

Class 14: Pathway Analysis from RNA-Seq

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Here we run through a complete RNASeq analysis from counts to pathways and biology insight...

Data Import

```
#!/ 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 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
```

```
#!/ message: FALSE  
metaFile <- "GSE37704_metadata.csv"  
countFile <- "GSE37704_featurecounts.csv"  
  
# Import metadata and take a peak  
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					

```
# Note we need to remove 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

```
# Filter count data where you have 0 read count across all samples.
to.keep.inds <- rowSums(countData) > 0
countData <- countData[to.keep.inds,]
```

Setup for DESeq

```
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

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

estimating size factors

estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing

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

```
summary(res)
```

out of 15975 with nonzero total read count

adjusted p-value < 0.1

LFC > 0 (up) : 4349, 27%

LFC < 0 (down) : 4396, 28%

outliers [1] : 0, 0%

low counts [2] : 1237, 7.7%

(mean count < 0)

[1] see 'cooksCutoff' argument of ?results

[2] see 'independentFiltering' argument of ?results

Running DESeq

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

using pre-existing size factors

estimating dispersions

found already estimated dispersions, replacing these

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing

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

```
head(res)
```

log2 fold change (MLE): condition hoxa1 kd vs control sirna

Wald test p-value: condition hoxa1 kd vs control sirna

DataFrame with 6 rows and 6 columns

	baseMean	log2FoldChange	lfcSE	stat	pvalue
	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>
ENSG00000279457	29.9136	0.1792571	0.3248216	0.551863	5.81042e-01
ENSG00000187634	183.2296	0.4264571	0.1402658	3.040350	2.36304e-03
ENSG00000188976	1651.1881	-0.6927205	0.0548465	-12.630158	1.43989e-36
ENSG00000187961	209.6379	0.7297556	0.1318599	5.534326	3.12428e-08
ENSG00000187583	47.2551	0.0405765	0.2718928	0.149237	8.81366e-01
ENSG00000187642	11.9798	0.5428105	0.5215599	1.040744	2.97994e-01
	padj				
	<numeric>				
ENSG00000279457	6.86555e-01				
ENSG00000187634	5.15718e-03				
ENSG00000188976	1.76549e-35				
ENSG00000187961	1.13413e-07				
ENSG00000187583	9.19031e-01				
ENSG00000187642	4.03379e-01				

Add gene annotation data(gene names etc.)

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

```
columns(org.Hs.eg.db)
```

[1]	"ACCNUM"	"ALIAS"	"ENSEMBL"	"ENSEMBLPROT"	"ENSEMBLTRANS"
[6]	"ENTREZID"	"ENZYME"	"EVIDENCE"	"EVIDENCEALL"	"GENENAME"
[11]	"GENETYPE"	"GO"	"GOALL"	"IPI"	"MAP"
[16]	"OMIM"	"ONTOLOGY"	"ONTOLOGYALL"	"PATH"	"PFAM"
[21]	"PMID"	"PROSITE"	"REFSEQ"	"SYMBOL"	"UCSCKG"
[26]	"UNIPROT"				

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

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

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

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

