class11: RNA-Seq continued

Ari_Fon (PID: A15390446)

2/22/2022

The 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)

##Read Data into R Studio

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

```
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
```

```
head(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
```

I always need to double check that hte columns of my countdata and my coldata (metadata) match.

```
metadata$id
```

```
## [1] "SRR1039508" "SRR1039509" "SRR1039512" "SRR1039513" "SRR1039516"
## [6] "SRR1039517" "SRR1039520" "SRR1039521"
```

```
colnames(counts)
```

```
## [1] "SRR1039508" "SRR1039509" "SRR1039512" "SRR1039513" "SRR1039516"
## [6] "SRR1039517" "SRR1039520" "SRR1039521"
```

```
metadata$id==colnames(counts)
```

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

I can use the `all()` function ot make sure all my values match (i.e. all values are TRUE)

```
all(metadata$id==colnames(counts))
```

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

2 Extract control and treated counts for comparison

First lets extract the control counts columns

```
control.ids <- metadata[metadata$dex == "control", "id"]
control.counts <- counts[,control.ids]
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
```

```
#Take the mean count value per gene (i.e. row)
control.mean <- rowMeans(control.counts)
head(control.mean)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          900.75            0.00          520.50          339.75           97.25
## ENSG00000000938
##            0.75
```

Now do the same thing for "treated" samples.

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

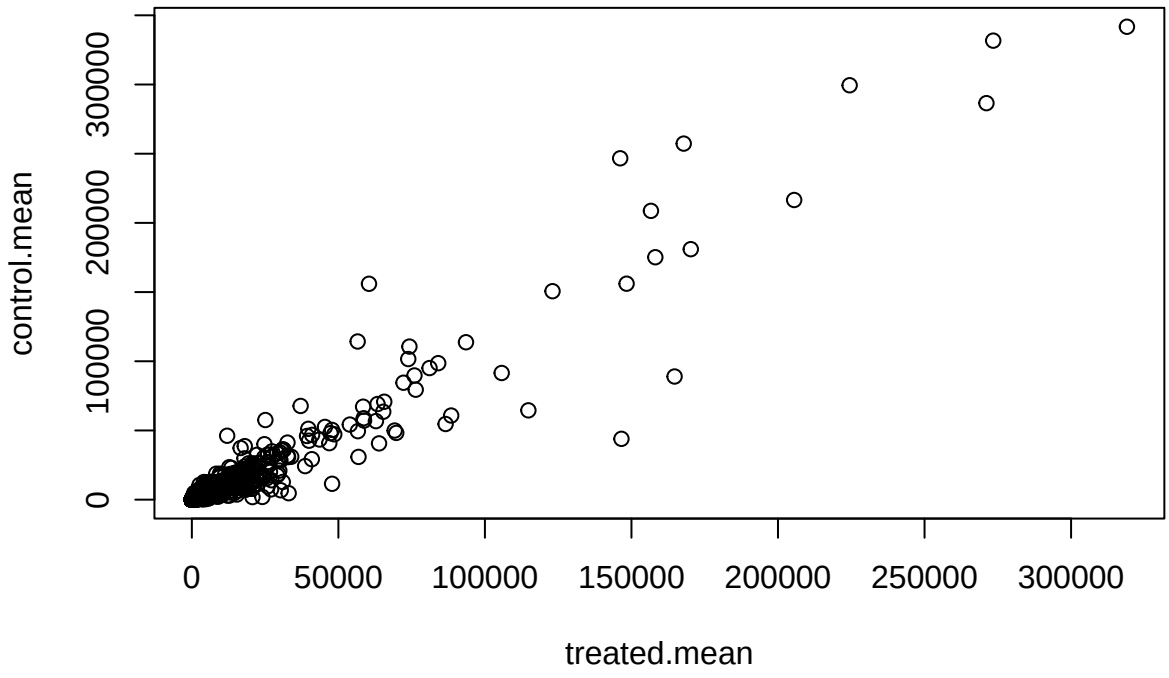
```
##                 SRR1039509 SRR1039513 SRR1039517 SRR1039521
## ENSG00000000003        486        445       1097        604
## ENSG00000000005          0          0          0          0
## ENSG00000000419        523        371        781        509
## ENSG00000000457        258        237    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

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

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          658.00            0.00          546.00          316.50           78.75
## ENSG00000000938
##            0.00
```

