Class 12: RNA-Seq Mini Project

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Differential Expression Analysis

```
library(DESeq2)
library(ggplot2)
library(AnnotationDbi)
library(org.Hs.eg.db)
```

Load the counts and metadata files.

```
metaFile <- "GSE37704_metadata.csv"
countFile <- "GSE37704_featurecounts.csv"

#import metadata
colData = read.csv(metaFile, row.names = 1)
head(colData)
```

```
##                condition
## SRR493366 control_sirna
## SRR493367 control_sirna
## SRR493368 control_sirna
## SRR493369      hoxa1_kd
## SRR493370      hoxa1_kd
## SRR493371      hoxa1_kd
```

```
#import countData
countData = read.csv(countFile, row.names = 1)
head(countData)
```

```
##                 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
```

Q1: Complete the code below to remove the first column from countData.

```
countData <- as.matrix(countData[,-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
```

Q2: Complete the code below to filter countData to exclude genes (ie. rows) that have 0 read counts across all samples (ie. columns).

```
counts <- countData[rowSums(countData) > 0, ]
head(counts)
```

```
##                 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
```

Running DESeq2

Set up the DESeqDataSet object required for the DESeq() function, then run the DESeq pipeline.

```
dds = DESeqDataSetFromMatrix(countData = counts,
                             colData = colData,
                             design =~condition)
```

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

```
dds = DESeq(dds)
```

```
## estimating size factors
```

```
## estimating dispersions
```

```
## gene-wise dispersion estimates
## mean-dispersion relationship
```

```
## final dispersion estimates
```

```
## fitting model and testing
```

```
dds
```

```
## class: DESeqDataSet
## dim: 15975 6
## metadata(1): version
## assays(4): counts mu H cooks
## rownames(15975): ENSG00000279457 ENSG00000187634 ... ENSG00000276345
##   ENSG00000271254
## rowData names(22): baseMean baseVar ... deviance maxCooks
## colnames(6): SRR493366 SRR493367 ... SRR493370 SRR493371
## colData names(2): condition sizeFactor
```

Get results for the HoxA1 knockdown versus control siRNA. These are located in the colData input, under the names "hoxa1_kd" and "control_sirna".

```
res = results(dds, contrast = c("condition", "hoxa1_kd", "control_sirna"))
```

Q3: 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.

