Class 12: RNASeq Analysis

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Here we will use the DESeq2 package for RNASeq analysis. The data for today's class 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 their data

We need two things for this analysis:

-**countData** (counts for everyy transcription/gene in each experiment) -**colData** (metadata that describes the expiremental setup)

```
countData <- read.csv("airway_scaledcounts.csv", row.names = 1)
head(countData)
```

	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 <- read.csv("airway_metadata.csv")
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(countData)
```

```
[1] 38694
```

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

```
table(metaData$dex)
```

```
control treated
      4       4
```

Another way

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

```
[1] 4
```

- Step 1. Calculate the mean of the control samples (i.e columns in `countData`)

(a) We need to find which columns in `countData` are "control" samples.

- look in the metadata (a.k.a. colData), $dex column

```
control.inds <- metaData$dex == "control"
```

(b) Extract all the control columns from `contData` and call it `control.counts`

```
control.counts <- countData[ , control.inds]
```

(c) Calculate the mean value across the rows of `control.counts` i.e. calculate the mean count values for each gene in the control samples.

```
control.means <- rowMeans(control.counts)
head(control.means)
```

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

- Step 2. Calculate the mean of the treated samples...

```
#(a) We need to find which columns in `countData` are "treated" samples.
#  - look in the metadata (a.k.a. colData), $dex column
treated.inds <- metaData$dex == "treated"
#(b) Extract all the treated columns from `contData` and call it `treated.counts`
treated.counts <- countData[ , treated.inds]
#(c) Calculate the mean value across the rows of `treated.counts` i.e. calculate the mean
treated.means <- rowMeans(treated.counts)
head(treated.means)
```

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

We now move control and treated mean count values. For ease or book-keeping I will combine our `meancount` data

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

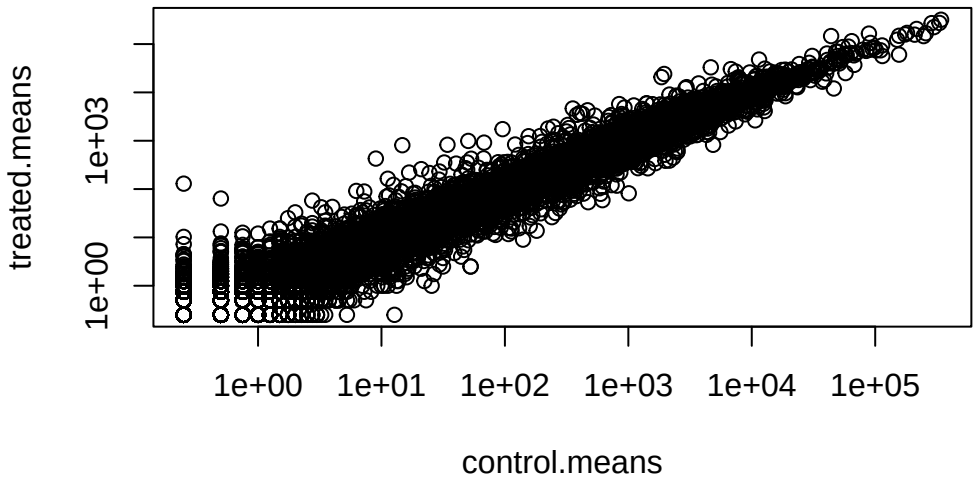
```
                control.means treated.means
ENSG00000000003        900.75        658.00
ENSG00000000005          0.00          0.00
ENSG00000000419        520.50        546.00
ENSG00000000457        339.75        316.50
```

```
ENSG00000000460          97.25          78.75
ENSG00000000938           0.75           0.00
```

```
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 use log transforms for skewed data such as this and because we really care most about relative changes in magnitude.

We must often use log2 as our transform as the math is easier to interpert than log10 or others.

If we have no change -i.e. some values in control and treated we will have a log2 value of zero

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

```
[1] 0
```

If I have double the amount i.e. 20 compared to 0 for example I will have a log2 fold-change of +1

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

```
[1] 1
```

If I have half the amount I will have a log2 fold-change of -1

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

```
[1] -1
```

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

```
[1] 2
```

```
meancounts$log2fc <- log2(meancounts$treated.means/meancounts$control.means)
head(meancounts)
```

```
                control.means treated.means      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
```

Q. How many genes are up regulated at the commin threshold of +2 log2FC values?

```
sum(meancounts$log2fc >= 2, na.rm=T)
```

```
[1] 1910
```

Hold on what about the stats! Yes these are big changes but are these changes significant!!

To do this properly we will turn to DESeq2 package.

