols, xlab="Log2(FoldChange)
```




```
inds <- (res2_omitna$padj<0.01) & (abs(res2_omitna$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"
sum(inds)
```

```
[1] 606
```

Gene annotation

```
library(AnnotationDbi)
library(org.Hs.eg.db)
```

```
columns(org.Hs.eg.db)
```

```
 [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
 [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
[11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
[16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
[21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
[26] "UNIPROT"
```

Q. Use the mapIDs() function multiple times to add SYMBOL, ENTREZID and GENENAME annotation to our results by completing the code below.

Add gene SYMBOL and ENTREZID

```
res$symbol = mapIds(org.Hs.eg.db,
                    keys=rownames(res),
                    keytype="ENSEMBL",
                    column= "SYMBOL",
                    multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$entrez = mapIds(org.Hs.eg.db,
                    keys=rownames(res),
                    keytype="ENSEMBL",
                    column="ENTREZID",
                    multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$name = mapIds(org.Hs.eg.db,
                    keys=rownames(res),
                    keytype="ENSEMBL",
                    column="GENENAME",
                    multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
log2 fold change (MLE): condition hoxa1 kd vs control sirna
Wald test p-value: condition hoxa1 kd vs control sirna
DataFrame with 6 rows and 9 columns
                 baseMean log2FoldChange     lfcSE       stat      pvalue
                <numeric>      <numeric> <numeric>  <numeric>   <numeric>
ENSG00000279457   29.9136      0.1792571 0.3248216   0.551863 5.81042e-01
ENSG00000187634  183.2296      0.4264571 0.1402658   3.040350 2.36304e-03
ENSG00000188976 1651.1881     -0.6927205 0.0548465 -12.630158 1.43990e-36
ENSG00000187961  209.6379      0.7297556 0.1318599   5.534326 3.12428e-08
ENSG00000187583   47.2551      0.0405765 0.2718928   0.149237 8.81366e-01
ENSG00000187642   11.9798      0.5428105 0.5215598   1.040744 2.97994e-01
                      padj      symbol      entrez                   name
                 <numeric> <character> <character>            <character>
ENSG00000279457 6.86555e-01          NA          NA                     NA
ENSG00000187634 5.15718e-03      SAMD11      148398 sterile alpha motif ..
ENSG00000188976 1.76549e-35       NOC2L       26155 NOC2 like nucleolar ..
ENSG00000187961 1.13413e-07      KLHL17      339451 kelch like family me..
ENSG00000187583 9.19031e-01     PLEKHN1       84069 pleckstrin homology ..
ENSG00000187642 4.03379e-01       PERM1       84808 PPARGC1 and ESRR ind..
```

Q. Finally for this section let's reorder these results by adjusted p-value and save them to a CSV file in your current project directory.

```
#res2 = res[order(res$pvalue),]
#write.csv(res2, file="deseq_results.csv")
```

Pathway Analysis

```
library(gage)
```

```
library(gageData)
library(pathview)
```

```
#############################################################################
Pathview is an open source software package distributed under GNU General
Public License version 3 (GPLv3). Details of GPLv3 is available at
http://www.gnu.org/licenses/gpl-3.0.html. Particullary, users are required to
formally cite the original Pathview paper (not just mention it) in publications
or products. For details, do citation("pathview") within R.

The pathview downloads and uses KEGG data. Non-academic uses may require a KEGG
license agreement (details at http://www.kegg.jp/kegg/legal.html).
#############################################################################
```

Input vector for `gage()`

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
```

Load up the KEGG gene sets

```
data(kegg.sets.hs)
```

Run pathway analysis with KEGG

```
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

```
head(keggres$less, 3)
```

```
                                           p.geomean stat.mean
hsa04110 Cell cycle                        8.995727e-06 -4.378644
hsa03030 DNA replication                   9.424076e-05 -3.951803
hsa05130 Pathogenic Escherichia coli infection 1.405864e-04 -3.765330
                                                  p.val       q.val
hsa04110 Cell cycle                        8.995727e-06 0.001889103
hsa03030 DNA replication                   9.424076e-05 0.009841047
hsa05130 Pathogenic Escherichia coli infection 1.405864e-04 0.009841047
                                           set.size         exp1
hsa04110 Cell cycle                             121 8.995727e-06
hsa03030 DNA replication                         36 9.424076e-05
hsa05130 Pathogenic Escherichia coli infection   53 1.405864e-04
```

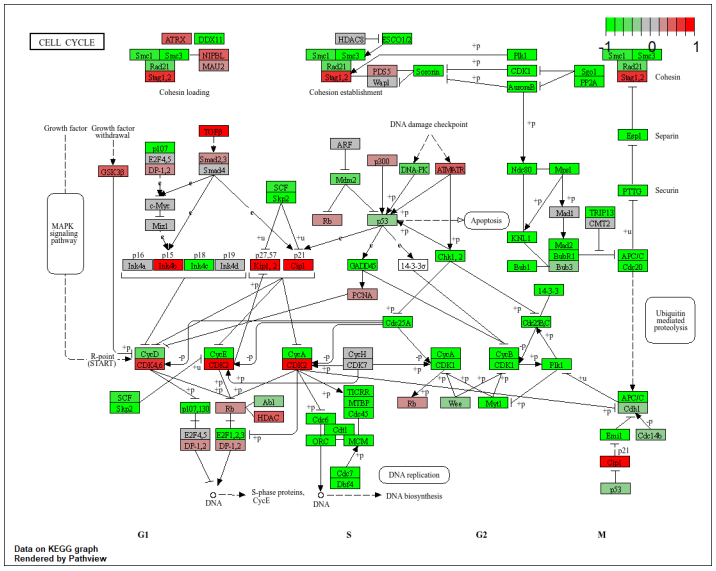
Cell cycle figure

