class14

Amit Subramanian

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Running Code

When you click the **Render** button a document will be generated that includes both content and the output of embedded code. You can embed code like this:

```
1 + 1
```

```
[1] 2
```

You can add options to executable code like this

```
[1] 4
```

The `echo: false` option disables the printing of code (only output is displayed).

Section 1. Differential Expression Analysis

```
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, table,
    tapply, union, unique, unsplit, which.max, which.min



Attaching package: 'S4Vectors'


The following object is masked from 'package:utils':

    findMatches


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
```

Let's load in our data 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 troublesome first column from countData.

```
# Removing the odd first $length col
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 (i.e. rows) where we have 0 read count across all samples (i.e. columns).

```
# Filter count data to have 0 read count across all samples
countData <- countData[-c(1,2,4,5),]
head(countData)
```

```
                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

Nice now lets setup the DESeqDataSet object required for the DESeq() function and then run the DESeq pipeline.

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

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

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

```
estimating size factors

estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

```r
dds
```

```
class: DESeqDataSet
dim: 19804 6
metadata(1): version
assays(4): counts mu H cooks
rownames(19804): ENSG00000279457 ENSG00000187634 ... ENSG00000277475
  ENSG00000268674
rowData names(22): baseMean baseVar ... deviance maxCooks
colnames(6): SRR493366 SRR493367 ... SRR493370 SRR493371
colData names(2): condition sizeFactor
```

Next, get results for the HoxA1 knockdown versus control siRNA.

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

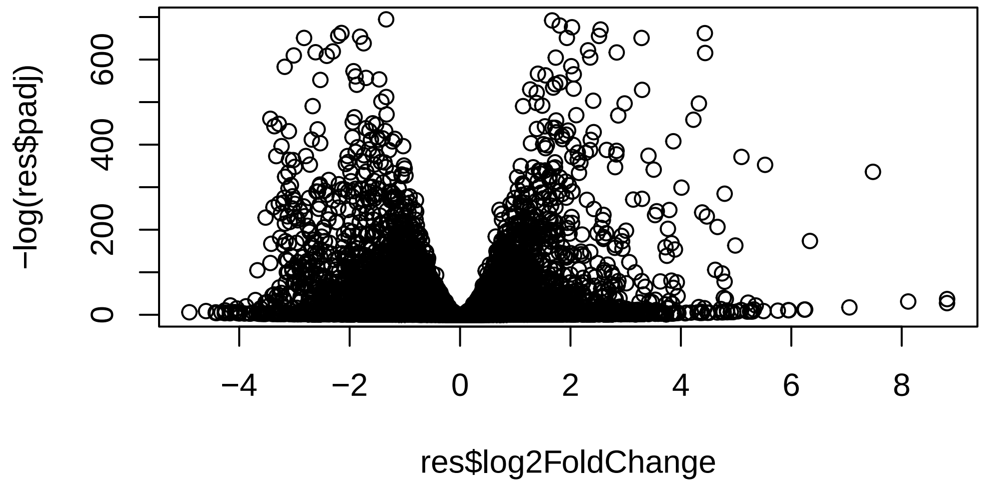
> Q3. Call the summary() function on your results to get a sense of how many genes are up or down-regulated at the default 0.1 p-value cutoff.

```r
summary(res)
```

```
out of 15975 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 4349, 27%
LFC < 0 (down)     : 4393, 27%
outliers [1]       : 0, 0%
low counts [2]     : 1221, 7.6%
(mean count < 0)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

Volcano Plot

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



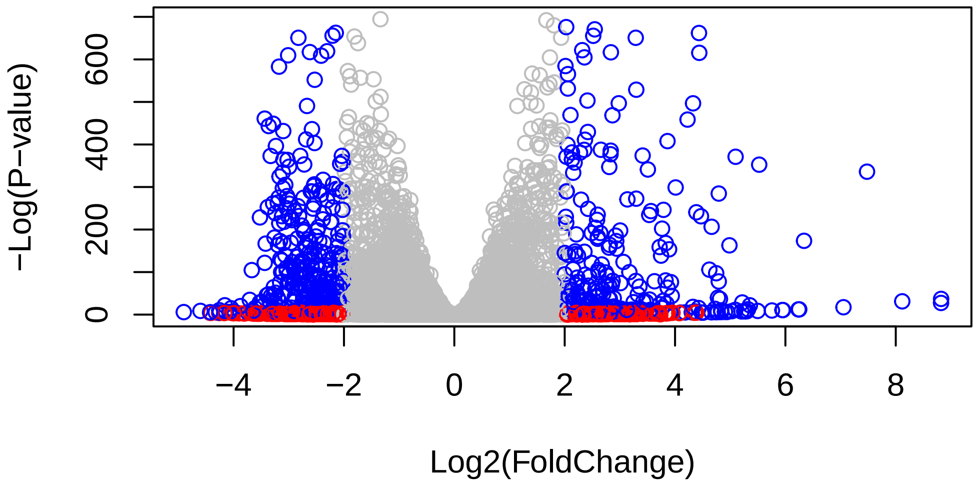
Q4. Improve this plot by completing the below code, which adds color and axis labels.