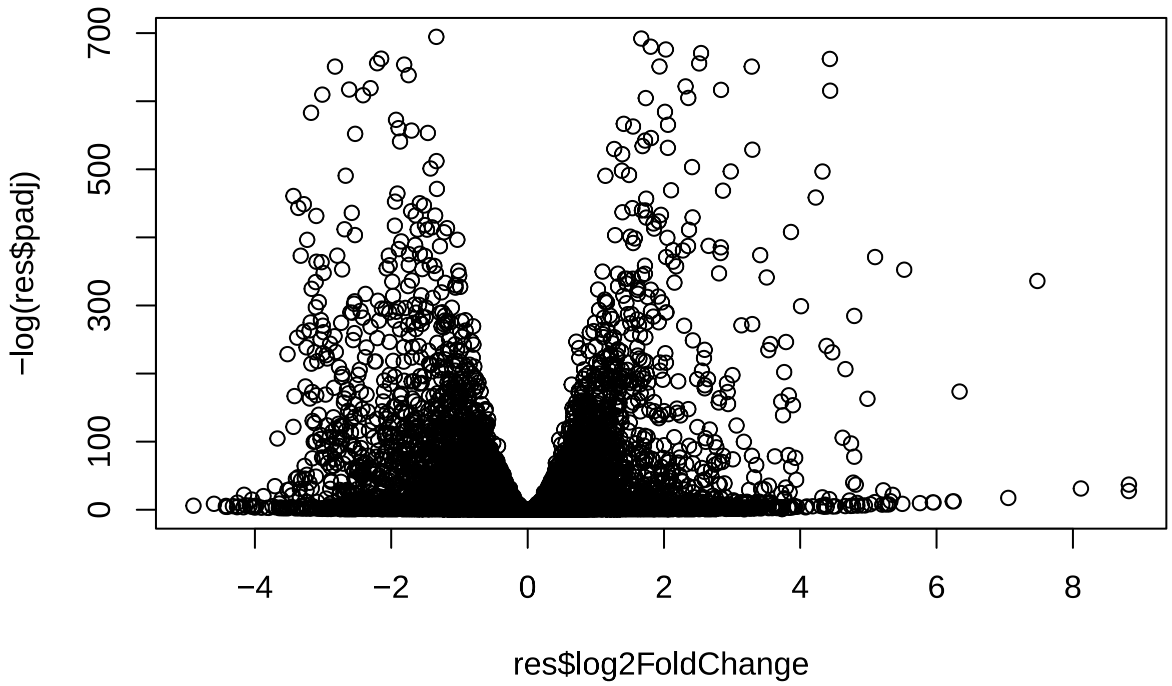
```
summary(res)
```

```
##
## 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

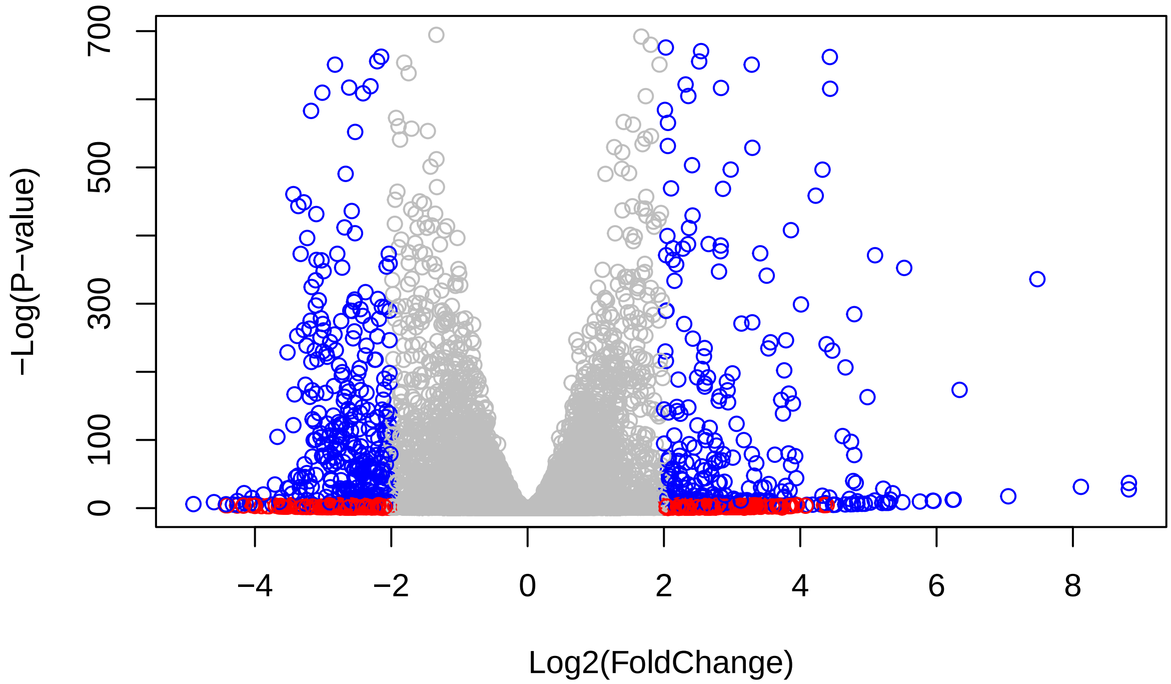
We'll make a volcano plot to visualize the data. A volcano plot is a plot of log2 fold change vs. -log adjusted p-value.