Now we can make plot comparing treated vs control

```
plot(treated.mean, control.mean)
```

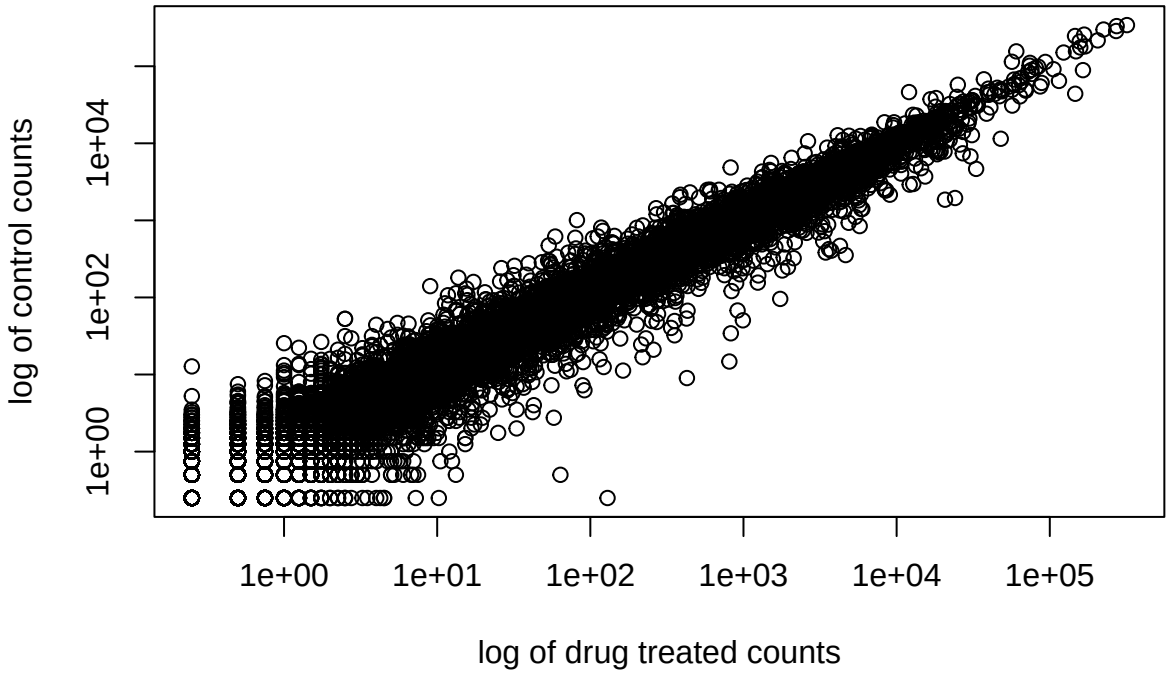


When we see data that is so skewed like this over quite a wide range of values we start to think of log transformations to make our analysis easier.

```
plot(treated.mean, control.mean, log="xy",
     xlab= "log of drug treated counts",
     ylab= "log of control counts")
```

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

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



We are after changes in gene expression: treated vs control and this would represent points (i.e. genes) that do not like on the diagonal.

