Class 14: RNASeq mini project

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Table of contents

Background

Here we work through a complete RNASeq analysis project. The input data comes from a knock-down experiment of a HOX gene.

Data Import

Reading the `counts` and `metadata` csv files Check on data structure

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

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(metadata)
```

```
         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
```

Some book-keeping is required as there looks to be a mis-match between counts columns and metadata rows.

```
ncol(counts)
```

```
[1] 7
```

```
nrow(metadata)
```

```
[1] 6
```

Looks like we need to get rid of the first "length" column of our `counts` object.

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

	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

```
ncol(cleancounts)
```

```
[1] 6
```

Remove zero count genes

There are lots of genes with zero counts. We can remove these for future analysis.

```
to.keep.inds <- rowSums(cleancounts) > 0
nonzero_counts <- cleancounts[to.keep.inds,]
```

DESeq Analysis

Load the package

```
library(DESeq2)
```

Setup DESeq object

```
dds <- DESeqDataSetFromMatrix(countData = nonzero_counts,
                              colData = metadata,
                              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
```

Get results

```
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 0.3248215   0.551863 5.81042e-01
ENSG00000187634  183.2296      0.4264571 0.1402658   3.040350 2.36304e-03
ENSG00000188976 1651.1881     -0.6927205 0.0548465 -12.630156 1.43993e-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 1.76553e-35
ENSG00000187961 1.13413e-07
ENSG00000187583 9.19031e-01
ENSG00000187642 4.03379e-01
```

Summary of results:

```
summary(res)
```

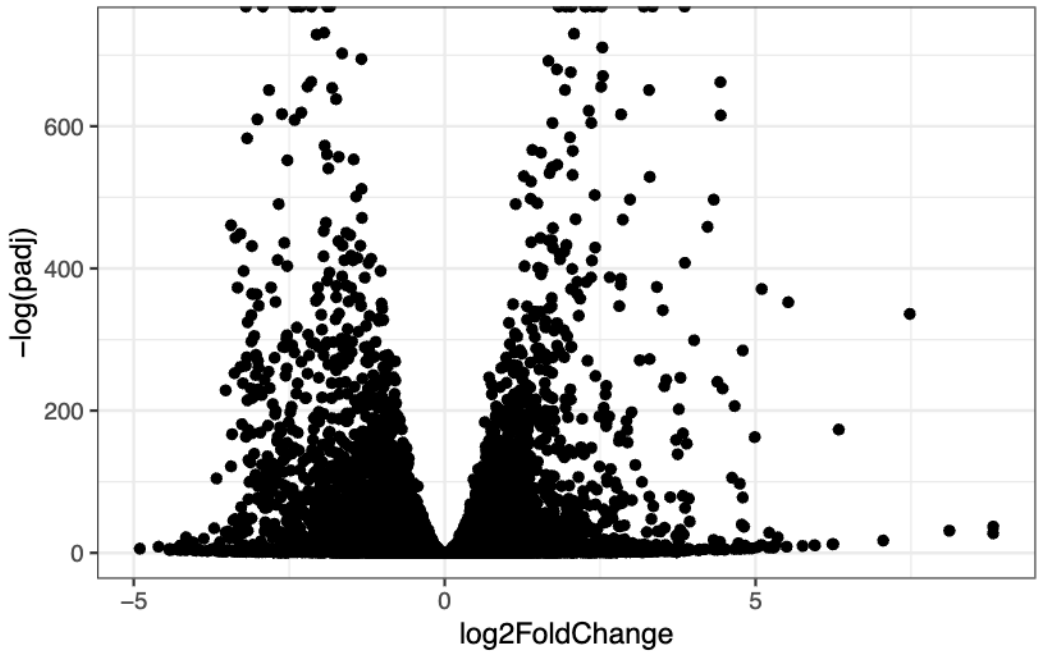
```
out of 15975 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 4349, 27%
LFC < 0 (down)     : 4396, 28%
outliers [1]       : 0, 0%
low counts [2]     : 1237, 7.7%
(mean count < 0)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results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

