Class 15 Transcriptomics and the analysis of RNA-Seq data

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#Background Our data for this hands-on session comes from a published RNA-seq experiment where airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects (Himes et al. 2014).

2. Import countData and colData

Read the countData and colData

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

Let's have a look at these

```
head(counts)
```

```
##                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
## ENSG00000000003        723        486        904        445       1170
## ENSG00000000005          0          0          0          0          0
## ENSG00000000419        467        523        616        371        582
## ENSG00000000457        347        258        364        237        318
## ENSG00000000460         96         81         73         66        118
## ENSG00000000938          0          0          1          0          2
##                 SRR1039517 SRR1039520 SRR1039521
## ENSG00000000003       1097        806        604
## ENSG00000000005          0          0          0
## ENSG00000000419        781        417        509
## ENSG00000000457        447        330        324
## ENSG00000000460         94        102         74
## ENSG00000000938          0          0          0
```

```
metadata
```

```
##           id     dex celltype     geo_id
## 1 SRR1039508 control   N61311 GSM1275862
## 2 SRR1039509 treated   N61311 GSM1275863
## 3 SRR1039512 control  N052611 GSM1275866
## 4 SRR1039513 treated  N052611 GSM1275867
## 5 SRR1039516 control  N080611 GSM1275870
## 6 SRR1039517 treated  N080611 GSM1275871
## 7 SRR1039520 control  N061011 GSM1275874
## 8 SRR1039521 treated  N061011 GSM1275875
```

Q1. How many genes are in this dataset?

```
nrow(counts)
```

```
## [1] 38694
```

- 38694 genes

Q2. How many 'control' cell lines do we have?

```
sum(metadata$dex == "control")
```

```
## [1] 4
```

- There are 4 control cell lines

#3. Toy differential gene expression

Lets perform some exploratory differential gene expression analysis. Note: this analysis is for demonstration only. NEVER do differential expression analysis this way!

Let's develop a bit of code that will first find the sample id for those labeled control and then calculate the mean counts per gene across these samples:

First I need to extract all the "control" columns. Then I will take the row wise mean to get the average count values for all genes in these four experiments.

```
control.inds <- metadata$dex == "control"
control.counts <- counts[ , control.inds]
head(control.counts)
```

```
##                 SRR1039508 SRR1039512 SRR1039516 SRR1039520
## ENSG00000000003        723        904       1170        806
## ENSG00000000005          0          0          0          0
## ENSG00000000419        467        616        582        417
## ENSG00000000457        347        364        318        330
## ENSG00000000460         96         73        118        102
## ENSG00000000938          0          1          2          0
```

```
control.mean <- rowMeans(control.counts)
```

Q3. How would you make the above code in either approach more robust?

- You should use rowMenas() instead of rowSums() + dividing by 4 because what if the dataset were updated? If the dataset were updated to have more than 4 control groups, the written code using rowSums() would prove not robust and would not work.

Q4. Follow the same procedure for the treated samples (i.e. calculate the mean per gene across drug treated samples and assign to a labeled vector called treated.mean)

Now do the same for the drug treated experiments (i.e. columns)

```
treated.inds <- metadata$dex == "treated"
treated.counts <- counts[ , treated.inds]
head(treated.counts)
```

```
##                 SRR1039509 SRR1039513 SRR1039517 SRR1039521
## ENSG00000000003        486        445       1097        604
## ENSG00000000005          0          0          0          0
## ENSG00000000419        523        371        781        509
## ENSG00000000457        258        237        447        324
## ENSG00000000460         81         66         94         74
## ENSG00000000938          0          0          0          0
```

```
treated.mean <- rowMeans(treated.counts)
```

We will combine our meancount data for bookkeeping purposes.

```
meancounts <- data.frame(control.mean, treated.mean)
```

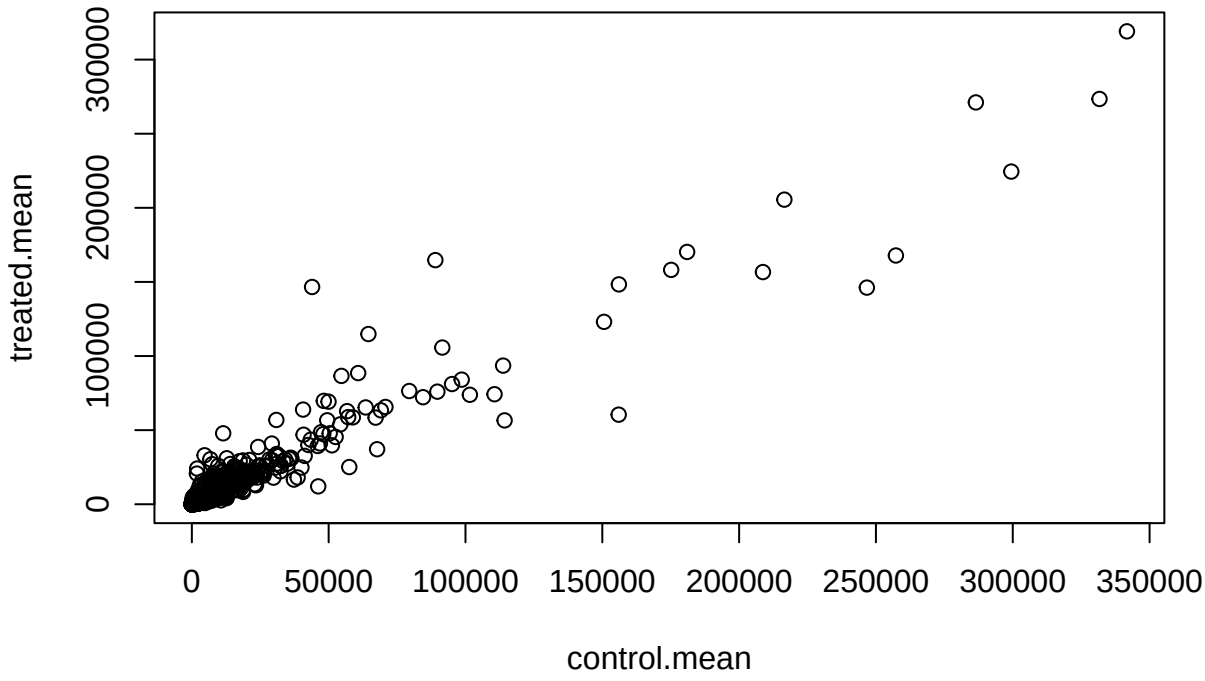
Now show the sum of the mean counts across all genes for each group:

```
colSums(meancounts)
```

```
## control.mean treated.mean
##     23005324     22196524
```