```
res$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, 10)
```

log2 fold change (MLE): condition hoxa1 kd vs control sirna

Wald test p-value: condition hoxa1 kd vs control sirna

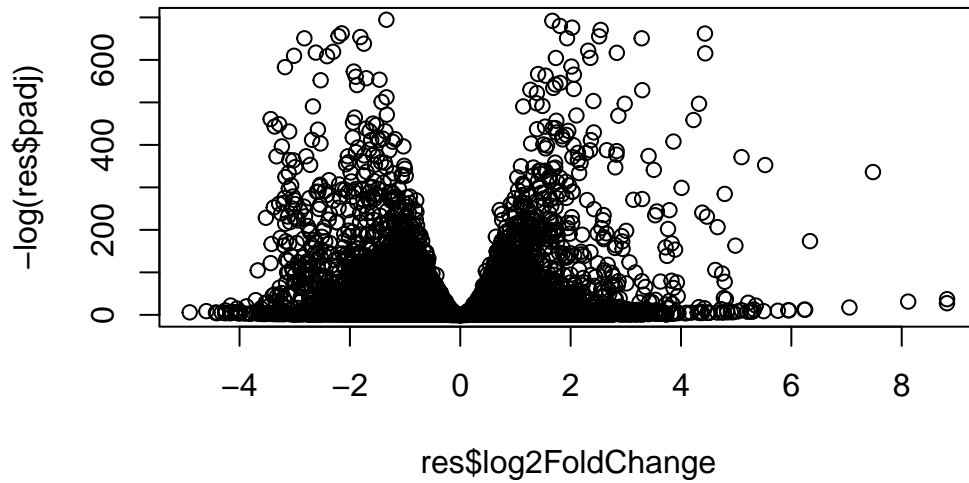
DataFrame with 10 rows and 9 columns

	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	genename	
	<numeric>	<character>	<character>	<character>	
ENSG00000279457	6.86555e-01	NA	NA	NA	
ENSG00000187634	5.15718e-03	SAMD11	148398	sterile alpha motif ..	
ENSG00000188976	1.76549e-35	NOC2L	26155	NOC2 like nucleolar ..	
ENSG00000187961	1.13413e-07	KLHL17	339451	kelch like family me..	
ENSG00000187583	9.19031e-01	PLEKHN1	84069	pleckstrin homology ..	
ENSG00000187642	4.03379e-01	PERM1	84808	PPARGC1 and ESRR ind..	
ENSG00000188290	1.30538e-24	HES4	57801	hes family bHLH tran..	
ENSG00000187608	2.37452e-02	ISG15	9636	ISG15 ubiquitin like..	
ENSG00000188157	4.21963e-16	AGRN	375790	agrin	
ENSG00000237330	NA	RNF223	401934	ring finger protein ..	

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


Results visualization

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

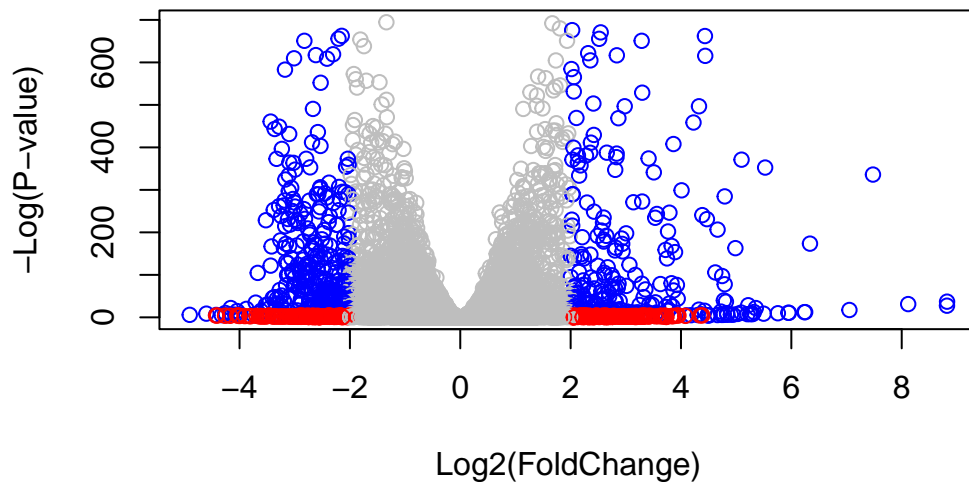


```
# Make a color vector for all genes
mycols <- rep("gray", nrow(res) )

# 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(
```



Save our results

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

Pathway analysis (KEGG, GO, Reactome)

```
library(pathview)
```

```
#####
Pathview is an open source software package distributed under GNU General
Public License version 3 (GPLv3). Details of GPLv3 is available at
http://www.gnu.org/licenses/gpl-3.0.html. Particullary, users are required to
formally cite the original Pathview paper (not just mention it) in publications
or products. For details, do citation("pathview") within R.
```

The pathview downloads and uses KEGG data. Non-academic uses may require a KEGG license agreement (details at <http://www.kegg.jp/kegg/legal.html>).

```
#####
```

```
library(gage)
```

```
library(gageData)
```

```
data(kegg.sets.hs)
```

```
data(sigmet.idx.hs)
```

```
# Focus on signaling and metabolic pathways only
```

```
kegg.sets.hs = kegg.sets.hs[sigmet.idx.hs]
```

```
# Examine the first 3 pathways
```

```
head(kegg.sets.hs, 3)
```

```
$`hsa00232 Caffeine metabolism`
```

```
[1] "10" "1544" "1548" "1549" "1553" "7498" "9"
```

```
$`hsa00983 Drug metabolism - other enzymes`
```

```
[1] "10" "1066" "10720" "10941" "151531" "1548" "1549" "1551"  
[9] "1553" "1576" "1577" "1806" "1807" "1890" "221223" "2990"  
[17] "3251" "3614" "3615" "3704" "51733" "54490" "54575" "54576"  
[25] "54577" "54578" "54579" "54600" "54657" "54658" "54659" "54963"  
[33] "574537" "64816" "7083" "7084" "7172" "7363" "7364" "7365"  
[41] "7366" "7367" "7371" "7372" "7378" "7498" "79799" "83549"  
[49] "8824" "8833" "9" "978"
```