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



Q4: Complete the code below to add colors and axis labels.

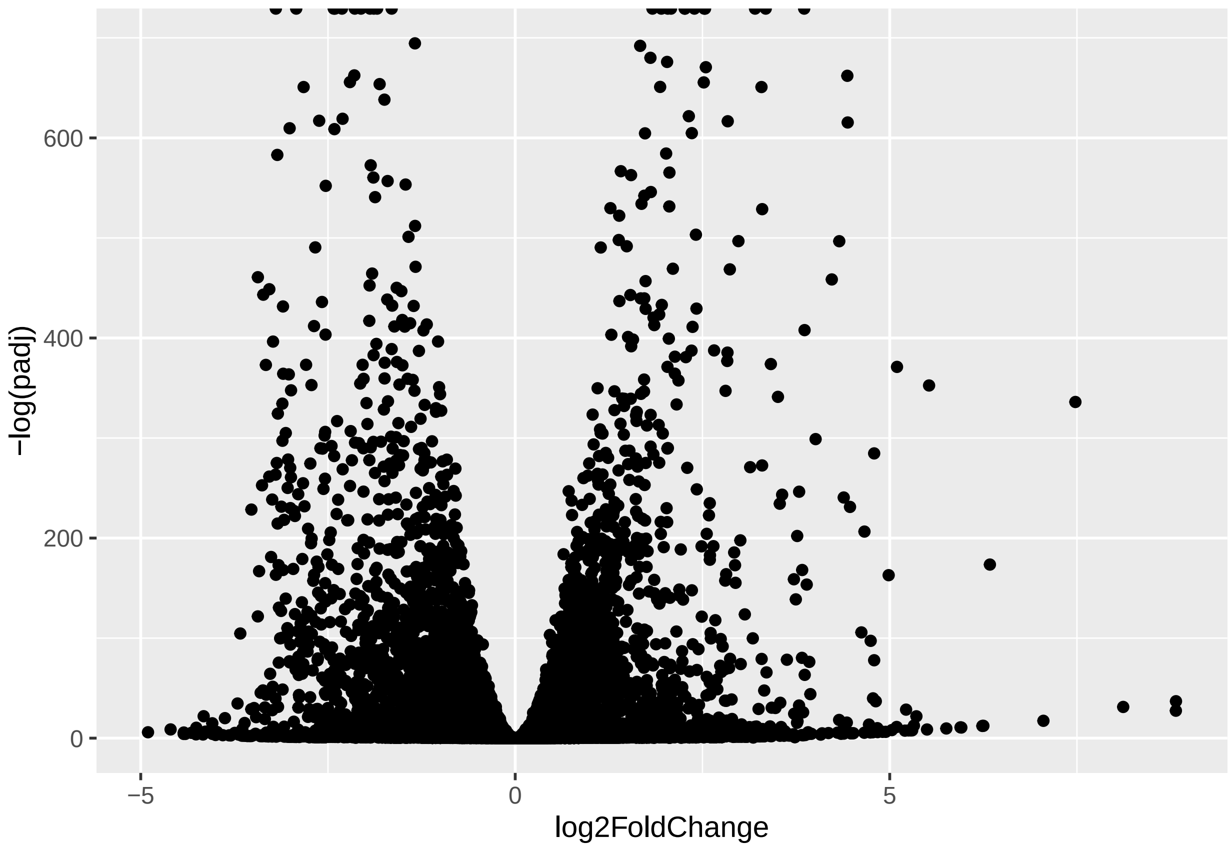
```
#make a color vector for all genes
mycols <- rep("gray", nrow(res) )