Data Visualization

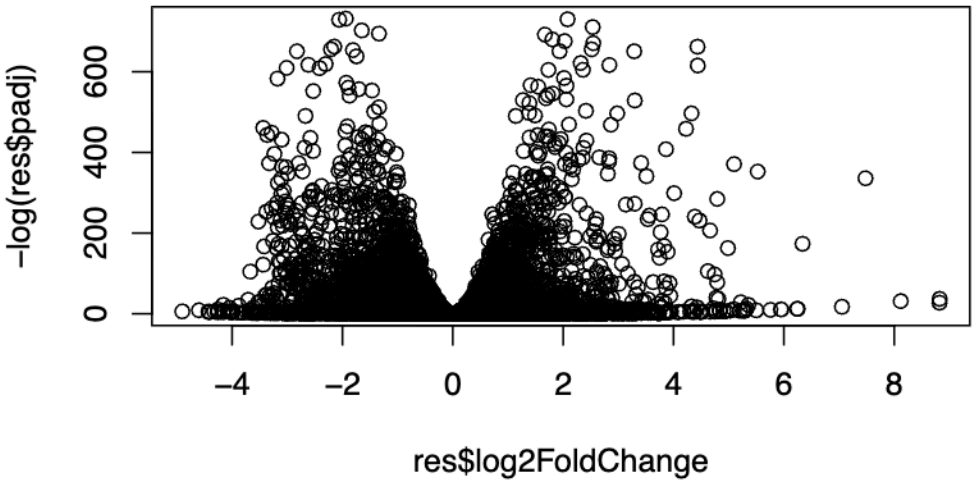
Volcano plot

```
library(ggplot2)

ggplot(res) +
  aes(log2FoldChange, -log(padj)) +
        geom_point() +
  theme_bw()
```

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

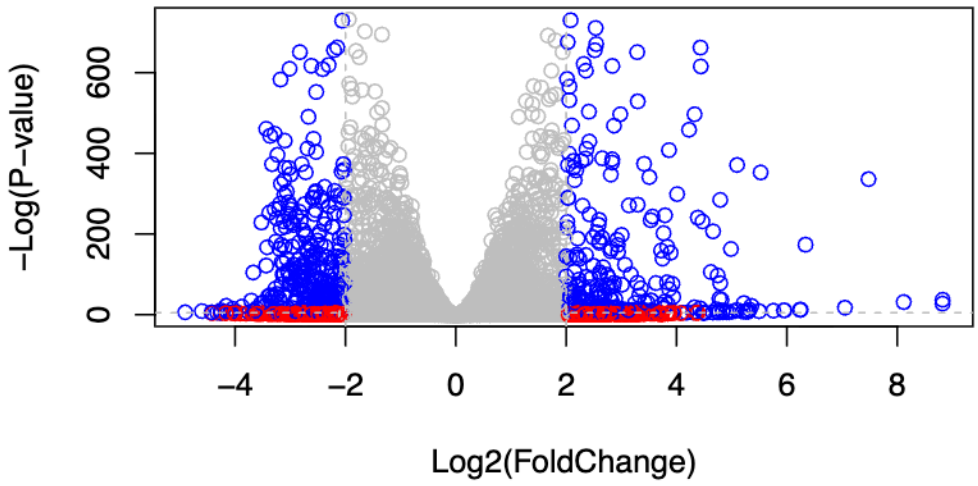



```
plot( res$log2FoldChange, -log(res$padj) )
```



Add threshfold lines for fold-change and P-value and color our subset of genes that makes these threshold cut-offs in the plot.

```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

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

# Volcano plot with custom colors
plot( res$log2FoldChange,  -log(res$padj),
 col=mycols, xlab="Log2(FoldChange)", ylab="-Log(P-value)")

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.01), col="gray", lty=2)
```



Add Annotation

Add gene symbols and entrez ids

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

```
res$entrez <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "ENTREZID"      # the format/DB to map to
      )

res$symbol <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "SYMBOL"      # the format/DB to map to
      )

res$GENENAME <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "GENENAME"      # the format/DB to map to
      )
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 0.3248215   0.551863 5.81042e-01
ENSG00000187634  183.2296      0.4264571 0.1402658   3.040350 2.36304e-03
ENSG00000188976 1651.1881     -0.6927205 0.0548465 -12.630156 1.43993e-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      entrez      symbol                GENENAME
                 <numeric> <character> <character>             <character>
ENSG00000279457 6.86555e-01          NA          NA                      NA
ENSG00000187634 5.15718e-03      148398      SAMD11 sterile alpha motif ..
ENSG00000188976 1.76553e-35       26155       NOC2L NOC2 like nucleolar ..
ENSG00000187961 1.13413e-07      339451      KLHL17 kelch like family me..
ENSG00000187583 9.19031e-01       84069     PLEKHN1 pleckstrin homology ..
ENSG00000187642 4.03379e-01       84808       PERM1 PPARGC1 and ESRR ind..
```

Pathway Analysis

Kegg pathway analysis

Run gage analysis

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

We need a named vector of fold-change values as inputs for gage.

```
foldchanges <- res$log2FoldChange
names(foldchanges) <- res$entrez
head(foldchanges)
```

```
       <NA>      148398       26155      339451       84069       84808
 0.17925708  0.42645712 -0.69272046  0.72975561  0.04057653  0.54281049
```

```
data(kegg.sets.hs)

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