> Q5 (a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples. Your plot should look something like the following.

```
plot(meancounts)
```



Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

- You would use `geom_point()`

Wait a sec. There are 60,000-some rows in this data, but I'm only seeing a few dozen dots at most outside of the big clump around the origin.

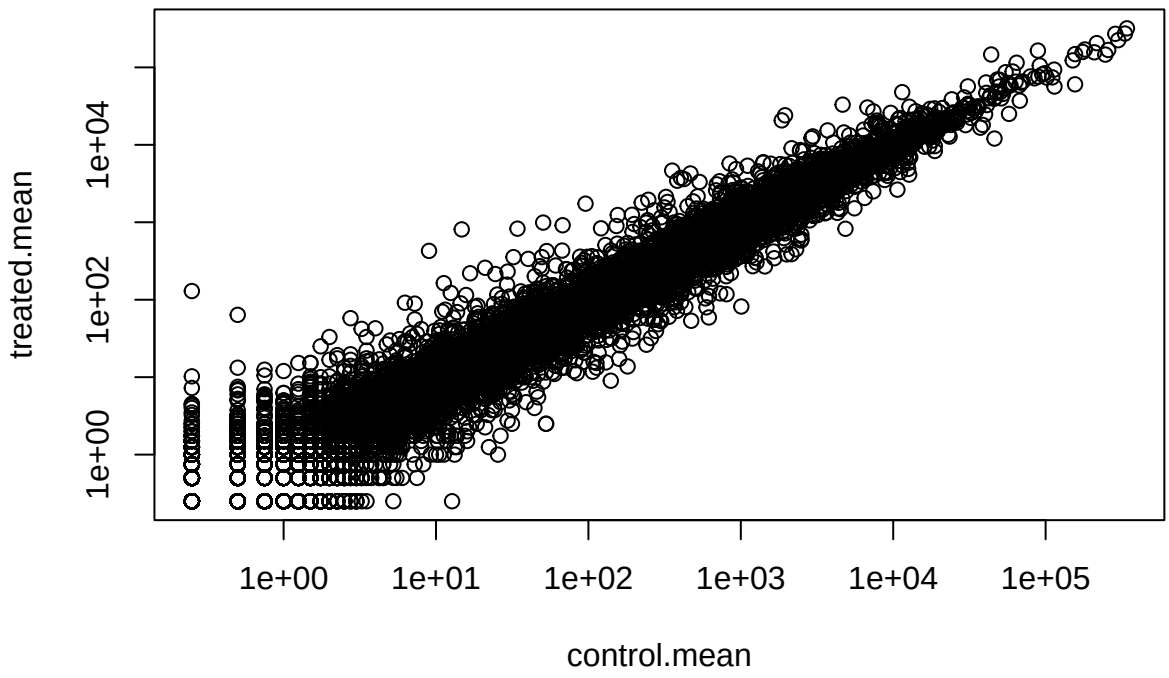
This plot indicates that we need to transform our data!

Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

```
plot(meancounts, log = "xy")
```

```
## Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 x values <= 0 omitted
## from logarithmic plot
```

```
## Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 y values <= 0 omitted
## from logarithmic plot
```



We often use log2 in this field because it has nice math properties that make interpretation easier.

Ex: if log2(somenumber)=0, this means that theres no change. Thus deviations from 0 indicate change (either positive or negative)

```
log2(10/10)
```

```
## [1] 0
```

```
log2(20/10)
```

```
## [1] 1
```

```
log2(40/10)
```

```
## [1] 2
```

```
log2(5/10)
```

```
## [1] -1
```

We see 0 values for no change and + values for increases and - values for decreases.This nice property leads us to work with **log2(fold-change)** all the time in the genomics and proteomics field.

Let's add the **log2(fold-change)** values to our `meancounts` dataframe.

We can find candidate differentially expressed genes by looking for genes with a large change between control and dex-treated samples. We usually look at the log2 of the fold change, because this has better mathematical properties.

Here we calculate log2foldchange, add it to our meancounts data.frame and inspect the results either with the head() or the View() function for example.

```
meancounts$log2fc <- log2(meancounts[,"treated.mean"]/
                          meancounts[,"control.mean"])
head(meancounts)
```

```
##                 control.mean treated.mean      log2fc
## ENSG00000000003       900.75       658.00 -0.45303916
## ENSG00000000005         0.00         0.00         NaN
## ENSG00000000419       520.50       546.00  0.06900279
## ENSG00000000457       339.75       316.50 -0.10226805
## ENSG00000000460        97.25        78.75 -0.30441833
## ENSG00000000938         0.75         0.00        -Inf
```

There are a couple of "weird" results. Namely, the NaN ("not a number") and -Inf (negative infinity) results.