#RED = genes with absolute fold change above 2
mycols[ abs(res$log2FoldChange) > 2 ] <- "red"

#BLUE = those with adjusted p-value less than 0.01
#AND absolute fold change more than 2
inds <- (res$padj < 0.01) & (abs(res$log2FoldChange) > 2 )
mycols[inds] <- "blue"

plot( res$log2FoldChange, -log(res$padj), col = mycols, xlab = "Log2(FoldChange)", ylab = "-Log(P-value
```



BONUS! Try summarizing our results with a ggplot.

```
tmp1 <- as.data.frame(res)

ggplot(tmp1) +
  aes(x = log2FoldChange, y = -log(padj)) +
  geom_point()
```

```
## Warning: Removed 1237 rows containing missing values (geom_point).
```



Adding Gene Annotation

Our results currently have information about Ensembl gene IDs. However, KEGG pathways use Entrez gene annotations!

Q5: Use the mapIDs() function multiple times to add SYMBOL, ENTREZID and GENENAME annotation to our results.

```
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"
```

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

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

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

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

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

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

```
head(res, 10)
```

```
## log2 fold change (MLE): condition hoxa1_kd vs control_sirna
## Wald test p-value: condition hoxa1 kd vs control sirna
## DataFrame with 10 rows and 9 columns
##                    baseMean log2FoldChange     lfcSE       stat      pvalue
##                   <numeric>      <numeric> <numeric>  <numeric>   <numeric>
## ENSG00000279457   29.913579      0.1792571 0.3248216   0.551863 5.81042e-01
## ENSG00000187634  183.229650      0.4264571 0.1402658   3.040350 2.36304e-03
## ENSG00000188976 1651.188076     -0.6927205 0.0548465 -12.630158 1.43990e-36
## ENSG00000187961  209.637938      0.7297556 0.1318599   5.534326 3.12428e-08
## ENSG00000187583   47.255123      0.0405765 0.2718928   0.149237 8.81366e-01
## ENSG00000187642   11.979750      0.5428105 0.5215598   1.040744 2.97994e-01
## ENSG00000188290  108.922128      2.0570638 0.1969053  10.446970 1.51282e-25
## ENSG00000187608  350.716868      0.2573837 0.1027266   2.505522 1.22271e-02
## ENSG00000188157 9128.439422      0.3899088 0.0467163   8.346304 7.04321e-17
## ENSG00000237330    0.158192      0.7859552 4.0804729   0.192614 8.47261e-01
##                        padj      symbol      entrez                   name
##                   <numeric> <character> <character>            <character>
## ENSG00000279457 6.86555e-01      WASH9P   102723897 WAS protein family h..
## 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..
## ENSG00000188290 1.30538e-24        HES4       57801 hes family bHLH tran..
## ENSG00000187608 2.37452e-02       ISG15        9636 ISG15 ubiquitin like..
## ENSG00000188157 4.21963e-16        AGRN      375790                  agrin
## ENSG00000237330          NA      RNF223      401934 ring finger protein ..
```

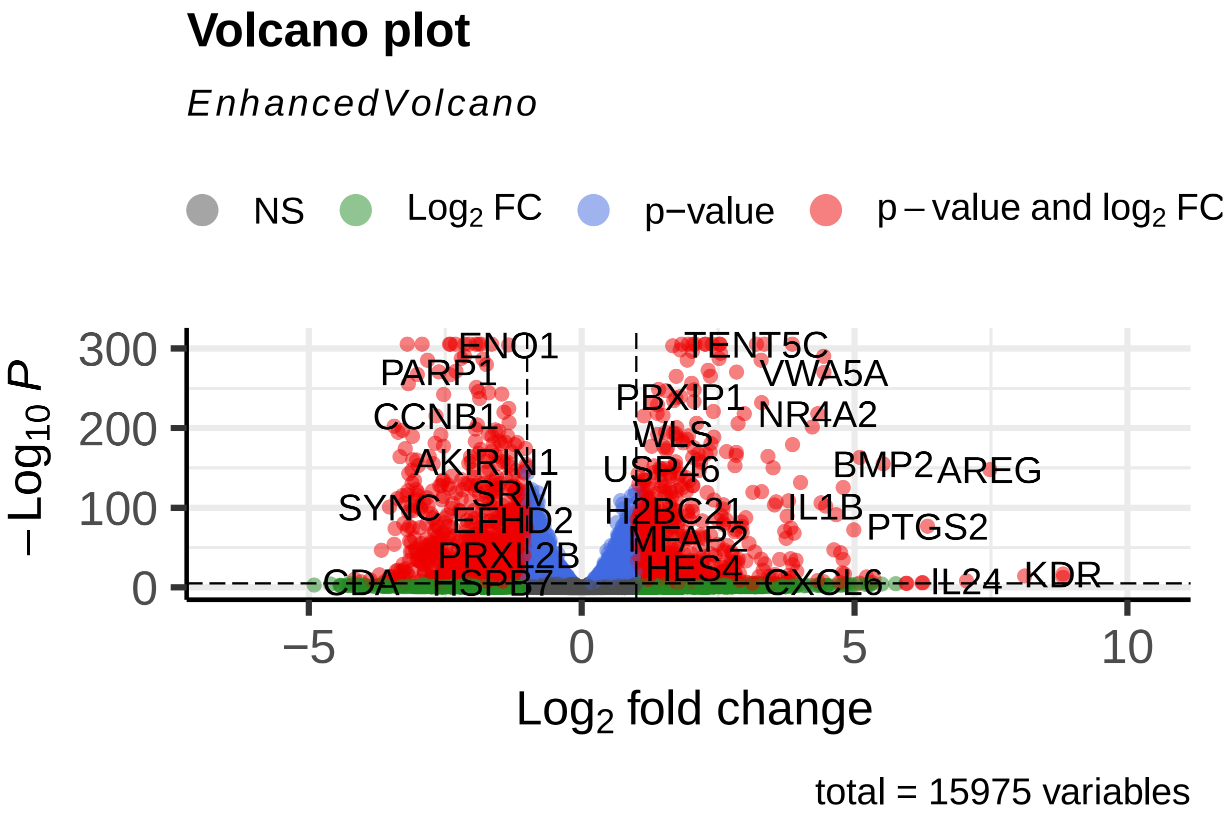
BONUS! Us EnhancedVolcano to visualize the results.