```
$`hsa00230 Purine metabolism`
```

```
[1] "100" "10201" "10606" "10621" "10622" "10623" "107" "10714"  
[9] "108" "10846" "109" "111" "11128" "11164" "112" "113"  
[17] "114" "115" "122481" "122622" "124583" "132" "158" "159"  
[25] "1633" "171568" "1716" "196883" "203" "204" "205" "221823"  
[33] "2272" "22978" "23649" "246721" "25885" "2618" "26289" "270"  
[41] "271" "27115" "272" "2766" "2977" "2982" "2983" "2984"  
[49] "2986" "2987" "29922" "3000" "30833" "30834" "318" "3251"  
[57] "353" "3614" "3615" "3704" "377841" "471" "4830" "4831"  
[65] "4832" "4833" "4860" "4881" "4882" "4907" "50484" "50940"  
[73] "51082" "51251" "51292" "5136" "5137" "5138" "5139" "5140"  
[81] "5141" "5142" "5143" "5144" "5145" "5146" "5147" "5148"  
[89] "5149" "5150" "5151" "5152" "5153" "5158" "5167" "5169"
```

```

[97] "51728" "5198" "5236" "5313" "5315" "53343" "54107" "5422"
[105] "5424" "5425" "5426" "5427" "5430" "5431" "5432" "5433"
[113] "5434" "5435" "5436" "5437" "5438" "5439" "5440" "5441"
[121] "5471" "548644" "55276" "5557" "5558" "55703" "55811" "55821"
[129] "5631" "5634" "56655" "56953" "56985" "57804" "58497" "6240"
[137] "6241" "64425" "646625" "654364" "661" "7498" "8382" "84172"
[145] "84265" "84284" "84618" "8622" "8654" "87178" "8833" "9060"
[153] "9061" "93034" "953" "9533" "954" "955" "956" "957"
[161] "9583" "9615"

```

```

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

```

```

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

```

```

attributes(keggres)

```

```

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

```

```

# Look at the first few down (less) pathways
head(keggres$less)

```

	p.geomean	stat.mean	p.val
hsa04110 Cell cycle	8.995727e-06	-4.378644	8.995727e-06
hsa03030 DNA replication	9.424076e-05	-3.951803	9.424076e-05
hsa03013 RNA transport	1.375901e-03	-3.028500	1.375901e-03
hsa03440 Homologous recombination	3.066756e-03	-2.852899	3.066756e-03
hsa04114 Oocyte meiosis	3.784520e-03	-2.698128	3.784520e-03
hsa00010 Glycolysis / Gluconeogenesis	8.961413e-03	-2.405398	8.961413e-03

	q.val	set.size	exp1
hsa04110 Cell cycle	0.001448312	121	8.995727e-06
hsa03030 DNA replication	0.007586381	36	9.424076e-05

hsa03013	RNA transport	0.073840037	144	1.375901e-03
hsa03440	Homologous recombination	0.121861535	28	3.066756e-03
hsa04114	Oocyte meiosis	0.121861535	102	3.784520e-03
hsa00010	Glycolysis / Gluconeogenesis	0.212222694	53	8.961413e-03

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

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04110.pathview.png

```
# 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/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04110.pathview.pdf