We like to work with log 2 values

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

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

```
log2(40/20)
```

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

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

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

```
log2(80/20)
```

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

Now lets calculate the log2 fold change

```
log2fc <- log2(treated.mean/control.mean)
```

Store my work so far

```
meancounts <- data.frame(control.mean, treated.mean, log2fc)
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
```

Filter our data to remove genes with zero expression values

```
c(10,20,0,40) == 0
```

```
## [1] FALSE FALSE  TRUE FALSE
```

```
which(c(10,20,0,40) == 0)
```

```
## [1] 3
```

```
z <- data.frame(x=c(10,0,30,40),
           y=c(10,0,30, 0))
z
```

```
##    x  y
## 1 10 10
## 2  0  0
## 3 30 30
## 4 40  0
```

```
z==0
```

```
##          x     y
## [1,] FALSE FALSE
## [2,]  TRUE  TRUE
## [3,] FALSE FALSE
## [4,] FALSE  TRUE
```

```
which(z==0)
```

```
## [1] 2 6 8
```

```
which(z==0, arr.ind=TRUE)
```

```
##      row col
## [1,]   2   1
## [2,]   2   2
## [3,]   4   2
```

```
i <- which(z==0, arr.ind=TRUE)
unique(i[,1])
```

```
## [1] 2 4
```

Now do it for our real dataset

```
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
```

How many genes do we have left?

```
nrow(mycounts)
```

```
## [1] 21817
```

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" genes...

```
sum(mycounts$log2fc > 2)
```

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

"Down" genes...

```
sum(mycounts$log2fc < -2)
```

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

We are missing the stats! Are these differences significant?

DESeq2 analysis

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

```
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

## 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
```

This package wants input in a specific way:

```
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
```

Now we can run the DESeq2 analysis

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

```
## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing
```

To get the results back in a usefulw ay, we can use the `results()` function

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

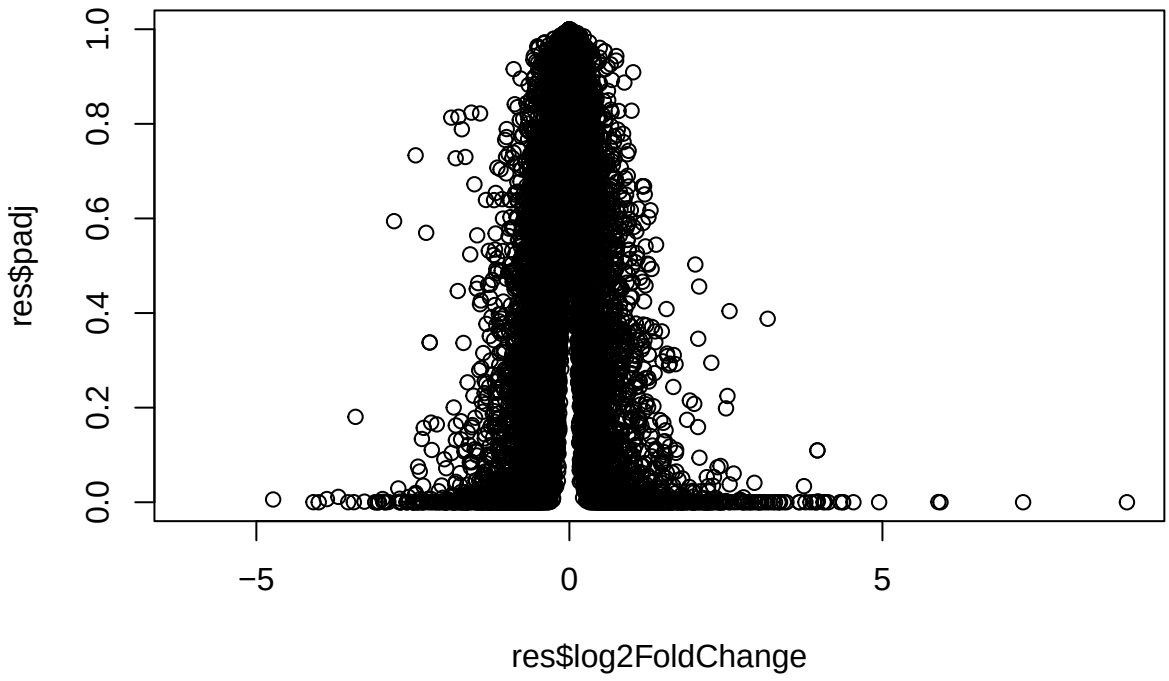
```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 38694 rows and 6 columns
##                  baseMean log2FoldChange     lfcSE      stat    pvalue
##                 <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  747.1942     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005    0.0000             NA        NA        NA        NA
## ENSG00000000419  520.1342      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457  322.6648      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460   87.6826     -0.1471420  0.257007 -0.572521 0.5669691
## ...                   ...            ...       ...       ...       ...
## ENSG00000283115  0.000000             NA        NA        NA        NA
```

```
## ENSG00000283116  0.000000             NA        NA        NA        NA
## ENSG00000283119  0.000000             NA        NA        NA        NA
## ENSG00000283120  0.974916      -0.668258   1.69456 -0.394354  0.693319
## ENSG00000283123  0.000000             NA        NA        NA        NA
##                      padj
##                 <numeric>
## ENSG00000000003  0.163035
## ENSG00000000005        NA
## ENSG00000000419  0.176032
## ENSG00000000457  0.961694
## ENSG00000000460  0.815849
## ...                   ...
## ENSG00000283115        NA
## ENSG00000283116        NA
## ENSG00000283119        NA
## ENSG00000283120        NA
## ENSG00000283123        NA
```