The NaN is returned when you divide by zero and try to take the log. The -Inf is returned when you try to take the log of zero. It turns out that there are a lot of genes with zero expression. Let's filter our data to remove these genes. Again inspect your result (and the intermediate steps) to see if things make sense to you

Let's just **exclude** these weird values for genes (i.e. rows) that we can't say anything about since we have no data for them:

```
zero.vals <- which(meancounts[,1:2]==0, arr.ind=TRUE)

to.rm <- unique(zero.vals[,1])
mycounts <- meancounts[-to.rm,]
head(mycounts)
```

```
##                 control.mean treated.mean      log2fc
## ENSG00000000003       900.75       658.00 -0.45303916
## ENSG00000000419       520.50       546.00  0.06900279
## ENSG00000000457       339.75       316.50 -0.10226805
## ENSG00000000460        97.25        78.75 -0.30441833
## ENSG00000000971      5219.00      6687.50  0.35769358
## ENSG00000001036      2327.00      1785.75 -0.38194109
```

> Q7. What is the purpose of the arr.ind argument in the which() function call above? Why would we then take the first column of the output and need to call the unique() function?

- The arr.ind argument, when set to TRUE, returns array indices (positions) of where x is in the array (in the table). The `arr.ind=TRUE` argument gets the columns and rows where the TRUE values are (i.e. the zero counts in our case)
- We then take the first column of the output and call the unique() function because we would like to ensure that we aren't double-counting any row

A common threshold used for calling something differentially expressed is a log2(FoldChange) of greater than 2 or less than -2. Let's filter the dataset both ways to see how many genes are up or down-regulated.

```
up.ind <- mycounts$log2fc > 2
down.ind <- mycounts$log2fc < (-2)
```

Q8. Using the up.ind vector above can you determine how many up regulated genes we have at the greater than 2 fc level?

```
sum(up.ind)
```

```
## [1] 250
```

- There are 250 up-regulated genes at the greater than 2fc level

Q9. Using the down.ind vector above can you determine how many down regulated genes we have at the greater than 2 fc level?

```
sum(down.ind)
```

```
## [1] 367
```

There are 367 down-regulated genes at the greater than 2 fc level

Q10. Do you trust these results? Why or why not?

- No. We're missing statistical analyses to help us ensure that the fold changes are significant. Even large fold-changes are not necessarily statistically significant. Thus, these current results may be misleading and inaccurate.
- We need to utilize the DESeq2 package in order to properly perform analysis.

#4. DESeq2 analysis – ADDING THE STATISTICS

Let's do this the right way. DESeq2 is an R package specifically for analyzing count-based NGS data like RNA-seq.

```
library(DESeq2)
```

```
## Loading required package: S4Vectors

## Loading required package: stats4

## Loading required package: BiocGenerics

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs
```

```
## The following objects are masked from 'package:base':
##
##     anyDuplicated, append, as.data.frame, basename, cbind, colnames,
##     dirname, do.call, duplicated, eval, evalq, Filter, Find, get, grep,
##     grepl, intersect, is.unsorted, lapply, Map, mapply, match, mget,
##     order, paste, pmax, pmax.int, pmin, pmin.int, Position, rank,
##     rbind, Reduce, rownames, sapply, setdiff, sort, table, tapply,
##     union, unique, unsplit, which.max, which.min

##
## Attaching package: 'S4Vectors'

## The following objects are masked from 'package:base':
##
##     expand.grid, I, unname

## Loading required package: IRanges

##
## Attaching package: 'IRanges'

## The following object is masked from 'package:grDevices':
##
##     windows

## Loading required package: GenomicRanges

## Loading required package: GenomeInfoDb

## Loading required package: SummarizedExperiment

## Loading required package: MatrixGenerics

## Loading required package: matrixStats

##
## Attaching package: 'MatrixGenerics'

## The following objects are masked from 'package:matrixStats':
##
##     colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
##     colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
##     colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
##     colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
##     colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
##     colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
##     colWeightedMeans, colWeightedMedians, colWeightedSds,
##     colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
##     rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
##     rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
##     rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
##     rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
##     rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
##     rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
##     rowWeightedSds, rowWeightedVars
```

```
## Loading required package: Biobase

## Welcome to Bioconductor
##
##     Vignettes contain introductory material; view with
##     'browseVignettes()'. To cite Bioconductor, see
##     'citation("Biobase")', and for packages 'citation("pkgname")'.

##
## Attaching package: 'Biobase'

## The following object is masked from 'package:MatrixGenerics':
##
##     rowMedians

## The following objects are masked from 'package:matrixStats':
##
##     anyMissing, rowMedians
```

```
citation("DESeq2")
```

```
##
##   Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change
##   and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550
##   (2014)
##
## A BibTeX entry for LaTeX users is
##
##   @Article{,
##     title = {Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2},
##     author = {Michael I. Love and Wolfgang Huber and Simon Anders},
##     year = {2014},
##     journal = {Genome Biology},
##     doi = {10.1186/s13059-014-0550-8},
##     volume = {15},
##     issue = {12},
##     pages = {550},
##   }
```

Importing data

DESeq works on a particular type of object called a DESeqDataSet.

The DESeqDataSet is a single object that contains input values, intermediate calculations like how things are normalized, and all results of a differential expression analysis.

You can construct a DESeqDataSet from (1) a count matrix, (2) a metadata file, and (3) a formula indicating the design of the experiment.

(3): tells DESeq2 which columns in the sample information table (colData) specify the experimental design (i.e. which groups the samples belong to) and how these factors should be used in the analysis. Essentially, this formula expresses how the counts for each gene depend on the variables in colData.

Take a look at metadata again. The thing we're interested in is the dex column, which tells us which samples are treated with dexamethasone versus which samples are untreated controls. We'll specify the design with a tilde, like this: design=~dex.