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

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

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04640.pathview.png

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04630.pathview.png

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa00140.pathview.png

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

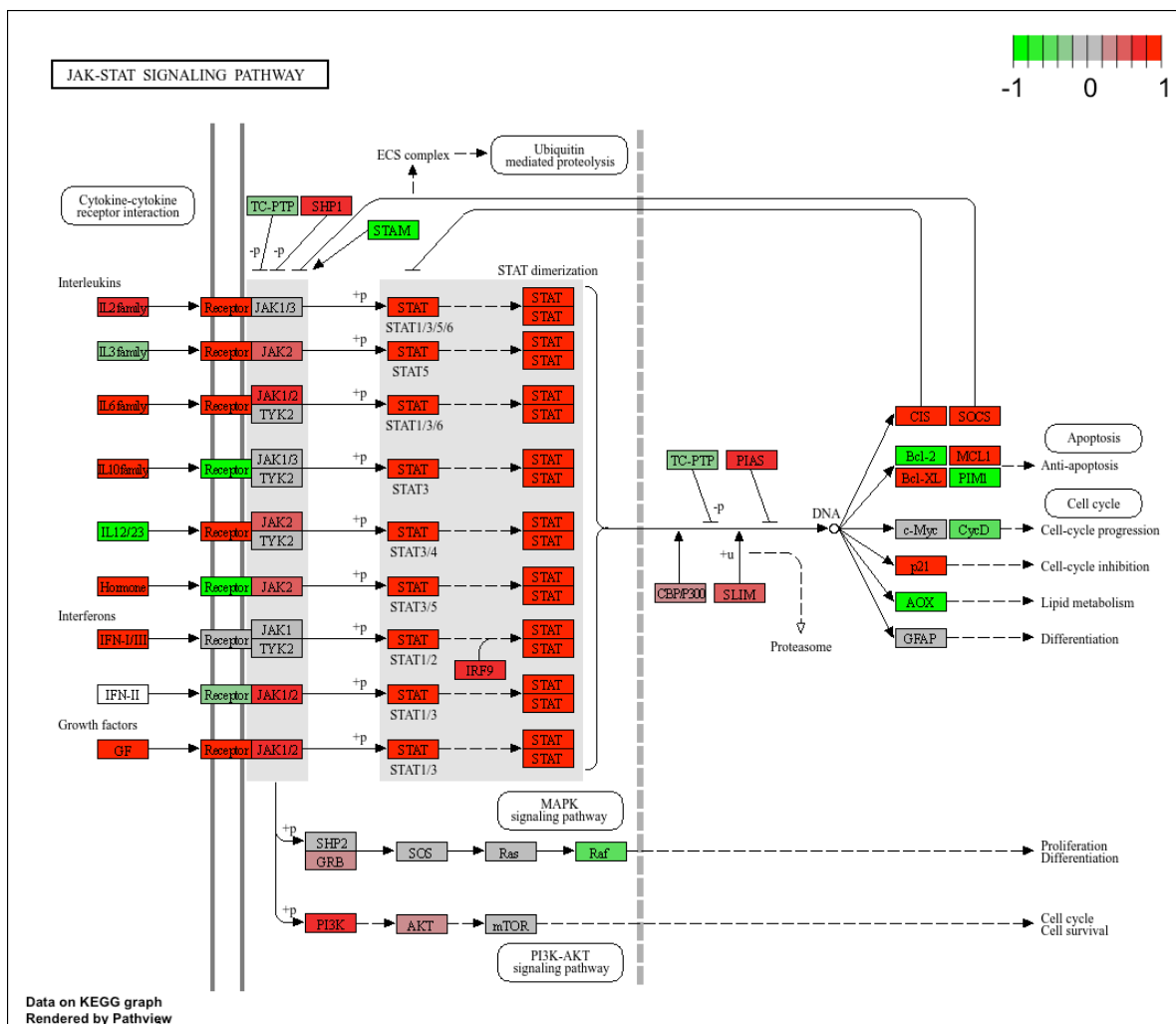
Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04142.pathview.png

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04330.pathview.png



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

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

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

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

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

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04640.pathview.png

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04630.pathview.png

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

Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa00140.pathview.png

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

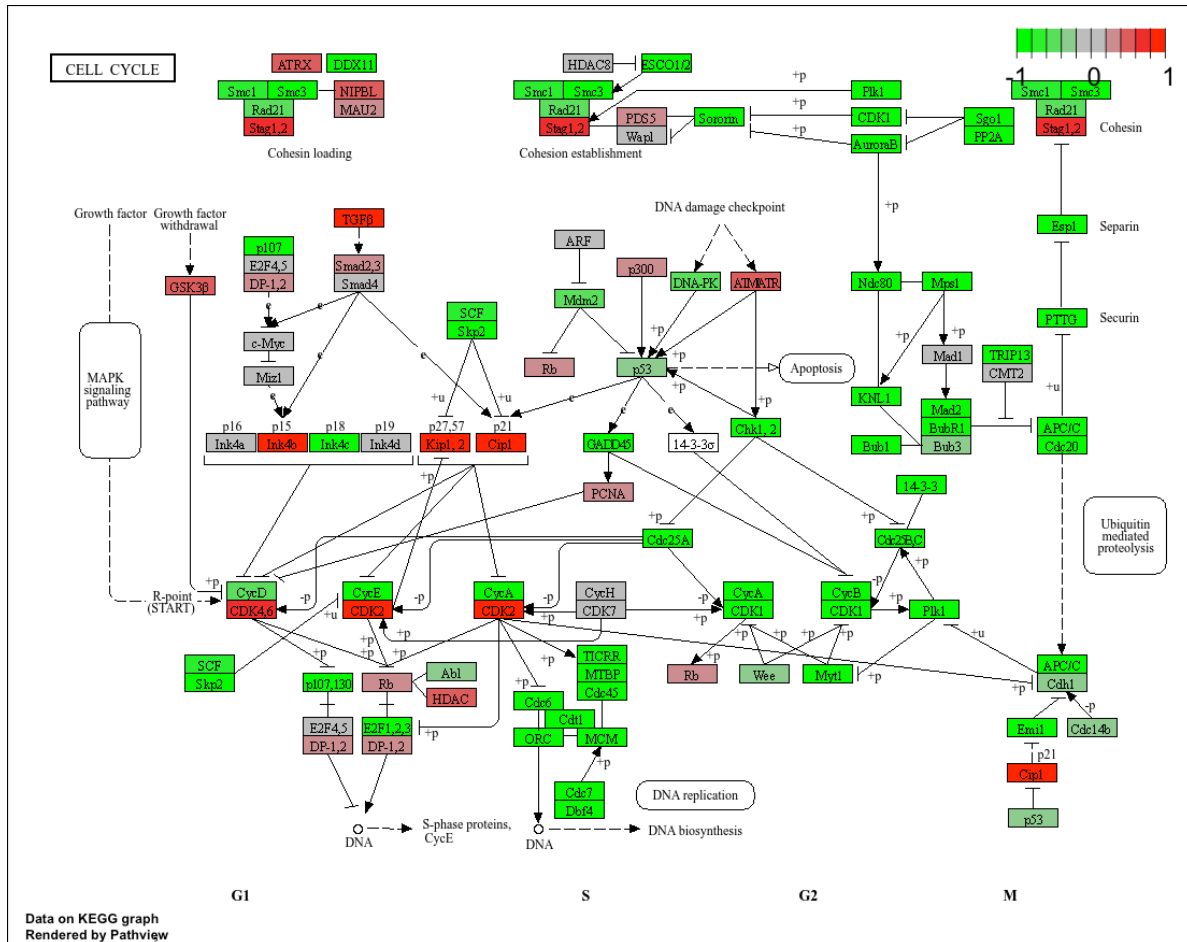
Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04142.pathview.png