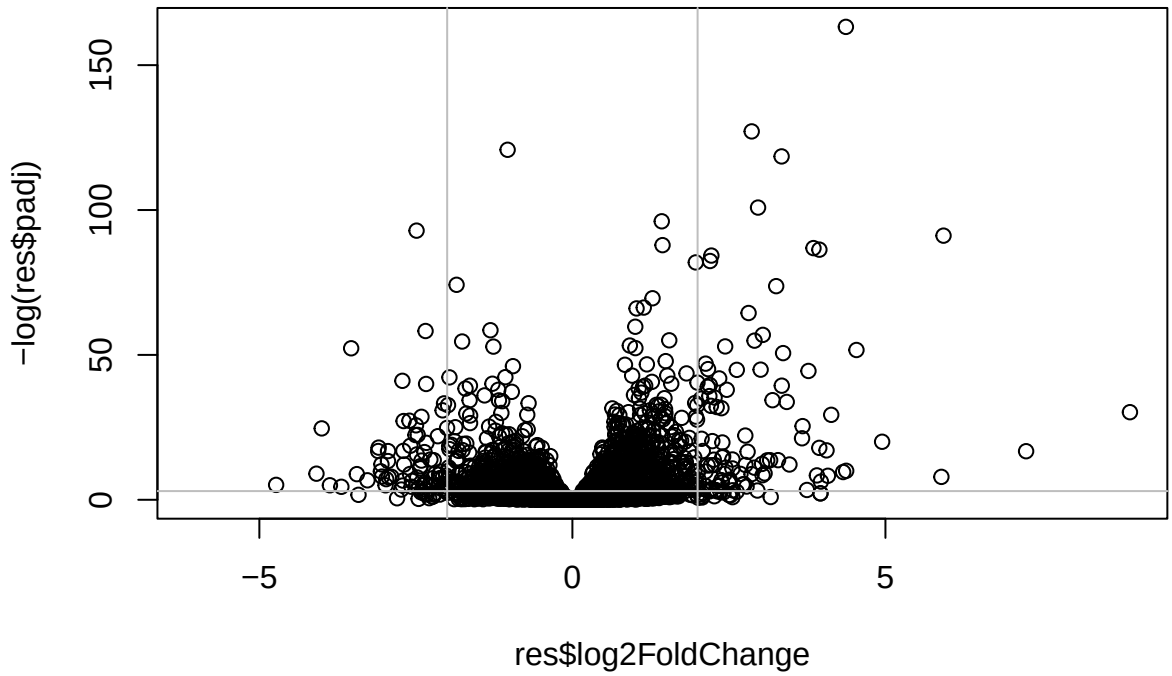
Volcano plots

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)
```



```
plot(res$log2FoldChange, -log(res$padj))
abline(h=-log(0.05), col="gray")
abline(v=c(-2,2), col="gray")
```

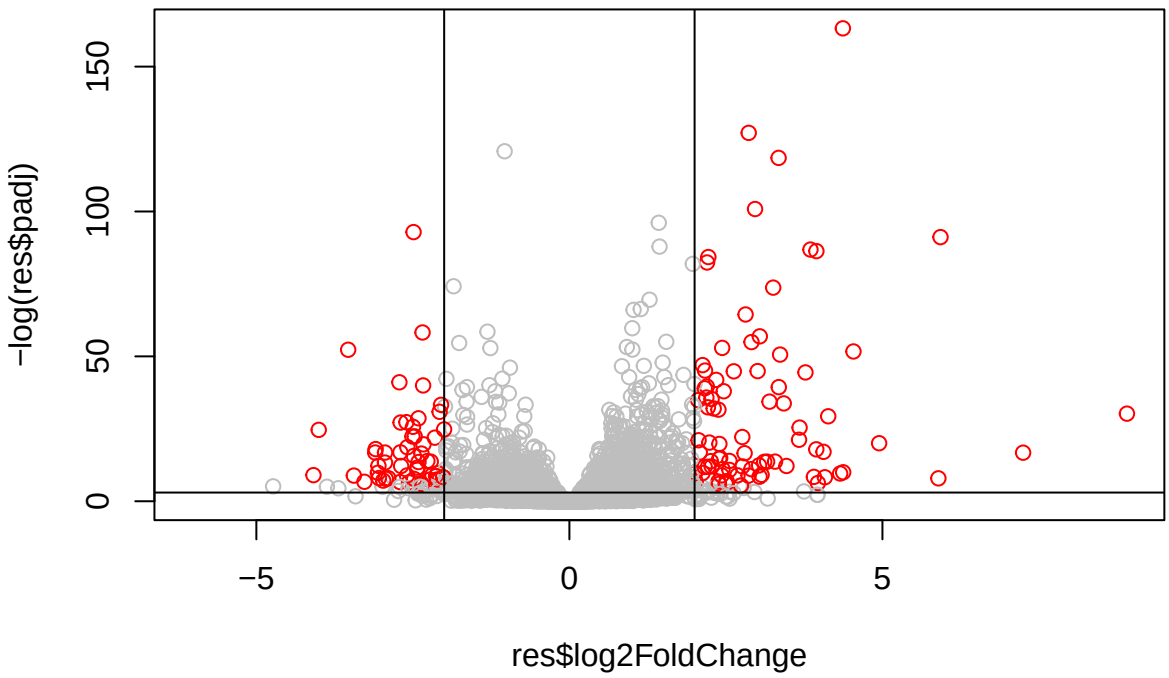


I want to polish this main results figure by adding color to the genes I will focus on next day

```
#I will start by making a gray vector for everything
mycols <- rep("gray", nrow(res))

#Now I will overwrite the small pasj values
mycols[res$padj < 0.005] <- "red"

#Now if my log2foldchange is small I will make them gray
mycols[ abs(res$log2FoldChange) < 2] <- "gray"
plot(res$log2FoldChange,-log(res$padj), col=mycols)
abline(h=-log(0.05))
abline(v=c(-2,2))
```



Adding annotation data

To help interpret our results we need to understand what the differentially expressed genes are. A first step here is to get the gene names (i.e. gene SYMBOLs).

For this I will install: - BiocManager::install("AnnotationDbi") - BiocManager::install("org.Hs.eg.db")

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

```
##
```

What DB identifiers can I look up?

```
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 will use the `mapIds()` function to translate between different ids.

```
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$symbol)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##        "TSPAN6"          "TNMD"          "DPM1"         "SCYL3"      "C1orf112"
## ENSG00000000938
##           "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$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 9 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              genename
```

```
##                 <numeric> <character> <character>           <character>
## ENSG00000000003  0.163035      TSPAN6        7105          tetraspanin 6
## ENSG00000000005        NA        TNMD       64102            tenomodulin
## ENSG00000000419  0.176032        DPM1        8813 dolichyl-phosphate m..
## ENSG00000000457  0.961694       SCYL3       57147 SCY1 like pseudokina..
## ENSG00000000460  0.815849    C1orf112       55732 chromosome 1 open re..
## ENSG00000000938        NA         FGR        2268 FGR proto-oncogene, ..
```

Patway analysis with R and Bioconductor

Here we use the GAGE package (which stands for generally Applicable Gene set Enrichment), to do KEGG pathway enrichment analysis on our RNA-Seq based differential expression results.