```
head(keggres$less, 2)
```

```
                           p.geomean stat.mean        p.val       q.val
hsa04110 Cell cycle      8.995727e-06 -4.378644 8.995727e-06 0.001889103
hsa03030 DNA replication 9.424076e-05 -3.951803 9.424076e-05 0.009841047
                         set.size         exp1
hsa04110 Cell cycle           121 8.995727e-06
hsa03030 DNA replication       36 9.424076e-05
```

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

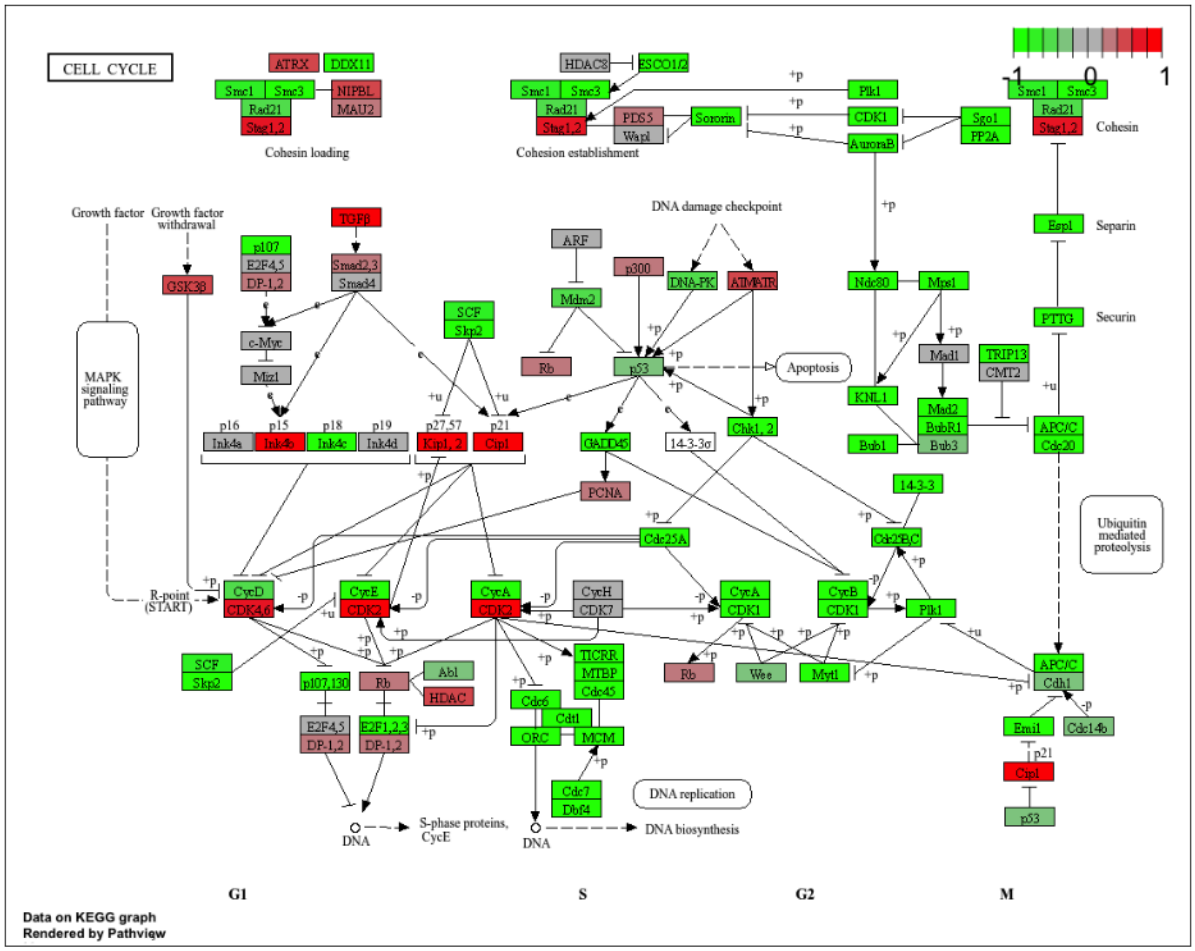
```
'select()' returned 1:1 mapping between keys and columns