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

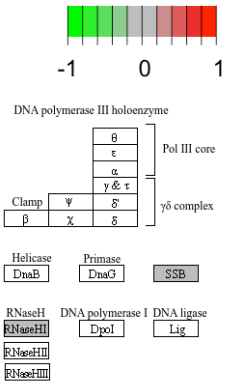
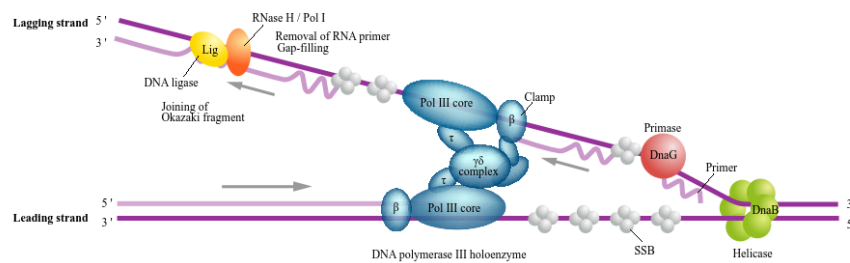
Info: Working in directory /Users/ericasanchez/Desktop/BIMM 143/Class 14

Info: Writing image file hsa04330.pathview.png

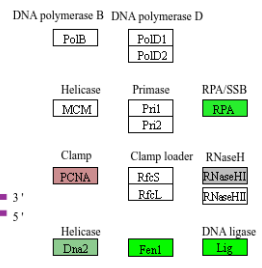
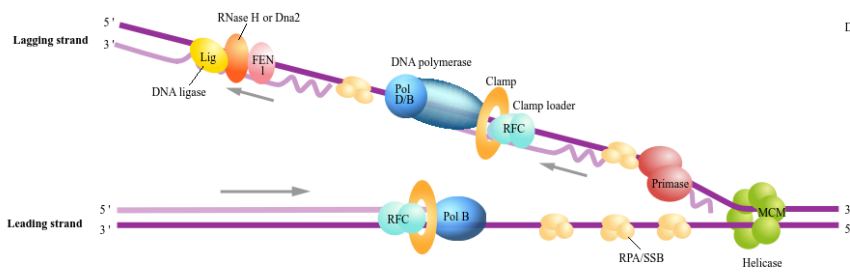


DNA REPLICATION

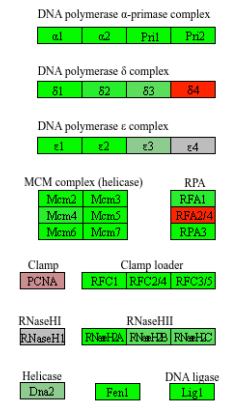
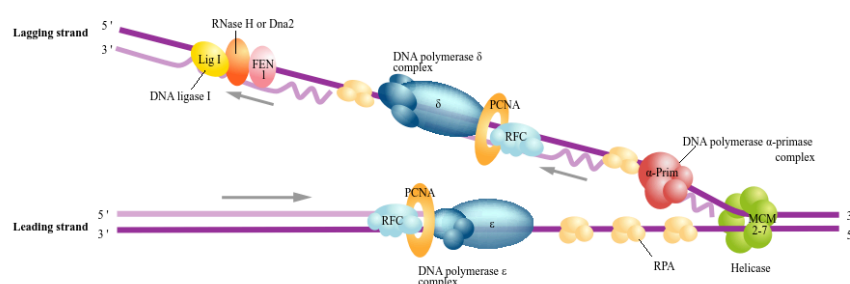
Replication complex (Bacteria)