```
pathview(foldchanges, pathway.id = "hsa04110")
```

```
'select()' returned 1:1 mapping between keys and columns
```

```
Info: Working in directory C:/Users/agree/Desktop/2024-2025 UCSD/Winter 2025/BIMM 143/RStudio
```

```
Info: Writing image file hsa04110.pathview.png
```



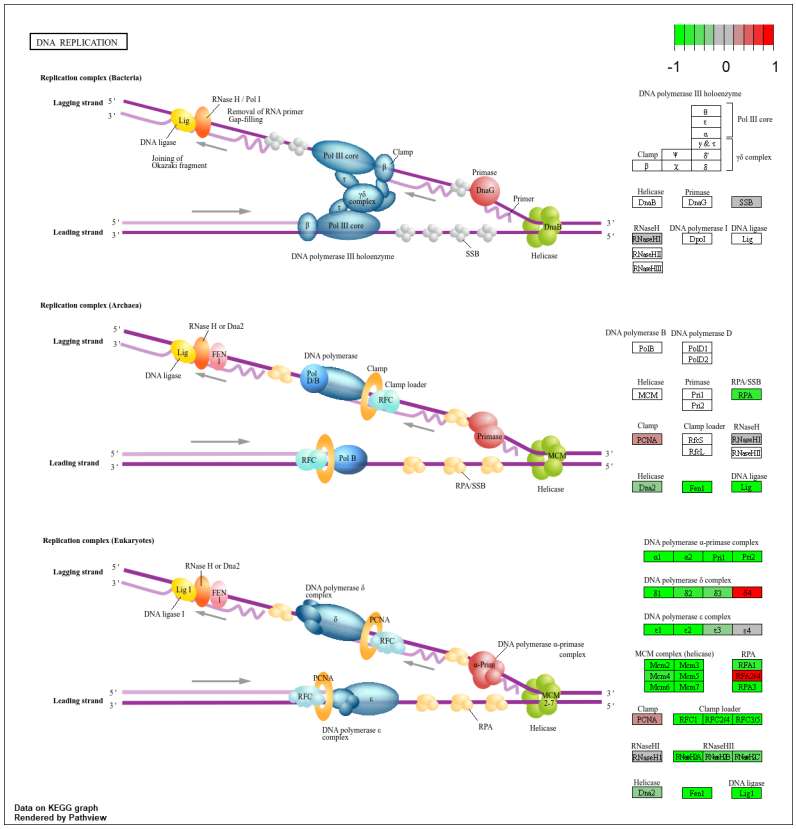
DNA replication figure

```
pathview(foldchanges, pathway.id = "hsa03030")
```

```
'select()' returned 1:1 mapping between keys and columns
```

```
Info: Working in directory C:/Users/agree/Desktop/2024-2025 UCSD/Winter 2025/BIMM 143/RStudio
```