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14

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

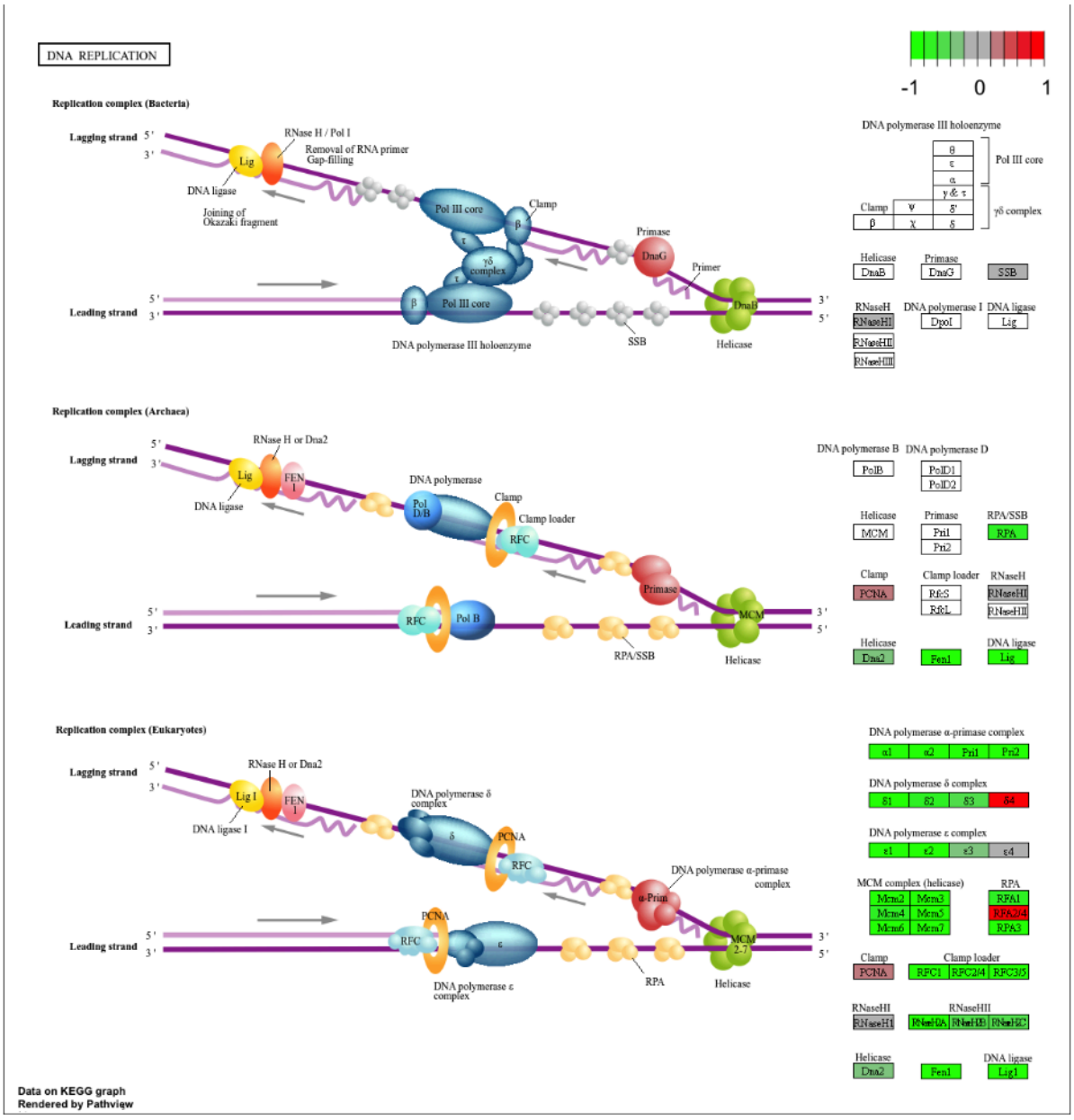
```
pathview(pathway.id = "hsa03030", gene.data = foldchanges)
```

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

```
Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14
```

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





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

hsa04110 and hsa03030 are seen above

```
## Focus on top 5 down-regulated pathways
keggrespathways <- rownames(keggres$less)[1:5]
```