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

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

# Color 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)" )
```



Adding gene annotation

Since we mapped and counted against the Ensembl annotation, our results only have information about Ensembl gene IDs. However, our pathway analysis downstream will use KEGG pathways, and genes in KEGG pathways are annotated with Entrez gene IDs. So lets add them as we did the last day.

> Q5. Use the mapIDs() function multiple times to add SYMBOL, ENTREZID and GENENAME annotation to our results by completing the code below

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

```
# Checking the column names
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
```

```r
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
```

```r
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 1.43989e-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 0.5215599   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 6.87080e-01          NA          NA                     NA
ENSG00000187634 5.16278e-03      SAMD11      148398 sterile alpha motif ..
ENSG00000188976 1.76740e-35       NOC2L       26155 NOC2 like nucleolar ..
ENSG00000187961 1.13536e-07      KLHL17      339451 kelch like family me..
ENSG00000187583 9.18988e-01     PLEKHN1       84069 pleckstrin homology ..
ENSG00000187642 4.03817e-01       PERM1       84808 PPARGC1 and ESRR ind..
ENSG00000188290 1.30680e-24        HES4       57801 hes family bHLH tran..
ENSG00000187608 2.37710e-02       ISG15        9636 ISG15 ubiquitin like..
ENSG00000188157 4.22421e-16        AGRN      375790                  agrin
ENSG00000237330          NA      RNF223      401934 ring finger protein ..
```

Q6. Finally for this section let's reorder these results by adjusted p-value and save them to a CSV file in your current project directory.

```r
res <- res[order(res$pvalue),]
write.csv(res, file = "deseq_results.csv")
```

Section 2. Pathway Analysis

Here we are going to use the gage package for pathway analysis. Once we have a list of enriched pathways, we're going to use the pathview package to draw pathway diagrams, shading the molecules in the pathway by their degree of up/down-regulation.

KEGG pathways

Installing the required bioconductor packages:

```r
# BiocManager::install( c("pathview", "gage", "gageData") )
```

Now we can load the packages and setup the KEGG data-sets we need.

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

```r
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"
```

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

```
     1266     54855      1465     51232      2034      2317
-2.422719  3.201955 -2.313738 -2.059631 -1.888019 -1.649792
```

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"
```

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

```
head(keggres$less)
```

```
                                         p.geomean stat.mean        p.val
hsa04110 Cell cycle                   7.077982e-06 -4.432593 7.077982e-06
hsa03030 DNA replication              9.424076e-05 -3.951803 9.424076e-05
hsa03013 RNA transport                1.160132e-03 -3.080629 1.160132e-03
hsa04114 Oocyte meiosis               2.563806e-03 -2.827297 2.563806e-03
hsa03440 Homologous recombination     3.066756e-03 -2.852899 3.066756e-03
hsa00010 Glycolysis / Gluconeogenesis 4.360092e-03 -2.663825 4.360092e-03
                                            q.val set.size         exp1
hsa04110 Cell cycle                   0.001160789      124 7.077982e-06
hsa03030 DNA replication              0.007727742       36 9.424076e-05
hsa03013 RNA transport                0.063420543      149 1.160132e-03
hsa04114 Oocyte meiosis               0.100589607      112 2.563806e-03
hsa03440 Homologous recombination     0.100589607       28 3.066756e-03
hsa00010 Glycolysis / Gluconeogenesis 0.119175854       65 4.360092e-03
```

Let's manually supply a pathway.id that we could see from the print out above.

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

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

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

We can change the display in various ways including generating a PDF graph.

```r
# A different PDF based output of the same data
pathview(gene.data=foldchanges, pathway.id="hsa04110", kegg.native=FALSE)
```

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

Warning: reconcile groups sharing member nodes!