```
library(EnhancedVolcano)
```

```
## Loading required package: ggrepel
## Registered S3 methods overwritten by 'ggalt':
##   method                  from
##   grid.draw.absoluteGrob  ggplot2
##   grobHeight.absoluteGrob ggplot2
##   grobWidth.absoluteGrob  ggplot2
##   grobX.absoluteGrob      ggplot2
##   grobY.absoluteGrob      ggplot2
```

```
tmp2 <- as.data.frame(res)

EnhancedVolcano(tmp2,
    lab = tmp2$symbol,
    x = 'log2FoldChange',
    y = 'pvalue')
```

```
## Warning: One or more p-values is 0. Converting to 10^-1 * current lowest non-
## zero p-value...
```



Q6: Reorder these results by adjusted p-value and save them to a CSV file in your current project directory

```
reorder = res[order(res$pvalue),]
write.csv(reorder, file = "deseq_results1.csv")
```

Pathway Analysis

Bring back the biology! This will help with the interpretation of our results. We try to answer the question: *which pathways and functions feature heavily in our differentially expressed genes?*

KEGG Pathways

```
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).
## ##############################################################################
```

```
library(gage)
```

```
##
```

```
library(gageData)

data(kegg.sets.hs)
data(sigmet.idx.hs)

#focus on signaling and metabolic pathways only
kegg.sets.hs = kegg.sets.hs[sigmet.idx.hs]

#examine the first 3 pathways
head(kegg.sets.hs, 3)
```

```
## $‘hsa00232 Caffeine metabolism‘
## [1] "10"   "1544" "1548" "1549" "1553" "7498" "9"
##
## $‘hsa00983 Drug metabolism - other enzymes‘
##  [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
##  [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
## [17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
## [25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
## [33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
## [41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
## [49] "8824"   "8833"   "9"      "978"
##
## $‘hsa00230 Purine metabolism‘
##   [1] "100"    "10201"  "10606"  "10621"  "10622"  "10623"  "107"    "10714"
##   [9] "108"    "10846"  "109"    "111"    "11128"  "11164"  "112"    "113"
##  [17] "114"    "115"    "122481" "122622" "124583" "132"    "158"    "159"
##  [25] "1633"   "171568" "1716"   "196883" "203"    "204"    "205"    "221823"
##  [33] "2272"   "22978"  "23649"  "246721" "25885"  "2618"   "26289"  "270"
##  [41] "271"    "27115"  "272"    "2766"   "2977"   "2982"   "2983"   "2984"
##  [49] "2986"   "2987"   "29922"  "3000"   "30833"  "30834"  "318"    "3251"
##  [57] "353"    "3614"   "3615"   "3704"   "377841" "471"    "4830"   "4831"
##  [65] "4832"   "4833"   "4860"   "4881"   "4882"   "4907"   "50484"  "50940"
##  [73] "51082"  "51251"  "51292"  "5136"   "5137"   "5138"   "5139"   "5140"
##  [81] "5141"   "5142"   "5143"   "5144"   "5145"   "5146"   "5147"   "5148"
##  [89] "5149"   "5150"   "5151"   "5152"   "5153"   "5158"   "5167"   "5169"
##  [97] "51728"  "5198"   "5236"   "5313"   "5315"   "53343"  "54107"  "5422"
## [105] "5424"   "5425"   "5426"   "5427"   "5430"   "5431"   "5432"   "5433"
## [113] "5434"   "5435"   "5436"   "5437"   "5438"   "5439"   "5440"   "5441"
## [121] "5471"   "548644" "55276"  "5557"   "5558"   "55703"  "55811"  "55821"
## [129] "5631"   "5634"   "56655"  "56953"  "56985"  "57804"  "58497"  "6240"
## [137] "6241"   "64425"  "646625" "654364" "661"    "7498"   "8382"   "84172"
## [145] "84265"  "84284"  "84618"  "8622"   "8654"   "87178"  "8833"   "9060"
## [153] "9061"   "93034"  "953"    "9533"   "954"    "955"    "956"    "957"
## [161] "9583"   "9615"
```

The gage() function requires a named vector of fold changes. The names are the Entrez gene IDs.

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