DESeq2 analysis

```
#/ message:false
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, aperm, 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
```

To use DESeq we need our input contData and colData in a specific format that DESeq wants:

```
dds <- DESeqDataSetFromMatrix(countData = countData,
                              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
```

To run the analysis I can now use the main DESeq2 function called `DESeq()` with `dds` as input.

```
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 out of this `dds` object we can use `results()` function from the package.

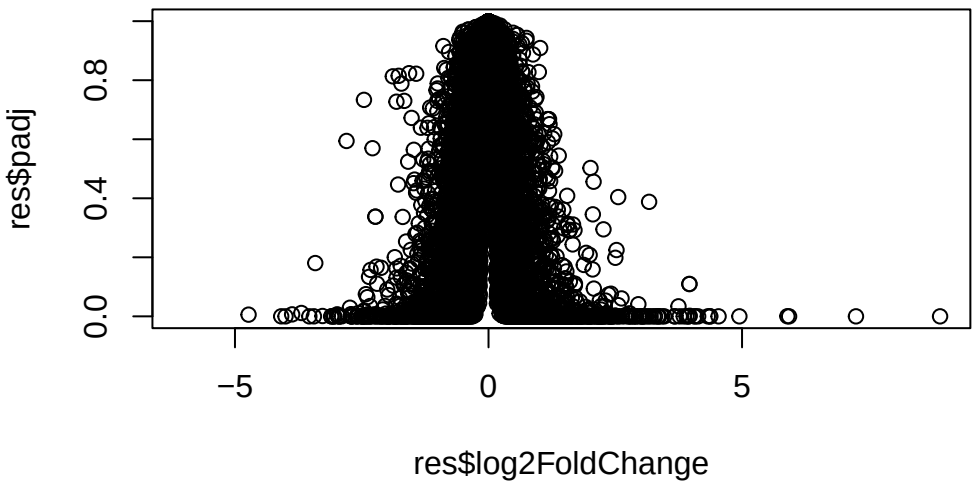
```
res <- results(dds)
head(res)
```

```
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
```

Volcano plot

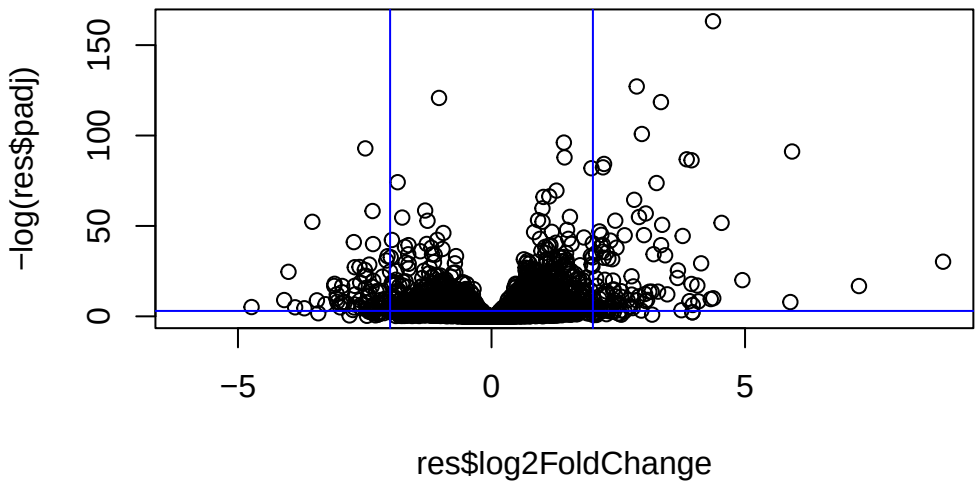
Let's make a final (for today) plot of log2 fold-change vs the adjusted P-value.

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



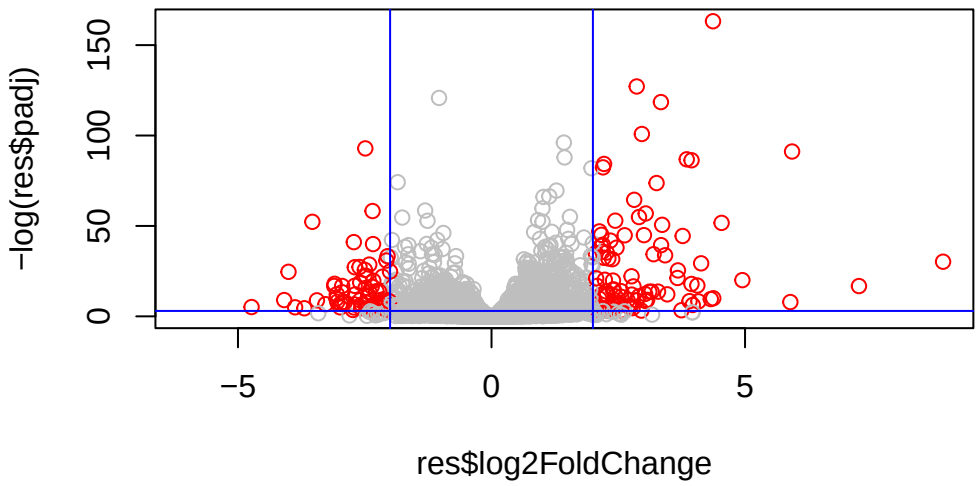
It is the low P-value that we care about and these are lost in the skewed plot above. Let's take the log of the $padj values for our plot

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



Finally we can make a color vector to use in the plot to be better highlight the genes we care about.