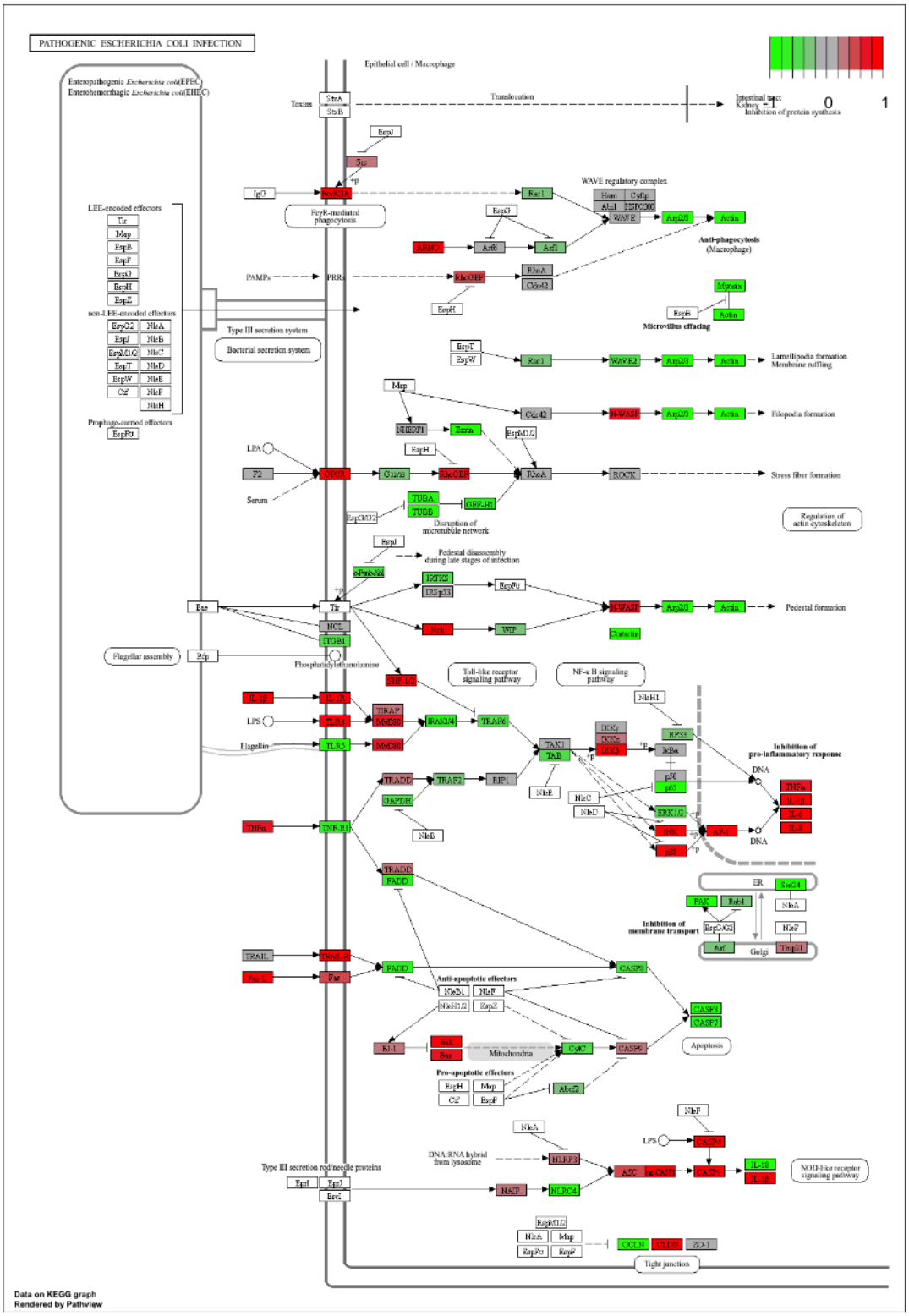
```
# Extract the 8 character long IDs part of each string
keggresids = substr(keggrespathways, start=1, stop=8)
keggresids
```