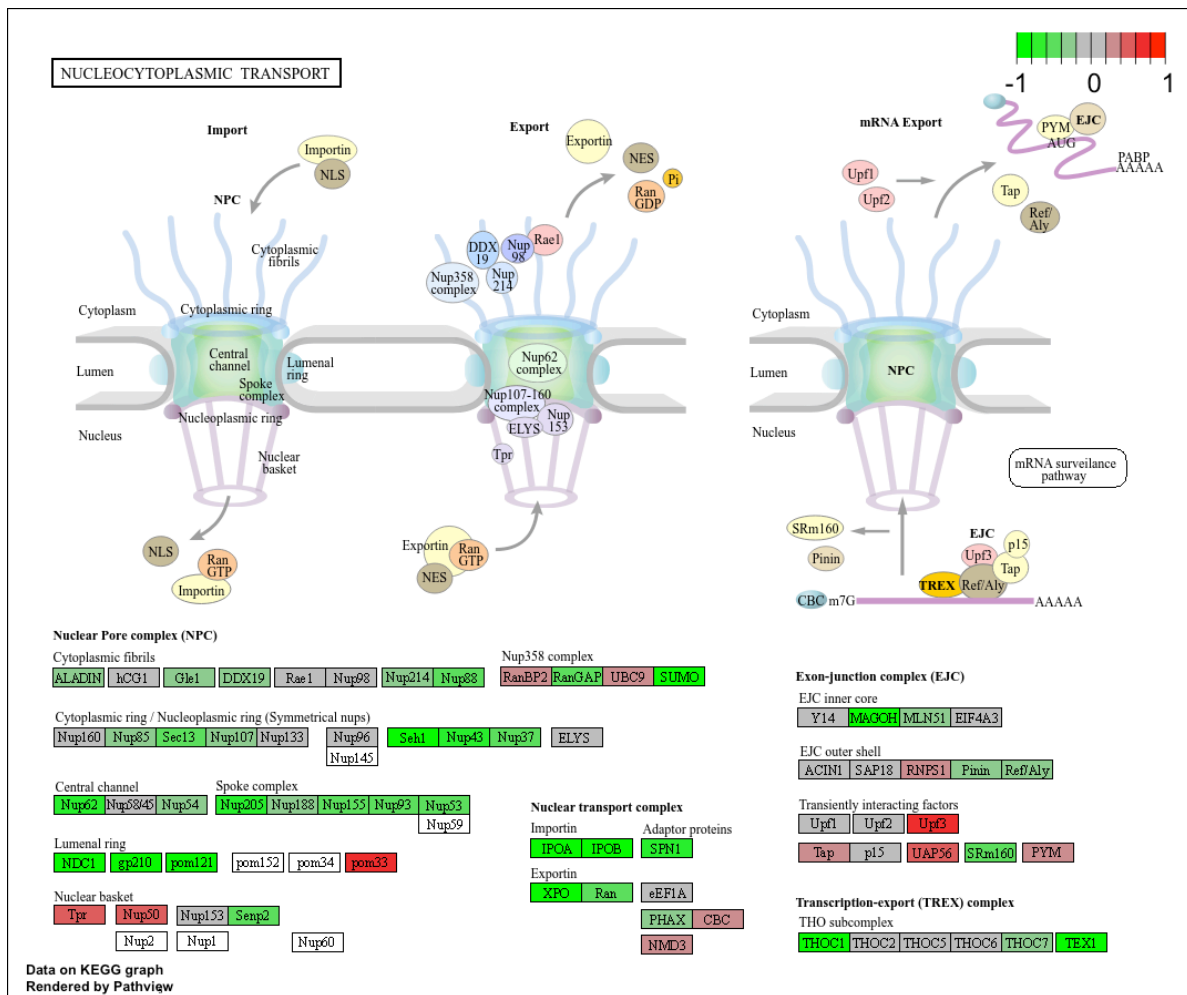
Replication complex (Archaea)

