

Class 13: Pathway Analysis from RNA-Seq Results

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Section 1. Differential Expression Analysis

Import metadata and take a look

```
colData <- read.csv("GSE37704_metadata.csv", 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 and take a look

```
countData <- read.csv("GSE37704_featurecounts.csv", 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					

Q. Complete the code below to remove the troublesome first column from countData

```
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

Q. 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 where you have 0 read count across all samples.
countData <- countData[rowSums(countData) > 0,]
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

Check to see if the samples in countData match to colData

```
all(colnames(countData) == rownames(colData))
```

```
[1] TRUE
```

Running DESeq2

```
library(DESeq2)
```

Warning: package 'matrixStats' was built under R version 4.2.2

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

estimating size factors

estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing

```
dds
```

```
class: DESeqDataSet  
dim: 15975 6  
metadata(1): version  
assays(4): counts mu H cooks  
rownames(15975): ENSG00000279457 ENSG00000187634 ... ENSG00000276345  
               ENSG00000271254  
rowData names(22): baseMean baseVar ... deviance maxCooks  
colnames(6): SRR493366 SRR493367 ... SRR493370 SRR493371  
colData names(2): condition sizeFactor
```

```
res <- results(dds, contrast=c("condition", "hoxa1_kd", "control_sirna"))
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	1.43990e-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	0.5215598	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				

Q. 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.

```
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%

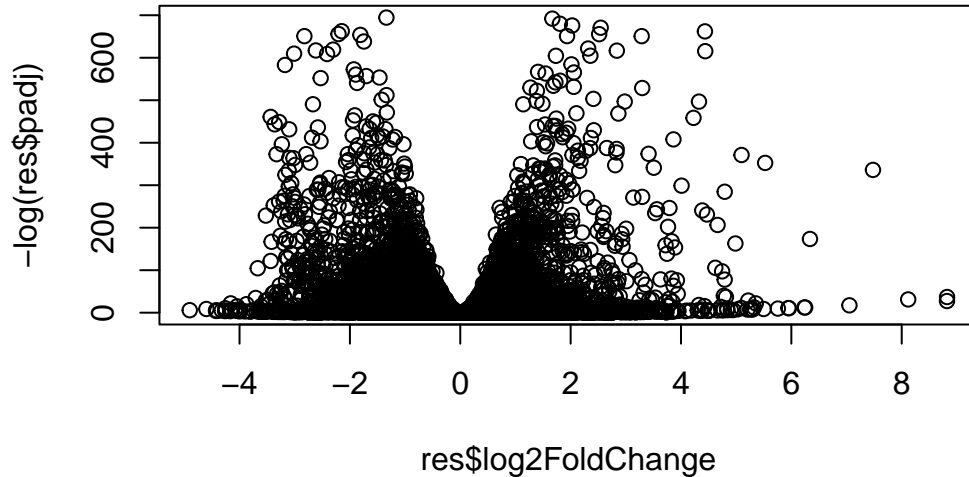
(mean count < 0)

[1] see 'cooksCutoff' argument of ?results

[2] see 'independentFiltering' argument of ?results

Volcano plot

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



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

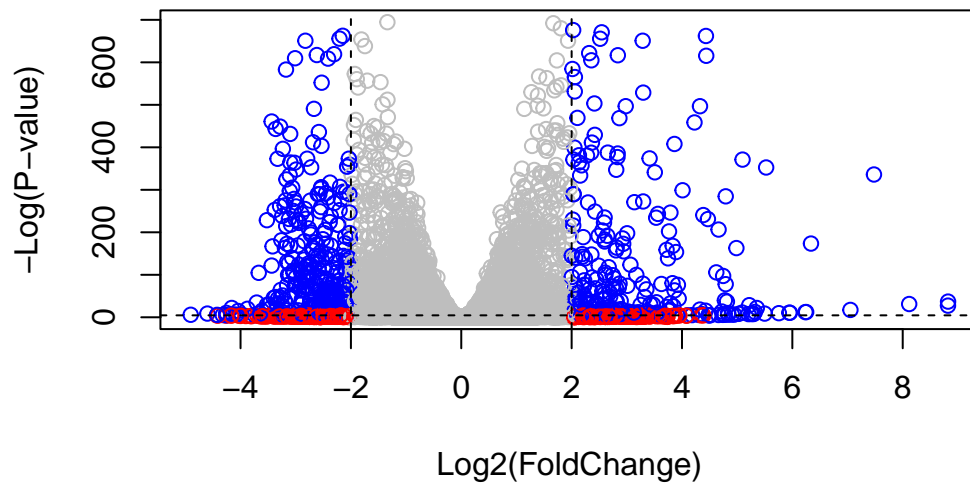
```
# Make a color vector for all genes
mycols <- rep("gray", nrow(res))