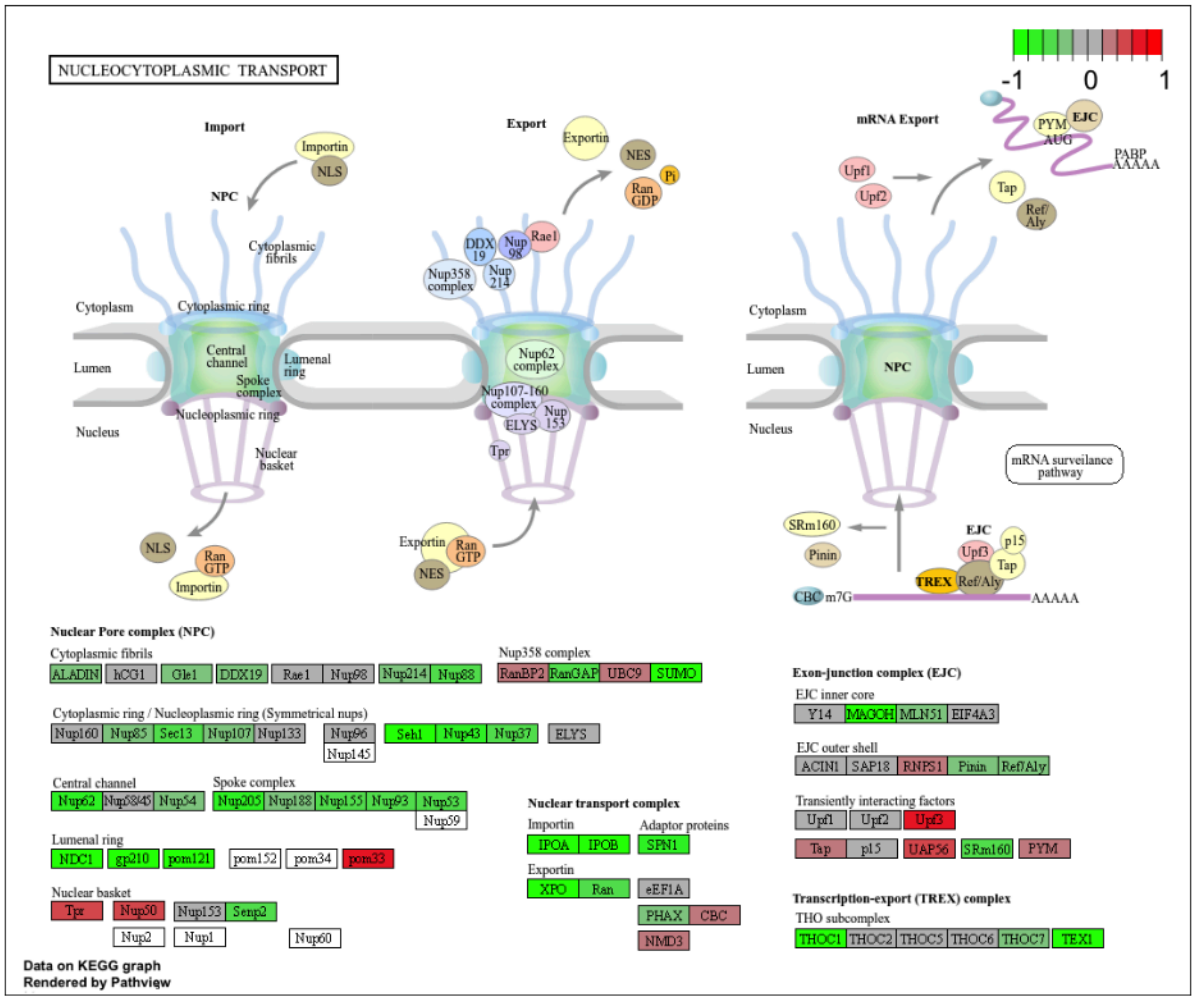
```
[1] "hsa04110" "hsa03030" "hsa05130" "hsa03013" "hsa03440"
```

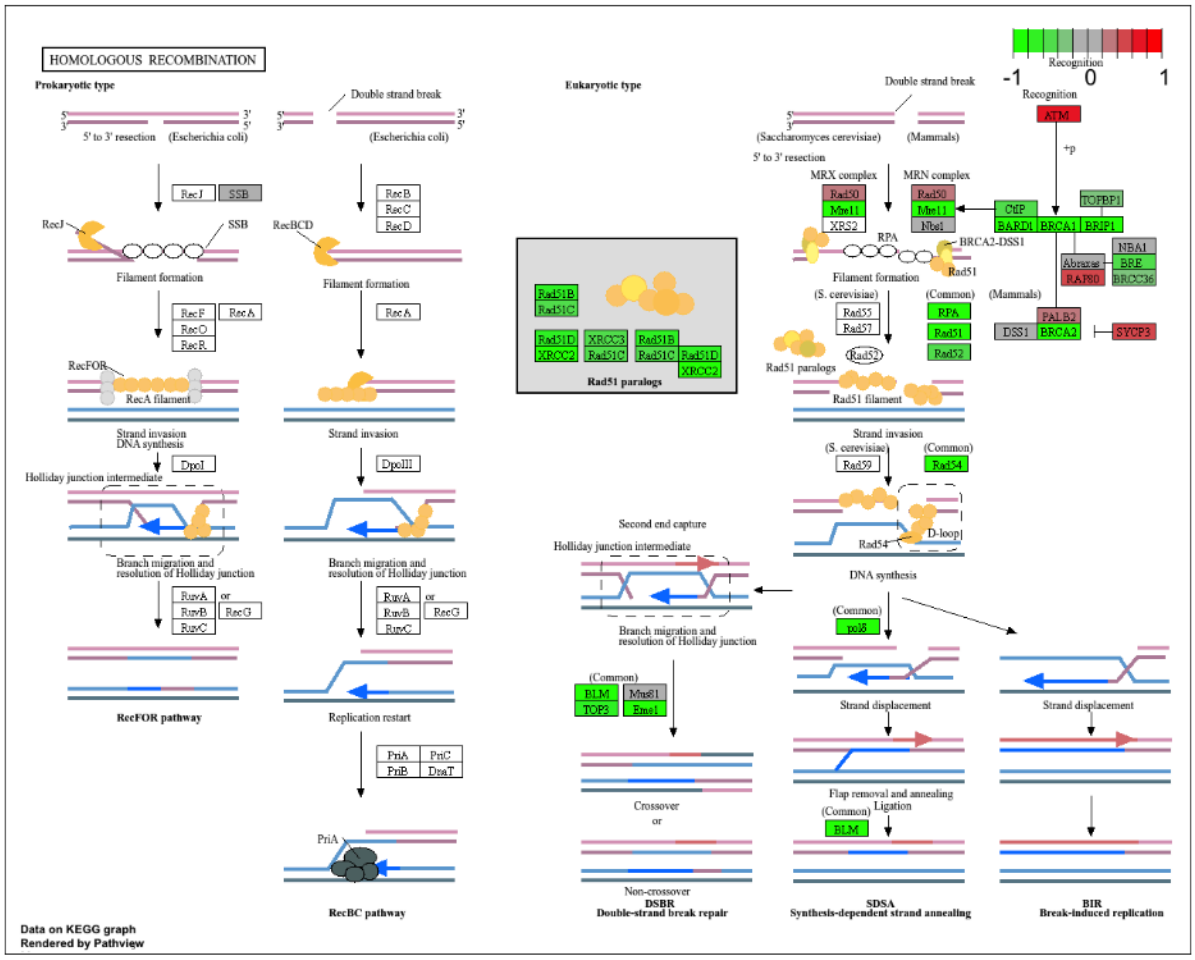
```
pathview(gene.data=foldchanges, pathway.id=keggresids, species="hsa")
```