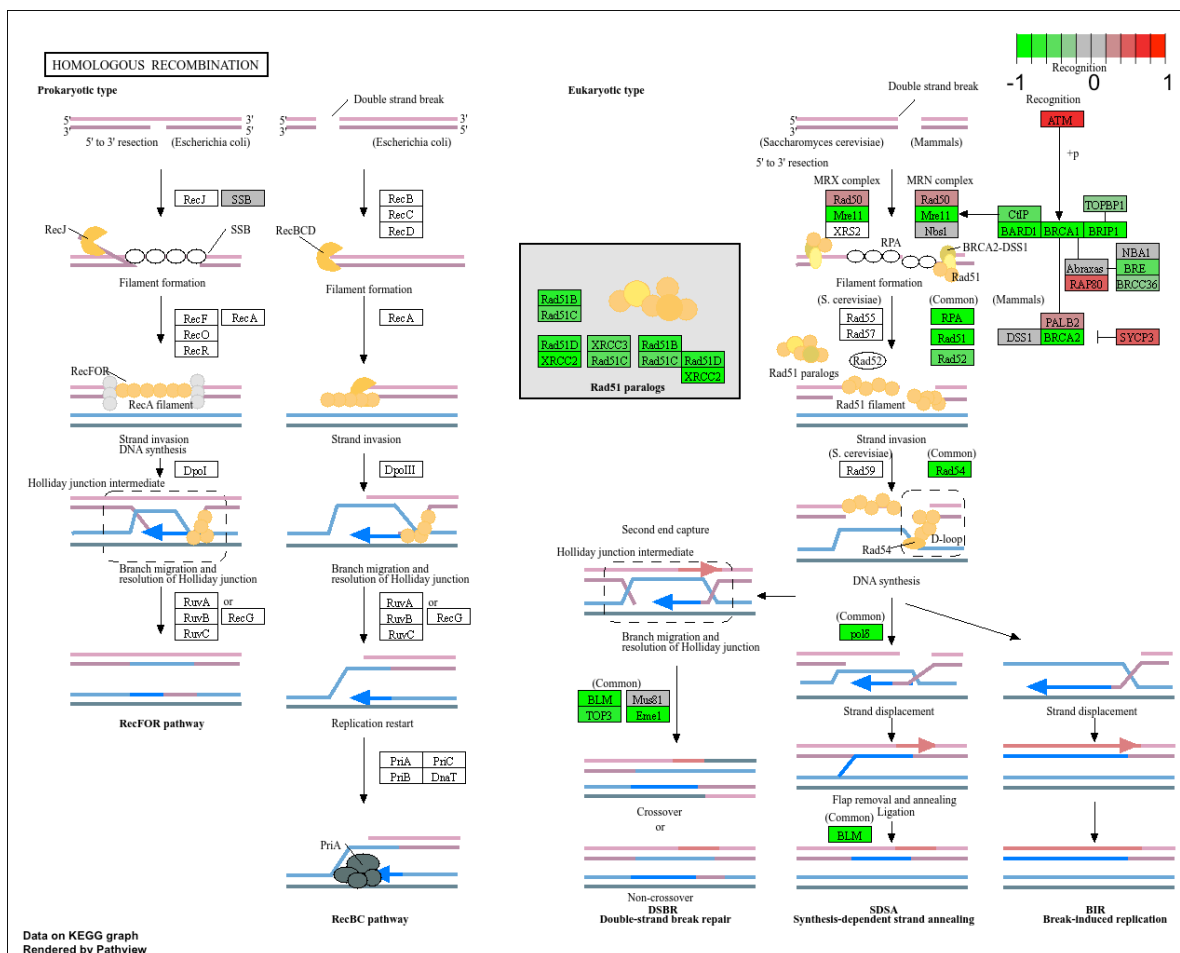


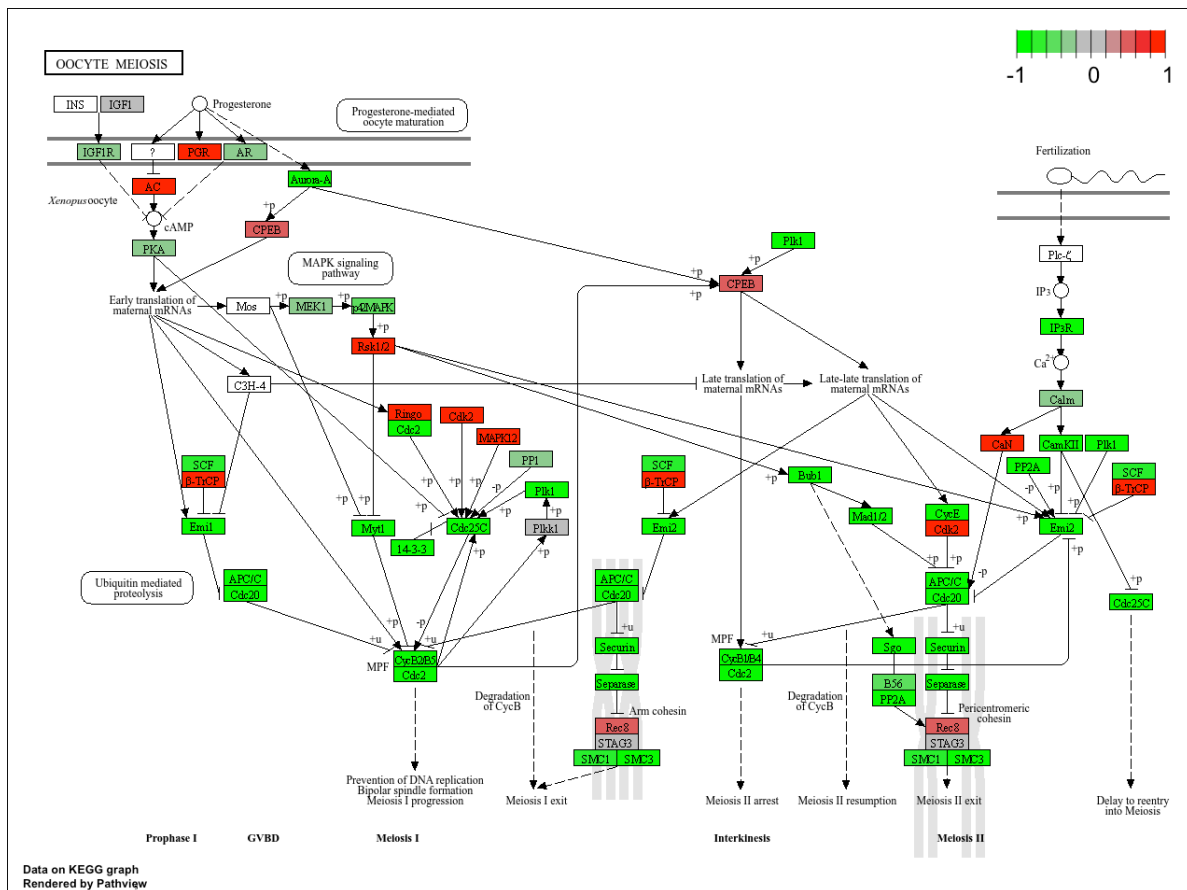
Replication complex (Eukaryotes)



Data on KEGG graph
Rendered by Pathview







Gene Ontology

```
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
G0:0007156 homophilic cell adhesion	8.519724e-05	3.824205	8.519724e-05
G0:0002009 morphogenesis of an epithelium	1.396681e-04	3.653886	1.396681e-04
G0:0048729 tissue morphogenesis	1.432451e-04	3.643242	1.432451e-04

G0:0007610	behavior	1.925222e-04	3.565432	1.925222e-04
G0:0060562	epithelial tube morphogenesis	5.932837e-04	3.261376	5.932837e-04
G0:0035295	tube development	5.953254e-04	3.253665	5.953254e-04

		q.val	set.size	exp1
G0:0007156	homophilic cell adhesion	0.1952430	113	8.519724e-05
G0:0002009	morphogenesis of an epithelium	0.1952430	339	1.396681e-04
G0:0048729	tissue morphogenesis	0.1952430	424	1.432451e-04
G0:0007610	behavior	0.1968058	426	1.925222e-04
G0:0060562	epithelial tube morphogenesis	0.3566193	257	5.932837e-04
G0:0035295	tube development	0.3566193	391	5.953254e-04

\$less

		p.geomean	stat.mean	p.val
G0:0048285	organelle fission	1.536227e-15	-8.063910	1.536227e-15
G0:0000280	nuclear division	4.286961e-15	-7.939217	4.286961e-15
G0:0007067	mitosis	4.286961e-15	-7.939217	4.286961e-15