Use the DESeqDataSetFromMatrix() function to build the required DESeqDataSet object and call it dds, short for our DESeqDataSet:

```
dds <- DESeqDataSetFromMatrix(countData=counts,
                              colData=metadata,
                              design=~dex)
```

```
## converting counts to integer mode

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

```
dds
```

```
## class: DESeqDataSet
## dim: 38694 8
## metadata(1): version
## assays(1): counts
## rownames(38694): ENSG00000000003 ENSG00000000005 ... ENSG00000283120
##   ENSG00000283123
## rowData names(0):
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
## colData names(4): id dex celltype geo_id
```

DESeq analysis

Let's run the DESeq analysis pipeline on the dataset, and reassign the resulting object back to the same variable. Note that before we start, dds is a bare-bones DESeqDataSet. The DESeq() function takes a DESeqDataSet and returns a DESeqDataSet, but with additional information filled in (including the differential expression results we are after). Notice how if we try to access these results before running the analysis, nothing exists.

Now we can run DESeq analysis:

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

```
## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing
```

Getting results

Since we've got a fairly simple design (single factor, two groups, treated versus control), we can get results out of the object simply by calling the results() function on the DESeqDataSet that has been run through the pipeline.

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

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 6 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005   0.000000             NA        NA        NA        NA
## ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
## ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
##                      padj
##                 <numeric>
## ENSG00000000003  0.163035
## ENSG00000000005        NA
## ENSG00000000419  0.176032
## ENSG00000000457  0.961694
## ENSG00000000460  0.815849
## ENSG00000000938        NA
```

Convert the res object to a data.frame with the as.data.frame() function and then pass it to View() to bring it up in a data viewer:

```
dat.fr.res <- as.data.frame(res)
View(dat.fr.res)
```

Now summarize some basic tallies using the summary function:

```
summary(res)
```

```
##
## out of 25258 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)       : 1563, 6.2%
## LFC < 0 (down)     : 1188, 4.7%
## outliers [1]       : 142, 0.56%
## low counts [2]     : 9971, 39%
## (mean count < 10)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

The results function contains a number of arguments to customize the results table. By default the argument alpha is set to 0.1. If the adjusted p value cutoff will be a value other than 0.1, alpha should be set to that value:

```
res05 <- results(dds, alpha=0.05)
summary(res05)
```

```
##
## out of 25258 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 1236, 4.9%
## LFC < 0 (down)     : 933, 3.7%
## outliers [1]       : 142, 0.56%
## low counts [2]     : 9033, 36%
## (mean count < 6)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

#5. Adding annotation data for our genes

Our result table so far only contains the Ensembl gene IDs. However, alternative gene names and extra annotation are usually required for informative interpretation of our results. In this section we will add this necessary annotation data to our results.

For this, we need two bioconductor packages:

```
#First, install packages
#BiocManager::install("AnnotationDbi")
#BiocManager::install("org.Hs.eg.db")

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

```
##
```

Let's then get a list of all available key types that we can use to map between:

```
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"
```

We can then use the mapIds() function to add individual columns to our results table:

We provide the row names of our results table as a key, and specify that keytype=ENSEMBL. The column argument tells the mapIds() function which information we want, and the multiVals argument tells the function what to do if there are multiple possible values for a single input value. Here we ask to just give us back the first one that occurs in the database.

```
res$symbol <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",   # The format of our genenames
                     column="SYMBOL",     # The new format we want to add
                     multiVals="first")
```

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

```
head(res)
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 7 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005   0.000000             NA        NA        NA        NA
## ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
## ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
##                      padj      symbol
##                 <numeric> <character>
## ENSG00000000003  0.163035      TSPAN6
## ENSG00000000005        NA        TNMD
## ENSG00000000419  0.176032        DPM1
## ENSG00000000457  0.961694       SCYL3
## ENSG00000000460  0.815849    C1orf112
## ENSG00000000938        NA         FGR
```

Q11. Run the mapIds() function two more times to add the Entrez ID and UniProt accession and GENENAME as new columns called res$entrez, res$uniprot and res$genename.

```
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$uniprot <- mapIds(org.Hs.eg.db,
                     keys=row.names(res),
                     keytype="ENSEMBL",
                     column="UNIPROT",
                     multiVals="first")
```

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

```
res$genename <- 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)
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 10 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005   0.000000             NA        NA        NA        NA
## ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
## ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
##                      padj      symbol      entrez     uniprot
##                 <numeric> <character> <character> <character>
## ENSG00000000003  0.163035      TSPAN6        7105  A0A024RCI0
## ENSG00000000005        NA        TNMD       64102      Q9H2S6
## ENSG00000000419  0.176032        DPM1        8813      O60762
## ENSG00000000457  0.961694       SCYL3       57147      Q8IZE3
## ENSG00000000460  0.815849    C1orf112       55732  A0A024R922
## ENSG00000000938        NA         FGR        2268      P09769
##                            genename
##                         <character>
## ENSG00000000003          tetraspanin 6
## ENSG00000000005            tenomodulin
## ENSG00000000419 dolichyl-phosphate m..
## ENSG00000000457 SCY1 like pseudokina..
## ENSG00000000460 chromosome 1 open re..
## ENSG00000000938 FGR proto-oncogene, ..
```

You can arrange and view the results by the adjusted p-value:

```
ord <- order( res$padj )
#View(res[ord,])
head(res[ord,])
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 10 columns
##                   baseMean log2FoldChange     lfcSE      stat      pvalue
##                  <numeric>      <numeric> <numeric> <numeric>   <numeric>
## ENSG00000152583   954.771        4.36836 0.2371268   18.4220 8.74490e-76
## ENSG00000179094   743.253        2.86389 0.1755693   16.3120 8.10784e-60
## ENSG00000116584  2277.913       -1.03470 0.0650984  -15.8944 6.92855e-57
## ENSG00000189221  2383.754        3.34154 0.2124058   15.7319 9.14433e-56
## ENSG00000120129  3440.704        2.96521 0.2036951   14.5571 5.26424e-48
## ENSG00000148175 13493.920        1.42717 0.1003890   14.2164 7.25128e-46
##                      padj      symbol      entrez     uniprot
##                 <numeric> <character> <character> <character>
## ENSG00000152583 1.32441e-71     SPARCL1        8404  A0A024RDE1
## ENSG00000179094 6.13966e-56        PER1        5187      O15534
## ENSG00000116584 3.49776e-53     ARHGEF2        9181      Q92974
```