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

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14

Info: Writing image file hsa04110.pathview.png

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

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14

Info: Writing image file hsa03030.pathview.png

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

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14

Info: Writing image file hsa05130.pathview.png

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

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14

Info: Writing image file hsa03013.pathview.png

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

Info: Working in directory /Users/josephgirgiss/Desktop/BIMM_143/class14
```

```
Info: Writing image file hsa03440.pathview.png
```


PATHOGENIC ESCHERICHIA COLI INFECTION
Epithelial cell / Macrophage
Enteropathogenic Escherichia coli(EPEC)
Enterohemorrhagic Escherichia coli(EHEC)
Toxins
Stx
Translocation
Intestinal tract
Kidney
Inhibition of protein synthesis
EspJ
FcγR-mediated phagocytosis
WAVE regulatory complex
Anti-phagocytosis (Macrophage)
LEE-encoded effectors
Tir
Map
EspB
EspF
EspG
EspH
EspZ
non-LEE-encoded effectors
EspG2
EspJ
EspM1/2
EspT
EspW
Cif
NleA
NleB
NleC
NleD
NleE
NleF
NleH
Prophage-carried effectors
EspFU
PAMPs
PRRs
Type III secretion system
Bacterial secretion system
Microvillus effacing
Lamellipodia formation
Membrane ruffling
Filopodia formation
LPA
F2
Serum
Stress fiber formation
Disruption of microtubule network
Regulation of actin cytoskeleton
Pedestal disassembly during late stages of infection
Pedestal formation
Ese
Flagellar assembly
Phosphatidylethanolamine
Toll-like receptor signaling pathway
NF-κB signaling pathway
Flagellin
LPS
Inhibition of pro-inflammatory response
DNA
Inhibition of membrane transport
ER
Golgi
Anti-apoptotic effectors
Pro-apoptotic effectors
Mitochondria
Apoptosis
Type III secretion rod/needle proteins
DNA/RNA hybrid from lysosome
NOD-like receptor signaling pathway
Tight junction
Data on KEGG graph
Rendered by Pathview


NUCLEOCYTOPLASMIC TRANSPORT
-1
0
1
Import
Importin
NLS
NPC
Cytoplasmic fibrils
Cytoplasm
Cytoplasmic ring
Central channel
Spoke complex
Lumenal ring
Lumen
Nucleoplasmic ring
Nucleus
Nuclear basket
NLS
Ran GTP
Importin
Export
Exportin
NES
Pi
Ran GDP
DDX 19
Nup 98
Rae1
Nup358 complex
Nup 214
Nup62 complex
Nup107-160 complex
ELYS
Nup 153
Tpr
Exportin
Ran GTP
NES
mRNA Export
PYM
EJC
AUG
PABP
AAAAA
Upf1
Upf2
Tap
Ref/ Aly
Cytoplasm
Lumen
NPC
Nucleus
mRNA surveilance pathway
SRm160
Pinin
EJC
Upf3
p15
Tap
TREX
Ref/Aly
CBC
m7G
AAAAA
Nuclear Pore complex (NPC)
Cytoplasmic fibrils
ALADIN
hCG1
Gle1
DDX19
Rae1
Nup98
Nup214
Nup88
Nup358 complex
RanBP2
RanGAP
UBC9
SUMO
Cytoplasmic ring / Nucleoplasmic ring (Symmetrical nups)
Nup160
Nup85
Sec13
Nup107
Nup133
Nup96
Nup145
Seh1
Nup43
Nup37
ELYS
Central channel
Nup62
Nup58/45
Nup54
Spoke complex
Nup205
Nup188
Nup155
Nup93
Nup53
Nup59
Lumenal ring
NDC1
gp210
pom121
pom152
pom34
pom33
Nuclear basket
Tpr
Nup50
Nup153
Senp2
Nup2
Nup1
Nup60
Nuclear transport complex
Importin
Adaptor proteins
IPOA
IPOB
SPN1
Exportin
XPO
Ran
eEF1A
PHAX
CBC
NMD3
Exon-junction complex (EJC)
EJC inner core
Y14
MAOOH
MLN51
EIF4A3
EJC outer shell
ACIN1
SAP18
RNPS1
Pinin
Ref/Aly
Transiently interacting factors
Upf1
Upf2
Upf3
Tap
p15
UAP56
SRm160
PYM
Transcription-export (TREX) complex
THO subcomplex
THOC1
THOC2
THOC5
THOC6
THOC7
TEX1
Data on KEGG graph
Rendered by Pathview



GO terms

Same analysis but using GO genesets rather than KEGG