     [,1] [,2]
[1,] "9"  "300"
[2,] "9"  "306"

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

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

Now, let's process our results a bit more to automagicaly pull out the top 5 upregulated pathways, then further process that just to get the pathway IDs needed by the pathview() function.

```r
## Focus on top 5 upregulated pathways here for demo purposes only
keggrespathways <- rownames(keggres$greater)[1:5]

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

```
[1] "hsa04740" "hsa04640" "hsa00140" "hsa04630" "hsa04976"
```

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

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

Info: Writing image file hsa04740.pathview.png

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

Info: Writing image file hsa04640.pathview.png

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

Info: Writing image file hsa00140.pathview.png

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

Info: Writing image file hsa04630.pathview.png

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

Info: Working in directory /Users/amit_sub/Documents/BIMM 143/class14

Info: Writing image file hsa04976.pathview.png
```

> Q7. Can you do the same procedure as above to plot the pathview figures for the top 5 down-reguled pathways?

```
# Focus on top 5 upregulated pathways here for demo purposes only
keggrespathways_down <- rownames(keggres$less)[1:5]

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

```
[1] "hsa04740" "hsa04640" "hsa00140" "hsa04630" "hsa04976"
```

Section 3. Gene Ontology (GO)

Let's focus on BP (a.k.a Biological Process) here.

```
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       1.734864e-05  4.210777 1.734864e-05
GO:0048729 tissue morphogenesis           5.407952e-05  3.888470 5.407952e-05
GO:0002009 morphogenesis of an epithelium 5.727599e-05  3.878706 5.727599e-05
GO:0030855 epithelial cell differentiation 2.053700e-04  3.554776 2.053700e-04
GO:0060562 epithelial tube morphogenesis  2.927804e-04  3.458463 2.927804e-04
GO:0048598 embryonic morphogenesis        2.959270e-04  3.446527 2.959270e-04
                                               q.val set.size         exp1
GO:0007156 homophilic cell adhesion       0.07584825      137 1.734864e-05
GO:0048729 tissue morphogenesis           0.08347021      483 5.407952e-05
GO:0002009 morphogenesis of an epithelium 0.08347021      382 5.727599e-05
GO:0030855 epithelial cell differentiation 0.16449701      299 2.053700e-04
GO:0060562 epithelial tube morphogenesis  0.16449701      289 2.927804e-04
GO:0048598 embryonic morphogenesis        0.16449701      498 2.959270e-04

$less
```

```
                                             p.geomean stat.mean        p.val
GO:0048285 organelle fission              6.626774e-16 -8.170439 6.626774e-16
GO:0000280 nuclear division               1.797050e-15 -8.051200 1.797050e-15
GO:0007067 mitosis                        1.797050e-15 -8.051200 1.797050e-15
GO:0000087 M phase of mitotic cell cycle 4.757263e-15 -7.915080 4.757263e-15
GO:0007059 chromosome segregation         1.081862e-11 -6.974546 1.081862e-11
GO:0051301 cell division                  8.718528e-11 -6.455491 8.718528e-11
                                                 q.val set.size         exp1
GO:0048285 organelle fission              2.618901e-12      386 6.626774e-16
GO:0000280 nuclear division               2.618901e-12      362 1.797050e-15
GO:0007067 mitosis                        2.618901e-12      362 1.797050e-15
GO:0000087 M phase of mitotic cell cycle 5.199689e-12      373 4.757263e-15
GO:0007059 chromosome segregation         9.459800e-09      146 1.081862e-11
GO:0051301 cell division                  6.352901e-08      479 8.718528e-11

$stats
                                           stat.mean     exp1
GO:0007156 homophilic cell adhesion         4.210777 4.210777
GO:0048729 tissue morphogenesis             3.888470 3.888470
GO:0002009 morphogenesis of an epithelium   3.878706 3.878706
GO:0030855 epithelial cell differentiation  3.554776 3.554776
GO:0060562 epithelial tube morphogenesis    3.458463 3.458463
GO:0048598 embryonic morphogenesis          3.446527 3.446527
```

Section 4. Reactome Analysis

```
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: 8146"
```

```
write.table(sig_genes, file="significant_genes.txt", row.names=FALSE, col.names=FALSE, quote=
```

> 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?

RHOBTB2 GTPase cycle has the most significant or least entities p-value of 1.83E-1.

There are some similarities such as cell cycle and gene expression.

The most significant pathways from Reactome are the signal transduction, a pathway from disease, one from gene expression, and from the cell cycle.

Reactome is not very reliable or accurate since it is just a starting point when it comes to analyzing these pathways.

The differences could be caused by experimental inaccuracies or mistakes within the data file.