```
## ENSG00000189221 3.46227e-52        MAOA        4128      P21397
## ENSG00000120129 1.59454e-44       DUSP1        1843      B4DU40
## ENSG00000148175 1.83034e-42        STOM        2040      F8VSL7
##                                genename
##                              <character>
## ENSG00000152583           SPARC like 1
## ENSG00000179094 period circadian reg..
## ENSG00000116584 Rho/Rac guanine nucl..
## ENSG00000189221    monoamine oxidase A
## ENSG00000120129 dual specificity pho..
## ENSG00000148175               stomatin
```

Finally, let's write out the ordered significant results with annotations:

```
write.csv(res[ord,], "deseq_results.csv")
```

#Save our results

Write out whole results dataset (including genes that don't change significantly)

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

Setting a cutoff for alpha:

Focus in on those genes with a small p-value (i.e. show a significant change):

```
res05 <- results(dds, alpha = 0.05)
summary(res05)
```

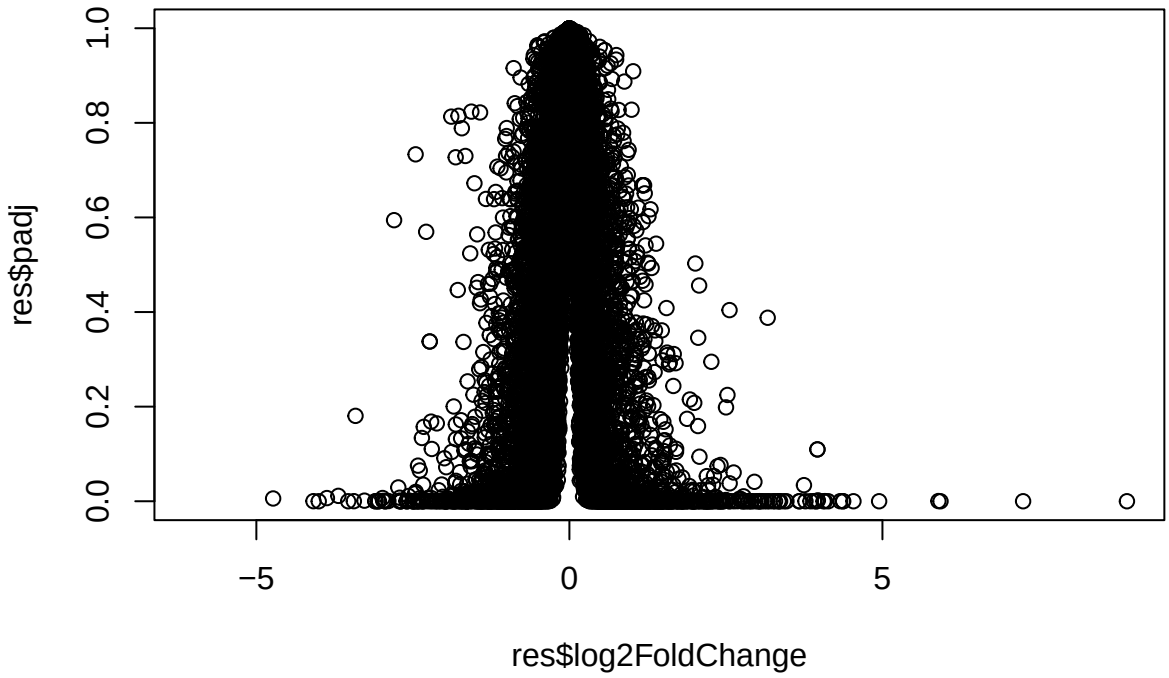
```
##
## out of 25258 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 1236, 4.9%
## LFC < 0 (down)     : 933, 3.7%
## outliers [1]       : 142, 0.56%
## low counts [2]     : 9033, 36%
## (mean count < 6)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

#6. Data Visualization

Volcano Plots

Let's make a commonly produced visualization from this data, namely a so-called Volcano plot. These summary figures are frequently used to highlight the proportion of genes that are both significantly regulated and display a high fold change.

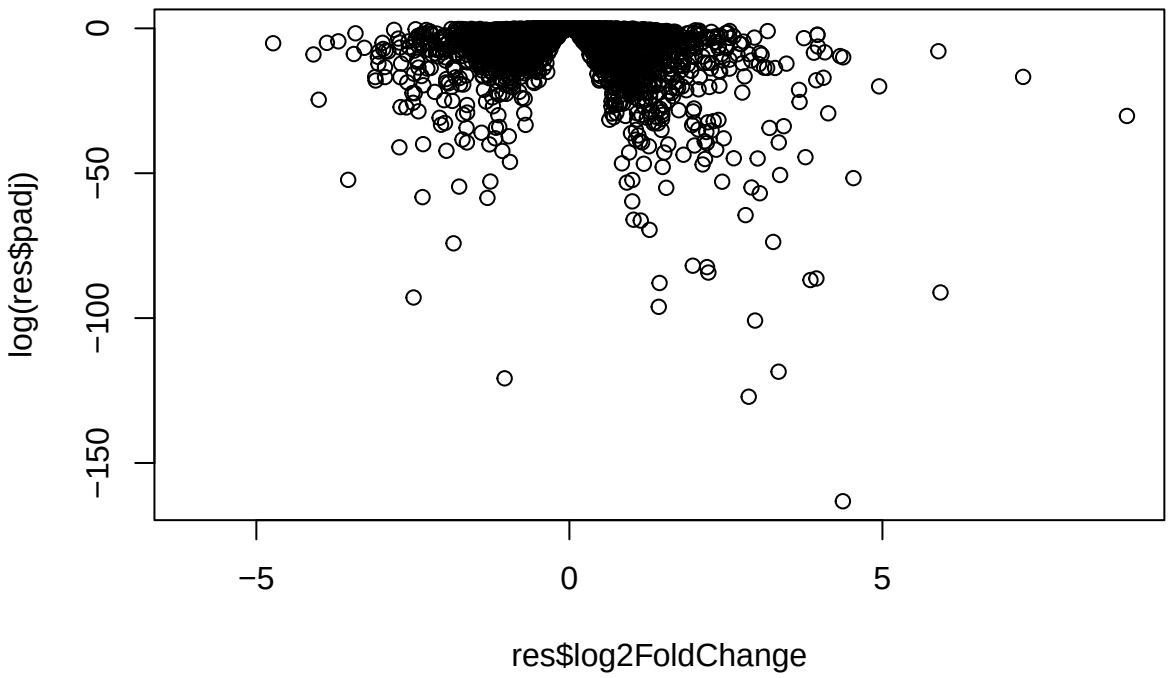
```
plot(res$log2FoldChange, res$padj)
```



This plot isn't too useful as all the small p-values are hidden at the bottom of the plot and we can't really see them. Taking the log will help.