```
mycols <- rep("gray", nrow(res))
mycols[abs(res$log2FoldChange) >= 2 ]<- "red"
mycols[res$padj > 0.05 ] <- "gray"
plot(res$log2FoldChange, -log(res$padj), col=mycols)
abline(v=c(+2,-2), col="blue")
abline(h=-log(0.05), col="blue")
```




```
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
```

Adding annotation data

We can use AnnotationDbi package to add annotation data such as gene identifiers from different sources to our results object.

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

We can translate (a.k.a. "map") between all these database id formats:

```
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"
```

```
head(rownames(res))
```

```
[1] "ENSG00000000003" "ENSG00000000005" "ENSG00000000419" "ENSG00000000457"
[5] "ENSG00000000460" "ENSG00000000938"
```

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

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

My IDs are stored as the rownames of `res`

```
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
```

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

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

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

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

```
res$genename <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our gene names
                     keytype="ENSEMBL",  # The format of our gene names
```

```
                     column="GENENAME",    # 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 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, ..
```

Save our results to date

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

Pathway analysis

We can use the KEGG database of biological pathways to get some more insight into our differentially expressed genes and the kinds of biology they are involved in.

```
#/message: false
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"
```

```
head(res$entrez)
```

```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         "7105"          "64102"           "8813"          "57147"          "55732"
ENSG00000000938
         "2268"
```

Make a new vector of fold-change values that I will use as an input for gage this will have the ENTREZ IDs as names.

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

```
x <-1:3
x
```

```
[1] 1 2 3
```

```
names(x) <-c("chandra","lisa","xinqiu")
x
```

```
chandra    lisa  xinqiu
      1       2       3
```

```
head(foldchanges)
```

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

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

```
attributes(keggres)
```

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

Look at top 3 “LESS”

```
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 use the **KEGG IDs** (“hsa05310”,etc.) of these pathways from gage to view our genes mapped to these pathways.

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

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

```
Info: Working in directory /Users/moisesg/Desktop/BIMM 143/class12
```

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

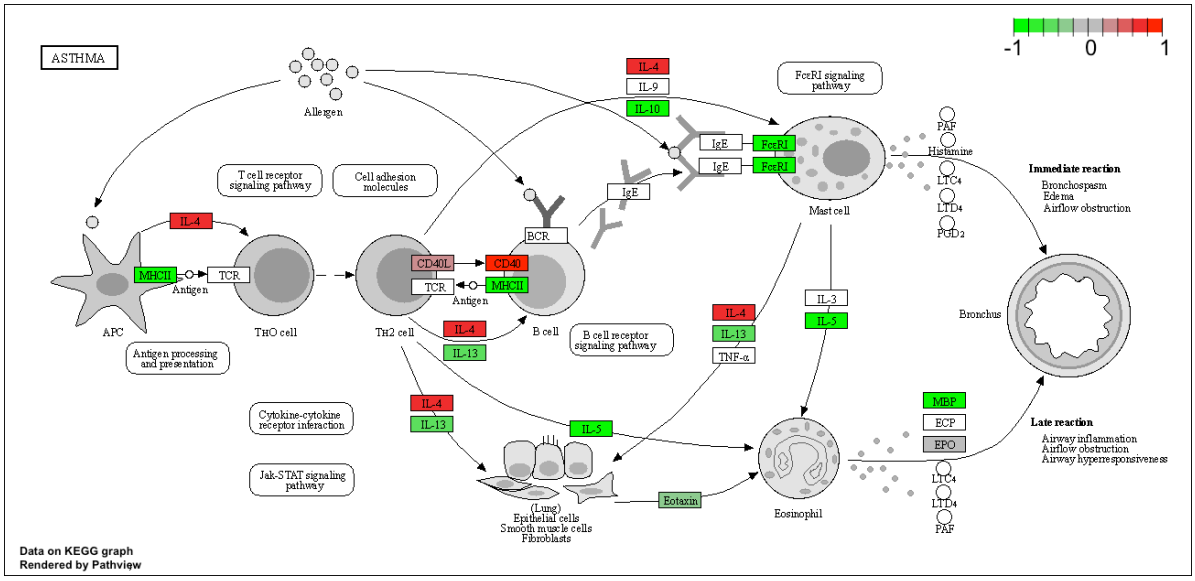


Figure 1: Asthma pathway from KEGG with our genes shown in color