```
##   102723897      148398       26155      339451       84069       84808
##  0.17925708  0.42645712 -0.69272046  0.72975561  0.04057653  0.54281049
```

Now run the gage analysis!

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

```
## $names
## [1] "greater" "less"    "stats"
```

Look at the first two down-regulated pathways.

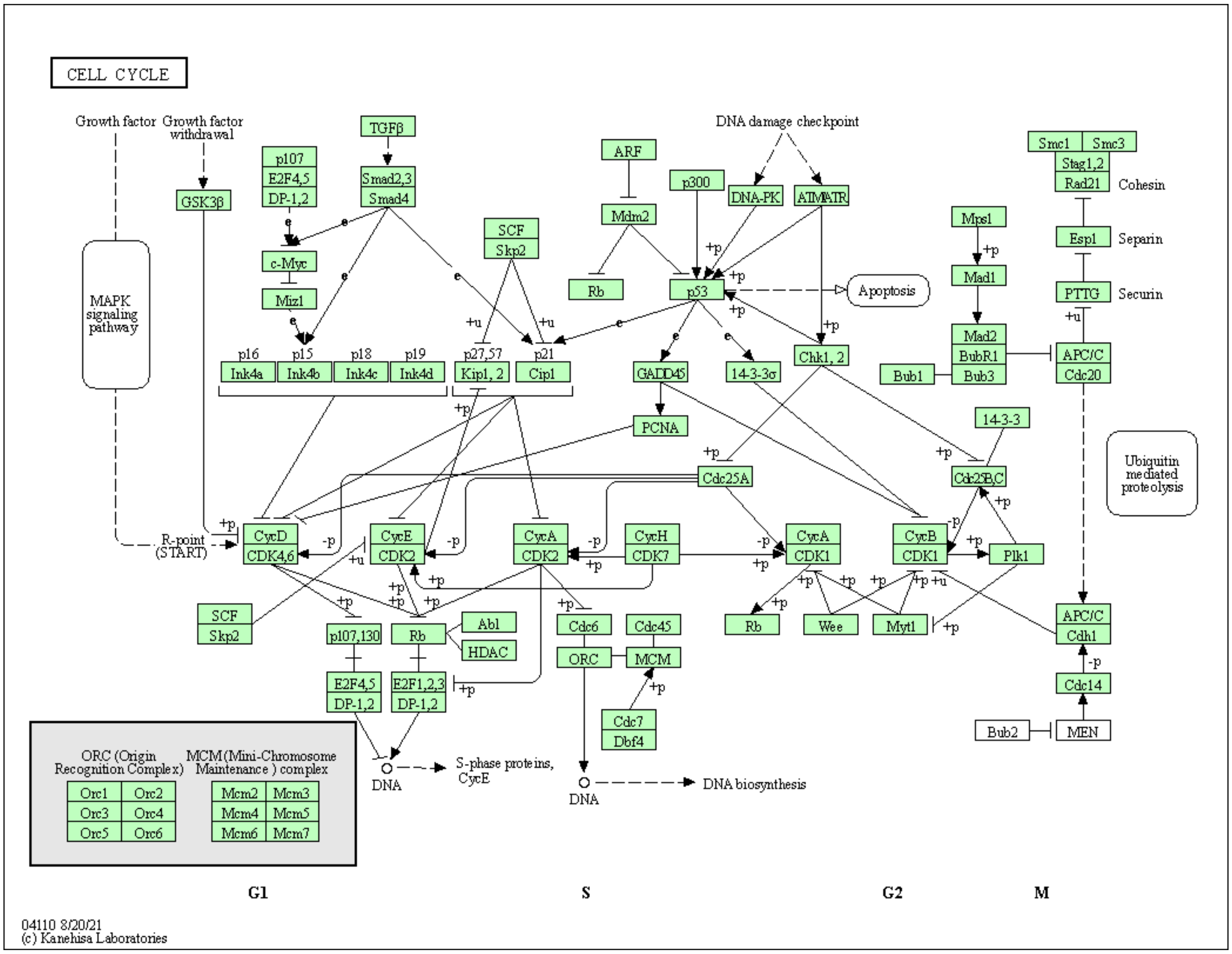
```
head(keggres$less, 2)
```