G0:0000087	M phase of mitotic cell cycle	1.169934e-14	-7.797496	1.169934e-14
G0:0007059	chromosome segregation	2.028624e-11	-6.878340	2.028624e-11
G0:0000236	mitotic prometaphase	1.729553e-10	-6.695966	1.729553e-10

		q.val	set.size	exp1
G0:0048285	organelle fission	5.843127e-12	376	1.536227e-15
G0:0000280	nuclear division	5.843127e-12	352	4.286961e-15
G0:0007067	mitosis	5.843127e-12	352	4.286961e-15
G0:0000087	M phase of mitotic cell cycle	1.195965e-11	362	1.169934e-14
G0:0007059	chromosome segregation	1.659009e-08	142	2.028624e-11
G0:0000236	mitotic prometaphase	1.178690e-07	84	1.729553e-10

\$stats

		stat.mean	exp1
G0:0007156	homophilic cell adhesion	3.824205	3.824205
G0:0002009	morphogenesis of an epithelium	3.653886	3.653886
G0:0048729	tissue morphogenesis	3.643242	3.643242
G0:0007610	behavior	3.565432	3.565432
G0:0060562	epithelial tube morphogenesis	3.261376	3.261376
G0:0035295	tube development	3.253665	3.253665

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: 8147"
```

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

Q: 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?

Cell cycle has the most significant “Entities p-value”. This matches the KEGG results. Database content can lead to differences between the two methods.