Lets improve it:

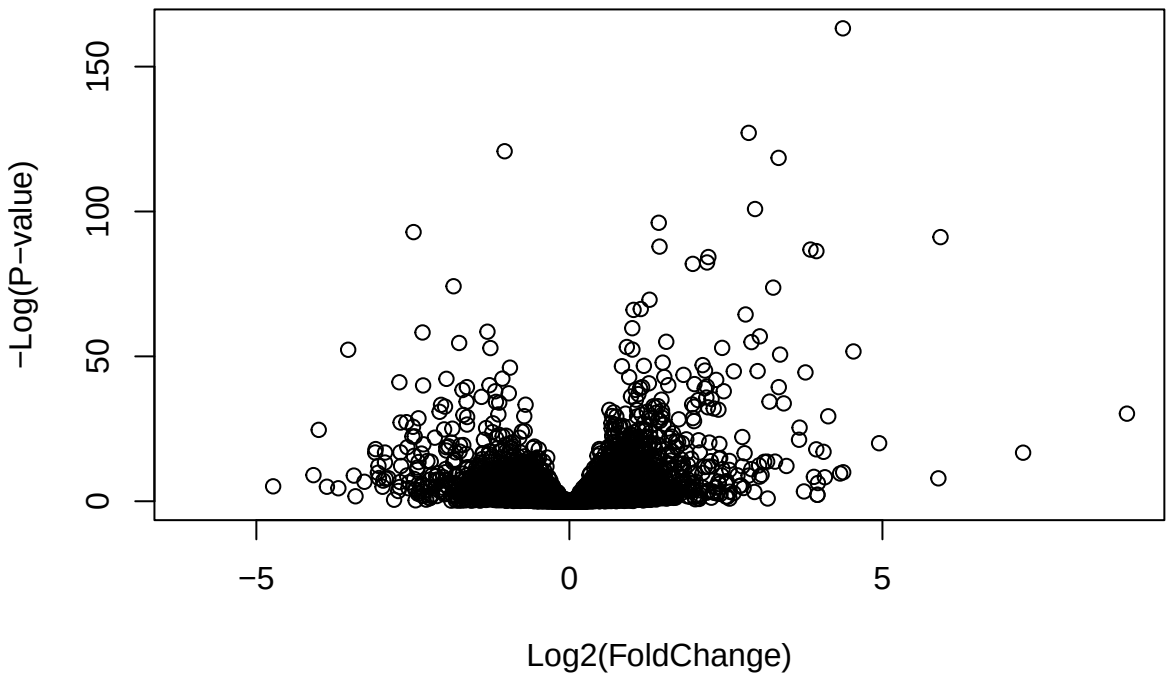
```
plot( res$log2FoldChange,log(res$padj))
```



And some more:

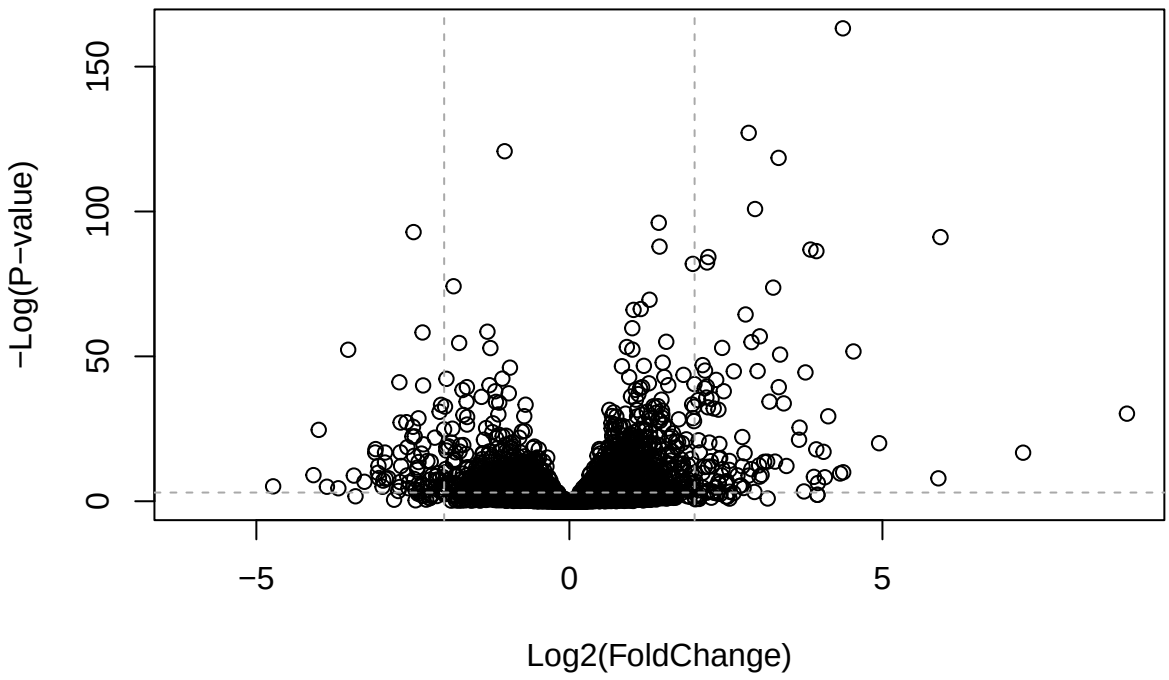
We can flip this p-value axis by adding a minus sign:

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



Now lets make this plot more useful (add some guidelines (with the abline() function) and color (with a custom color vector) highlighting genes that have padj<0.05 and the absolute log2FoldChange>2):

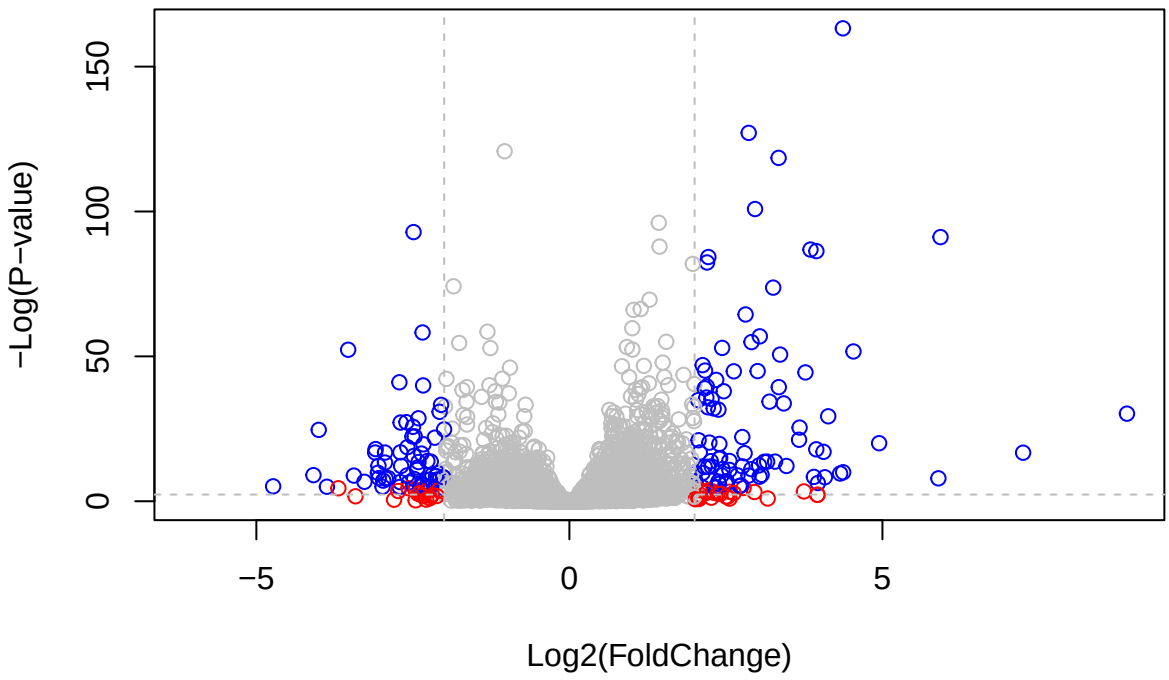
```
plot( res$log2FoldChange,  -log(res$padj),
 ylab="-Log(P-value)", xlab="Log2(FoldChange)")

# Add some cut-off lines
abline(v=c(-2,2), col="darkgray", lty=2)
abline(h=-log(0.05), col="darkgray", lty=2)
```



Finally let's add some color to this plot to draw attention to the genes (i.e. points) we care about - that is those with large fold-change and low p-values (i.e. high -log(p-values))

To color the points we will setup a custom color vector indicating transcripts with large fold change and significant differences between conditions:

```
# 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, ylab="-Log(P-value)", xlab="Log2(FoldChange)" )

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.1), col="gray", lty=2)
```



NEXT CLASS 11/18: For even more customization you might find the EnhancedVolcano bioconductor package useful (Note. It uses ggplot under the hood):

First we will add the more understandable gene symbol names to our full results object res as we will use this to label the most interesting genes in our final plot.

11/18: We've added gene labels. Now let's make another volcano plot with some gene labels

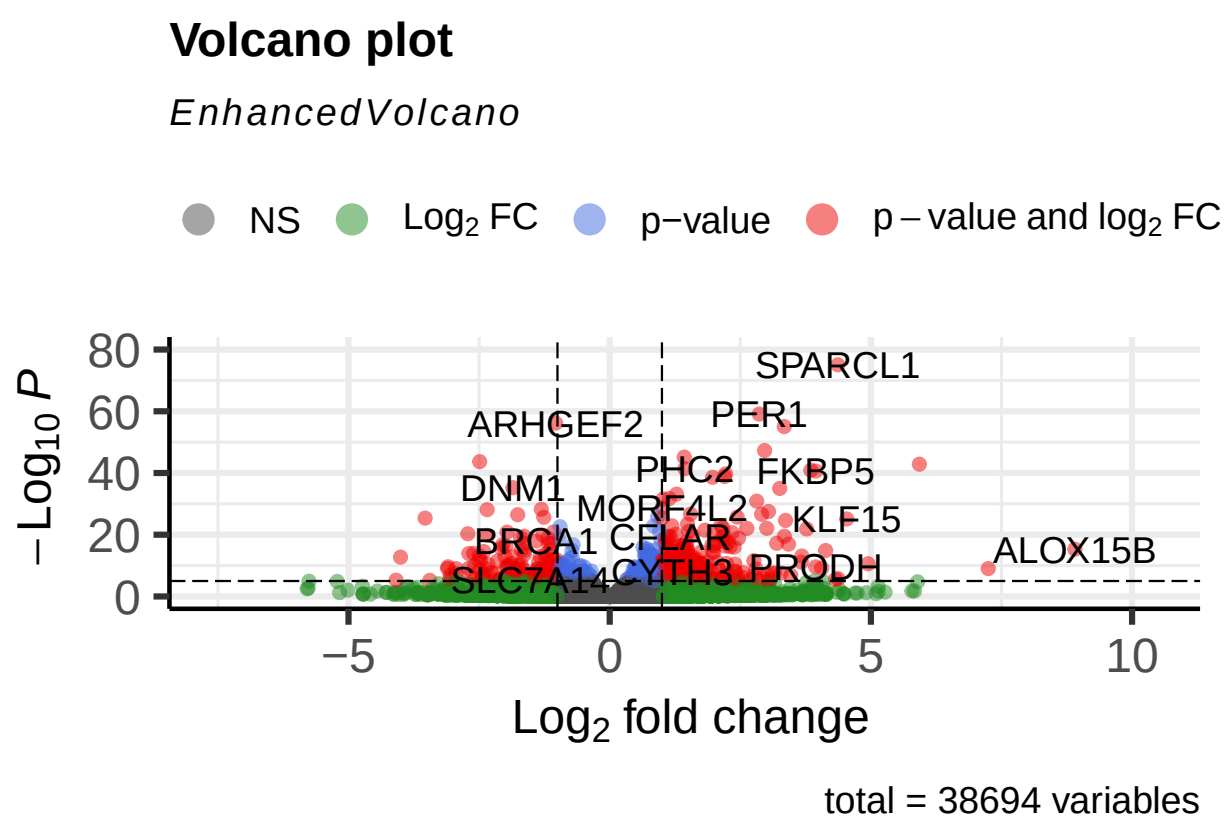
For this we can use the *EnhancedVolcano* package:

```
#BiocManager::install("EnhancedVolcano")

#^ installed but we haven't added gene symbols yet so we'll get back to this next class

library(EnhancedVolcano)
```

```
## Loading required package: ggplot2

## 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
```