I need to install the gage package along with the pathview package for generating pathway figures from my results.

Now load up the packages and have a peak at the first two pathways in KEGG.

```
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"
```

Recall that vectors can have a names attribute that helps with bookkeeping just like colnames and rownames.

```
x <- c(40,70,20)
names(x) <- c("lisa", "xinqiu", "barry")
x
```

```
##   lisa xinqiu  barry
##     40     70     20
```

We need a vector of fold-change labeled with the names of our genes in ENTREZ format.

```
foldchange <- res$log2FoldChange
names(foldchange) <- res$entrez
```

Now we can run the GAGE analysis passing in our foldchange vector and the KEGG genesets we are interested in.

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

Lets have a look at what is contained in this `keggres` results object (i.e its attributes)

```
attributes(keggres)
```

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

```
# 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
```

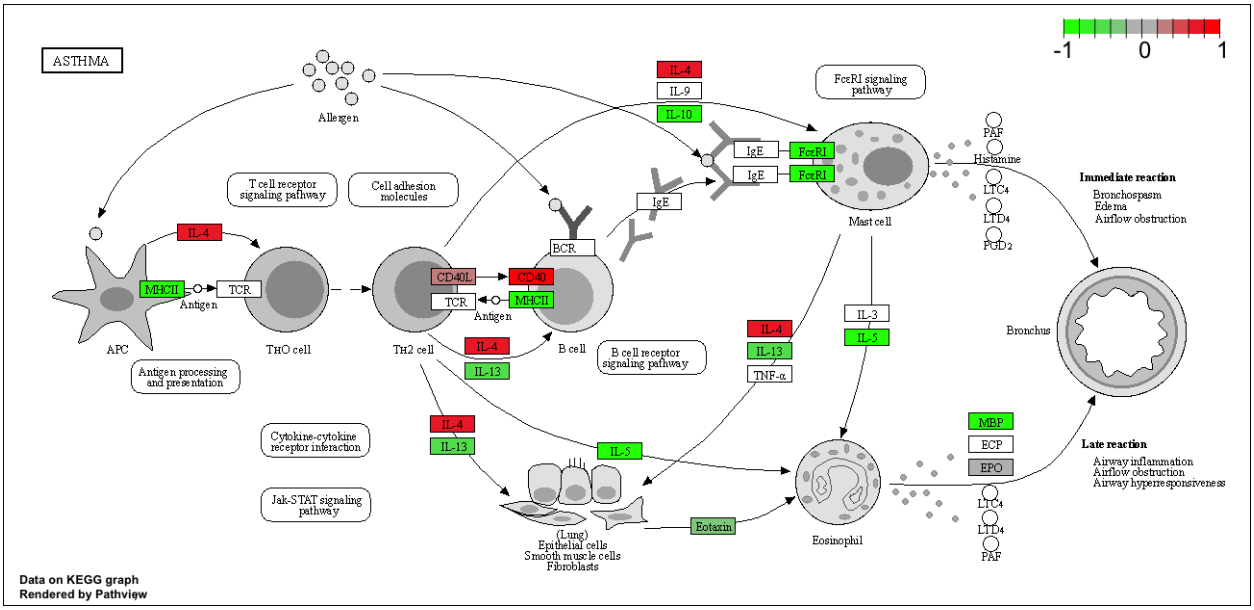
Now I can map my foldchange results onto any KEGG pathway. I will do this manually first by selecting one of the pathway IDS from above

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

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

```
## Info: Working in directory /Users/Ari_Fon/Desktop/BIMM143 /class11
```

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



Final step is to save our results.

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