```
Info: Writing image file hsa03030.pathview.png
```



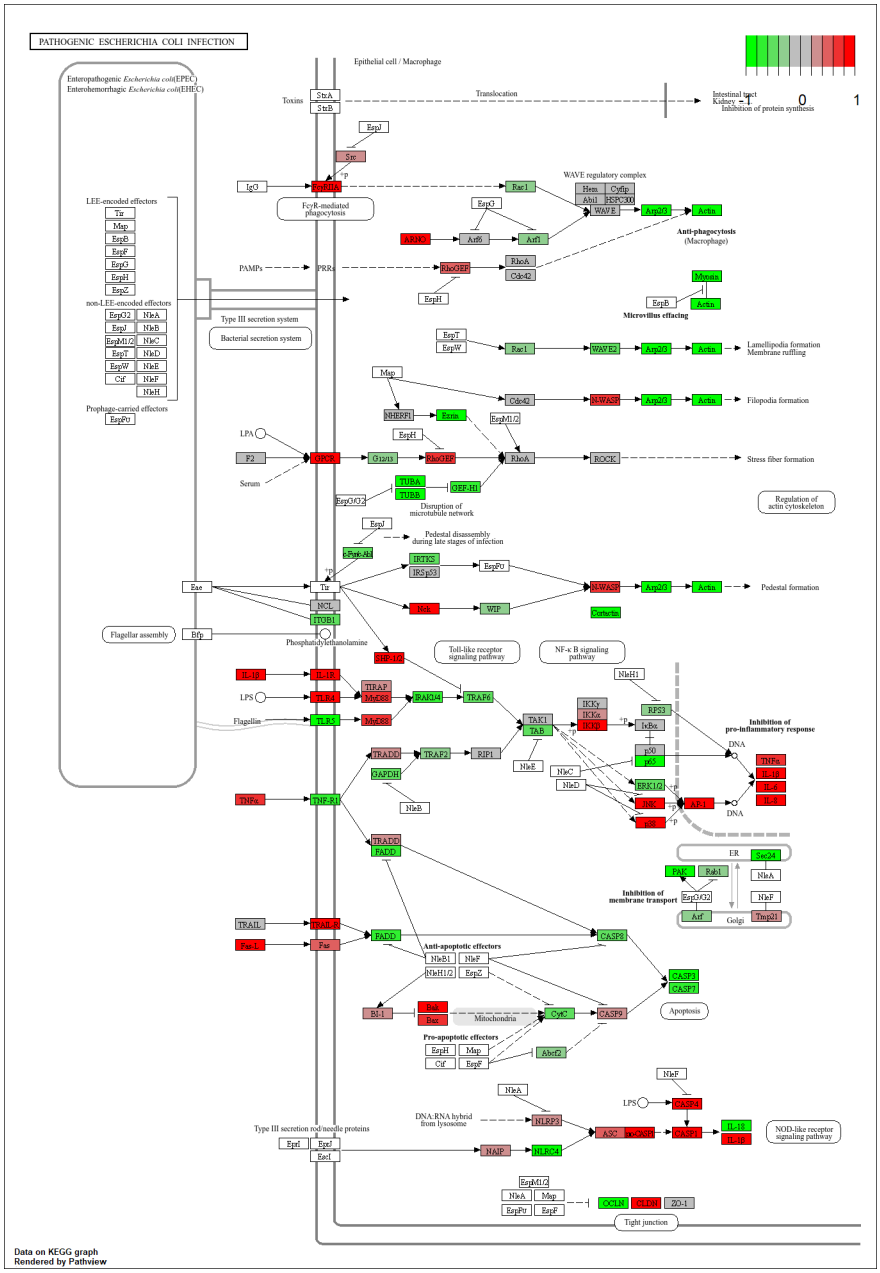
Pathogenic Escherichia coli infection

```
pathview(foldchanges, pathway.id = "hsa05130")
```

```
'select()' returned 1:1 mapping between keys and columns
```

```
Info: Working in directory C:/Users/agree/Desktop/2024-2025 UCSD/Winter 2025/BIMM 143/RStudio
```

```
Info: Writing image file hsa05130.pathview.png
```



Q. Can you do the same procedure as above to plot the pathview figures for the top 5 down-reguled pathways?

Gene Ontology analysis

Run pathway analysis with GO

```
data(go.sets.hs)
data(go.subs.hs)

# Focus on Biological Process subset of GO
gobpsets = go.sets.hs[go.subs.hs$BP]

gobpres = gage(foldchanges, gsets=gobpsets, same.dir=TRUE)

head(gobpres$less)
```

```
                                      p.geomean stat.mean        p.val
GO:0048285 organelle fission          1.536227e-15 -8.063910 1.536227e-15
GO:0000280 nuclear division           4.286961e-15 -7.939217 4.286961e-15
GO:0007067 mitosis                    4.286961e-15 -7.939217 4.286961e-15
GO:0000087 M phase of mitotic cell cycle 1.169934e-14 -7.797496 1.169934e-14
GO:0007059 chromosome segregation     2.028624e-11 -6.878340 2.028624e-11
GO:0000236 mitotic prometaphase       1.729553e-10 -6.695966 1.729553e-10
                                             q.val set.size         exp1
GO:0048285 organelle fission          5.841698e-12      376 1.536227e-15
GO:0000280 nuclear division           5.841698e-12      352 4.286961e-15
GO:0007067 mitosis                    5.841698e-12      352 4.286961e-15
GO:0000087 M phase of mitotic cell cycle 1.195672e-11      362 1.169934e-14
GO:0007059 chromosome segregation     1.658603e-08      142 2.028624e-11
GO:0000236 mitotic prometaphase       1.178402e-07       84 1.729553e-10
```

Reactome Analysis

```
sig_genes <- res[res$padj <= 0.05 & !is.na(res$padj), "symbol"]
print(paste("Total number of significant genes:", length(sig_genes)))
```

```
[1] "Total number of significant genes: 8147"
```

```
write.table(sig_genes, file="significant_genes.txt", row.names=FALSE, col.names=FALSE, quote=
```

> Q: What pathway has the most significant "Entities p-value"? Do the most significant pathways listed match your previous KEGG results? What factors could cause differences between the two methods?

Ans. Cellular response to starvation has the most significant "Entities p-value". The most significant pathways listed do not match my previous KEGG results. The differences between the two methods could be due to the input into reactome being both up and down regulated genes. However, KEGGS results uses only down regulated genes.