Now plot:

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

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



#7. Pathway Analysis

Now we will find out how to derive biological (and hopefully) mechanistic insight from the subset of our most interesting genes highlighted in these types of plots:

Pathway analysis (also known as gene set analysis or over-representation analysis), aims to reduce the complexity of interpreting gene lists via mapping the listed genes to known (i.e. annotated) biological pathways, processes and functions.

Pathway analysis with R and Bioconductor

In this analysis, we check for coordinated differential expression over gene sets from KEGG pathways instead of changes of individual genes. The assumption here is that consistent perturbations over a given pathway (gene set) may suggest mechanistic changes.

Once we have a list of enriched pathways from gage we will use the pathview package to draw pathway diagrams, coloring the molecules in the pathway by their degree of up/down-regulation.

First do a one time install of these required bioconductor packages:

```
# Run in your R console (i.e. not your Rmarkdown doc!)
#BiocManager::install( c("pathview", "gage", "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).
## ##############################################################################
```

```
library(gage)
```

```
##
```

```
library(gageData)

data(kegg.sets.hs)

# Examine the first 2 pathways in this kegg set for humans
head(kegg.sets.hs, 2)
```

```
## $`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"
```

The main **gage()** function requires a named vector of fold changes, where the names of the values are the Entrez gene IDs

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

```
##        7105       64102        8813       57147       55732        2268
## -0.35070302          NA  0.20610777  0.02452695 -0.14714205 -1.73228897
```

Now, let's run the gage pathway analysis.:

```
# Get the results
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

Now lets look at the object returned from gage():

```
attributes(keggres)
```

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

This separates out results by "greater" and "less" i.e. those that are up-regulated and those that are down-regulated.

Lets look at the first few down (less) pathway results:

```
# Look at the first three down (less) pathways
head(keggres$less, 3)
```

```
##                                       p.geomean stat.mean        p.val
## hsa05332 Graft-versus-host disease 0.0004250461 -3.473346 0.0004250461
## hsa04940 Type I diabetes mellitus  0.0017820293 -3.002352 0.0017820293
## hsa05310 Asthma                    0.0020045888 -3.009050 0.0020045888
##                                         q.val set.size         exp1
## hsa05332 Graft-versus-host disease 0.09053483       40 0.0004250461
## hsa04940 Type I diabetes mellitus  0.14232581       42 0.0017820293
## hsa05310 Asthma                    0.14232581       29 0.0020045888
```

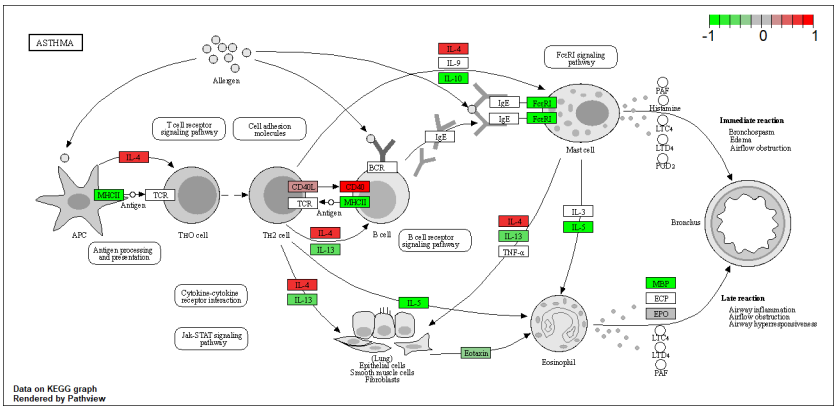
Now, let's try out the pathview() function from the pathview package to make a pathway plot with our RNA-Seq expression results shown in color:

```
pathview(gene.data=foldchanges, pathway.id="hsa05310")
```

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

```
## Info: Working in directory C:/Users/anita/Documents/Bimm143/Week4/bimm143/class15
```

```
## Info: Writing image file hsa05310.pathview.png
```



You can play with the other input arguments to pathview() to change the display in various ways including generating a PDF graph. For example:

```
# A different PDF based output of the same data
pathview(gene.data=foldchanges, pathway.id="hsa05310", kegg.native=FALSE)
```

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

```
## Info: Working in directory C:/Users/anita/Documents/Bimm143/Week4/bimm143/class15
```

```
## Info: Writing image file hsa05310.pathview.pdf
```