```
##                               p.geomean stat.mean        p.val       q.val
## hsa04110 Cell cycle        8.995727e-06 -4.378644 8.995727e-06 0.001448312
## hsa03030 DNA replication 9.424076e-05 -3.951803 9.424076e-05 0.007586381
##                          set.size         exp1
## hsa04110 Cell cycle           121 8.995727e-06
## hsa03030 DNA replication       36 9.424076e-05
```

Now use the pathview() function to make a pathway plot.

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

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

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



Above is the pathway hsa04110 - Cell cycle.

Focus on the top 5 up-regulated pathways:

```
keggrespathways <- rownames(keggres$greater)[1:5]

#extract the 8-character IDs of each string
keggresids = substr(keggrespathways, start = 1, stop = 8)
keggresids
```

```
## [1] "hsa04640" "hsa04630" "hsa00140" "hsa04142" "hsa04330"
```

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

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa04640.pathview.png

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa04630.pathview.png

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa00140.pathview.png

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa04142.pathview.png

## Info: some node width is different from others, and hence adjusted!

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa04330.pathview.png
```

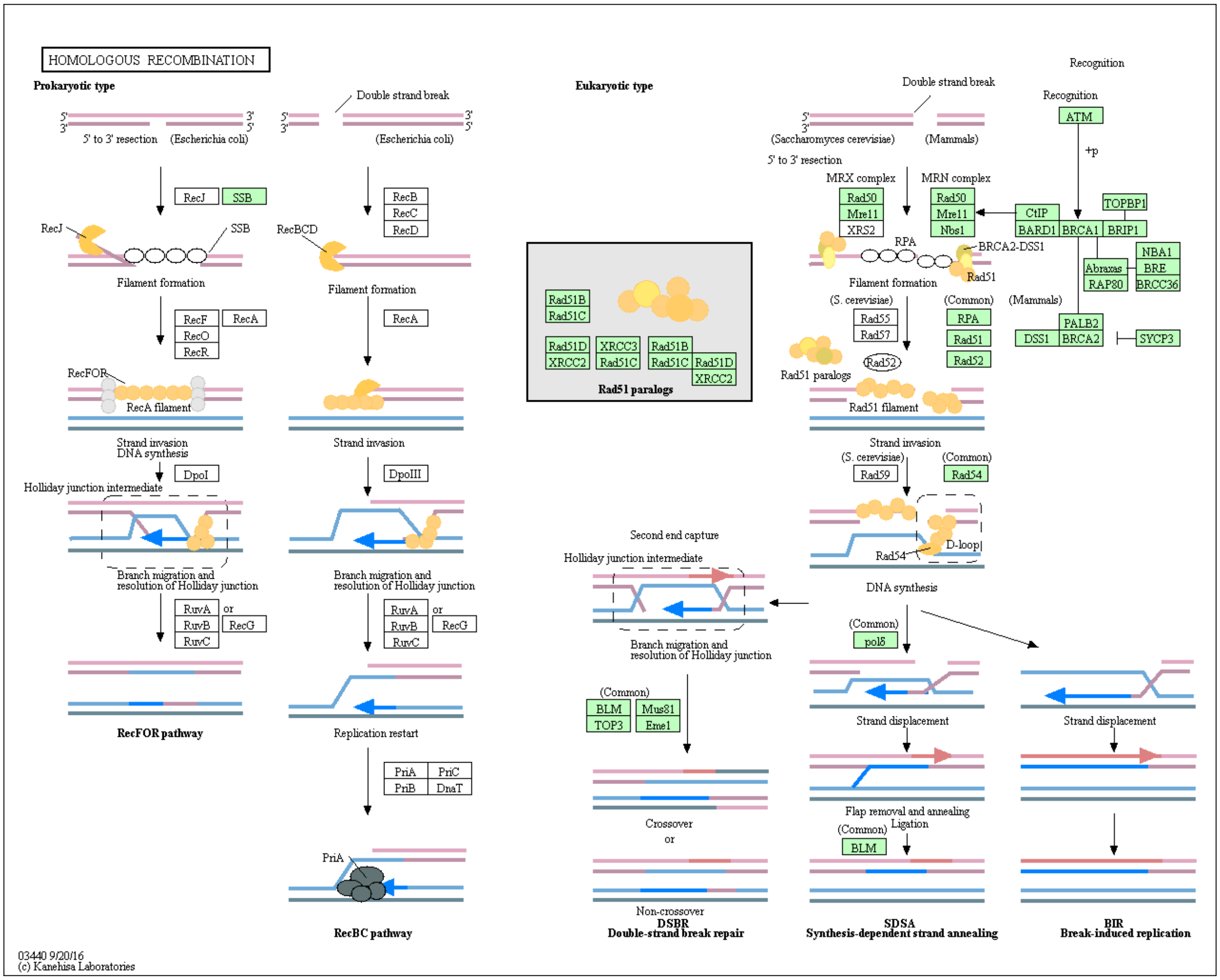
Q7: Do the same for the top 5 down-regulated pathways.

```r
keggresdown <- rownames(keggres$less)[1:5]

keggresdownids = substr(keggresdown, start = 1, stop = 8)
keggresdownids
```

```
## [1] "hsa04110" "hsa03030" "hsa03013" "hsa03440" "hsa04114"
```