```
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

Lots of folks like the reactome web interface. We can also run this as an R function but lets look at the web interface first <https://reactome.org/PathwayBrowser/#TOOL=AT >

The website wants a text file with one gene symbol per line of the genes you want to map to pathways.

```
sig_genes <- res[res$padj <= 0.05 & !is.na(res$padj), "symbol"]
head(sig_genes)
```

```
ENSG00000187634 ENSG00000188976 ENSG00000187961 ENSG00000188290 ENSG00000187608
       "SAMD11"          "NOC2L"         "KLHL17"           "HES4"          "ISG15"
ENSG00000188157
         "AGRN"
```

```
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?

```
# Top KEGG upregulated pathway (lowest p-value)
kegg_top_up <- rownames(keggres$greater)[ which.min(keggres$greater[ , "p.val"]) ]

# Top KEGG downregulated pathway (lowest p-value)
kegg_top_down <- rownames(keggres$less)[ which.min(keggres$less[ , "p.val"]) ]

kegg_top_up
```

```
[1] "hsa04060 Cytokine-cytokine receptor interaction"
```

```
kegg_top_down
```

```
[1] "hsa04110 Cell cycle"
```

```
# Top GO BP upregulated term
go_top_up <- rownames(gobpres$greater)[ which.min(gobpres$greater[ , "p.val"]) ]

# Top GO BP downregulated term
go_top_down <- rownames(gobpres$less)[ which.min(gobpres$less[ , "p.val"]) ]

go_top_up
```

```
[1] "GO:0007156 homophilic cell adhesion"
```

```
go_top_down
```

```
[1] "GO:0048285 organelle fission"
```

```
top_up_pval <- head(rownames(keggres$greater),1)
top_down_pval <- head(rownames(keggres$less), 1)
top_up_pval
```

```
[1] "hsa04060 Cytokine-cytokine receptor interaction"
```

```
top_down_pval
```

```
[1] "hsa04110 Cell cycle"
```

```
go_top_up_pval <- head(rownames(gobpres$greater),1)
go_top_down_pval <- head(rownames(gobpres$less), 1)
go_top_up_pval
```

```
[1] "GO:0007156 homophilic cell adhesion"
```

```
go_top_down_pval
```

```
[1] "GO:0048285 organelle fission"
```

Cytokine-cytokine receptor interaction and homophilic cell adhesion are the most significant up-regulated gene for Kegg and for Go, respectively. Cell cycle and organelle fission are the most significant down-regulated gene for Kegg and for Go, respectively. While both the up-regulated gene reflect enhanced cell–cell communication and coordinated cellular responses, and both the down-regulated genes are related to cell-cycle progression, differences in gene "hits" may arise from differences in how each method categorizes biology (Kegg = general pathway, GO = function-based) as well as different gene set sizes.

Save our results

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