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

```
## 'select()' returned 1:1 mapping between keys and columns
## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

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

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

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

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa03013.pathview.png

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

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

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

## Info: Working in directory C:/Users/div/Documents/bimm143/labs/class12

## Info: Writing image file hsa04114.pathview.png
```



Above is pathway hsa03440 - homologous recombination.

Gene Ontology

Using gene ontology, we can follow a similar procedure as above.

```
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)

lapply(gobpres, head)
```

```
## $greater
##                                              p.geomean stat.mean        p.val
## GO:0007156 homophilic cell adhesion       8.519724e-05  3.824205 8.519724e-05
## GO:0002009 morphogenesis of an epithelium 1.396681e-04  3.653886 1.396681e-04
## GO:0048729 tissue morphogenesis           1.432451e-04  3.643242 1.432451e-04
## GO:0007610 behavior                       2.195494e-04  3.530241 2.195494e-04
## GO:0060562 epithelial tube morphogenesis  5.932837e-04  3.261376 5.932837e-04
## GO:0035295 tube development               5.953254e-04  3.253665 5.953254e-04
##                                               q.val set.size         exp1
## GO:0007156 homophilic cell adhesion       0.1951953      113 8.519724e-05
## GO:0002009 morphogenesis of an epithelium 0.1951953      339 1.396681e-04
## GO:0048729 tissue morphogenesis           0.1951953      424 1.432451e-04
## GO:0007610 behavior                       0.2243795      427 2.195494e-04
## GO:0060562 epithelial tube morphogenesis  0.3711390      257 5.932837e-04
## GO:0035295 tube development               0.3711390      391 5.953254e-04
##
## $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
##
## $stats
##                                           stat.mean     exp1
## GO:0007156 homophilic cell adhesion        3.824205 3.824205
## GO:0002009 morphogenesis of an epithelium  3.653886 3.653886
## GO:0048729 tissue morphogenesis            3.643242 3.643242
## GO:0007610 behavior                        3.530241 3.530241
## GO:0060562 epithelial tube morphogenesis   3.261376 3.261376
## GO:0035295 tube development                3.253665 3.253665
```

Reactome Analysis

Reactome is database consisting of biological molecules and their relation to pathways and processes. Let's conduct over-representation enrichment analysis and pathway-topology analysis with Reactome using the previous list of significant genes generated from our differential expression results above.

Ouput the significant genes (ie. pval <= 0.05) to a plain text file.

```
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 = FA
```

To perform the analysis, go to the Reactome site (https://reactome.org/PathwayBrowser/#TOOL=AT). Upload the sig__genes file, set parameters to "Project to Humans", and analyze!

Q8: 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?

The "endosomal/vacuolar pathway" has the most significant Entities p-value. The significant pathways are somewhat similar since they both include cell cycle pathways, though other processes differ. This difference may be due to the method in which the over-representation analysis was conducted, such as the threshold value for what counts as a differentially expressed gene.

Go Online

Gene Set Gene Ontology (GO) Enrichment is a method to determine over-represented or under-represented GO terms for a given set of genes. GO terms are formal structured controlled vocabularies (ontologies) for gene products in terms of their biological function. The goal of this analysis is to determine the biological process the given set of genes are associated with.

Go online (http://www.geneontology.org/page/go-enrichment-analysis) to perform Gene Set GO Enrichment.

Q9: 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?

The "detection of chemical stimulus involved in sensory perception" pathway had the most significant p-value. Most of the pathways don't match the KEGG results, which may be due to the reasons stated in Q8, that the process of analysis differs.

Save Results

```
write.csv(res, file = "deseq_results2.csv")
```