# 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)")
```

```
# Cut-off lines
abline(v = c(-2,2), col = "black", lty = 2)
abline(h = -log(0.01), col = "black", lty = 2)
```



Adding gene annotation

Q. 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")

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")

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

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

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	1.43990e-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	0.5215598	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	6.86555e-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 ..

Q. 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.

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

Section 2. Pathway Analysis

```
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"
```

The ‘gage()’ function wants only a vector of importance input that has names in ENTREZ ID format

```
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
```

Let’s run the gage pathway analysis

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

Look at the object returned from 'gage()' function.

```
attributes(keggres)
```

```
$names
```

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

Let's look at the first few down (less) pathway results:

```
# 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

Look at hsa04110 Cell cycle

```
library(pathview)
```

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

Look at hsa03030 DNA replication

```
pathview(gene.data = foldchanges, pathway.id = "hsa03030")
```

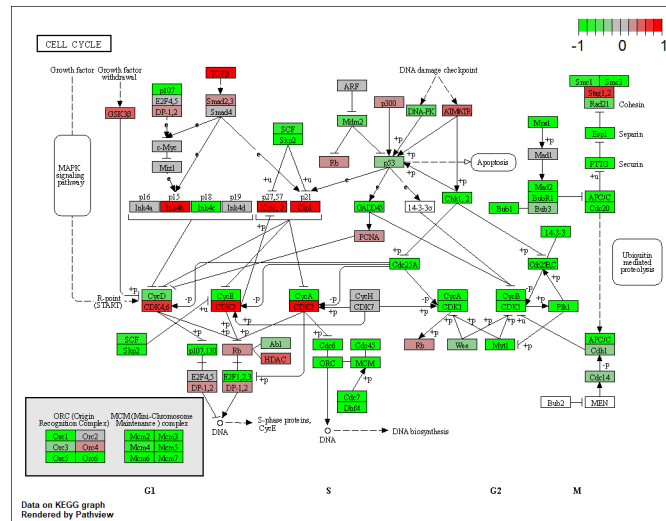


Figure 1: The Cell cycle pathway with genes are colored

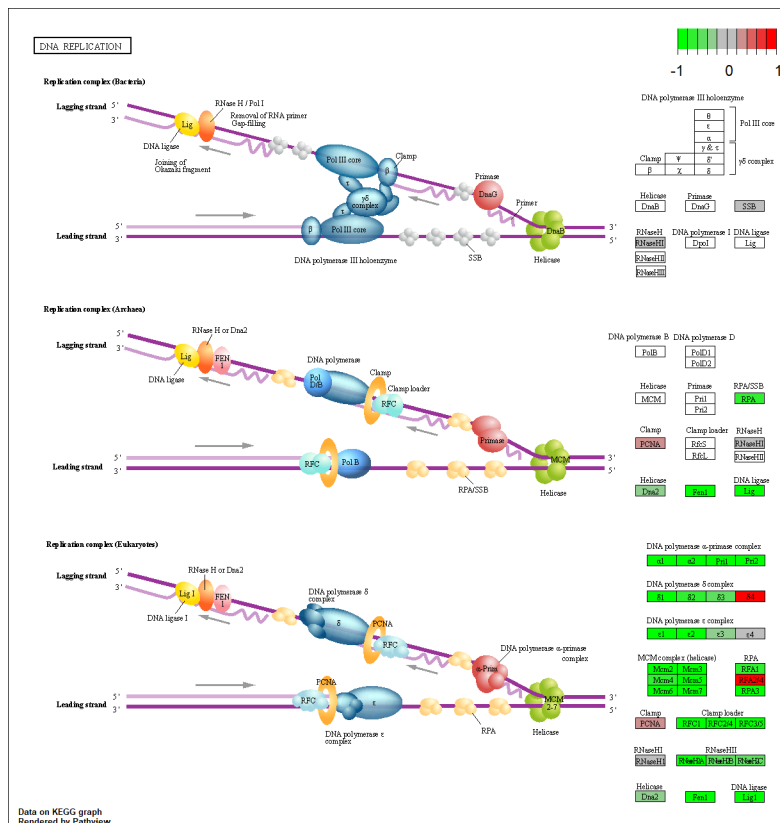


Figure 2: The DNA replication pathway with genes are colored

Now, focus on top 5 upregulated pathways

```
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"
```

Draw plots for all the top 5 pathways

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

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

```
keggrespathways_down <- rownames(keggres$less)[1:5]

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

```
[1] "hsa04110" "hsa03030" "hsa03013" "hsa03440" "hsa04114"
```

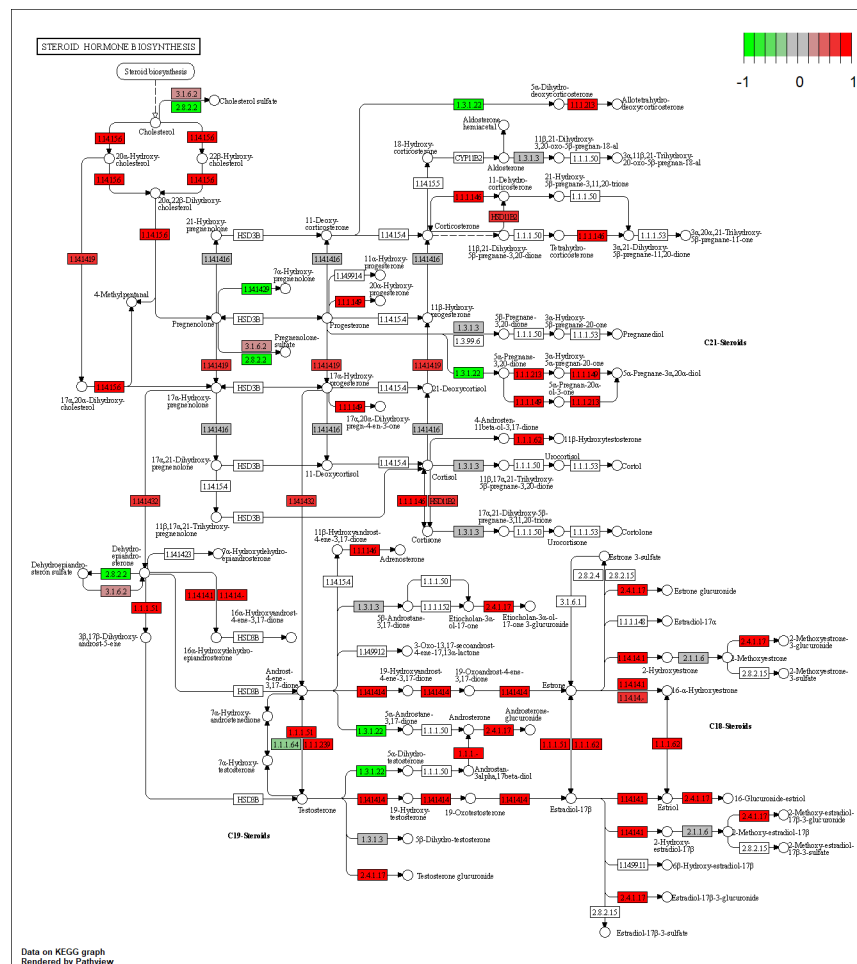
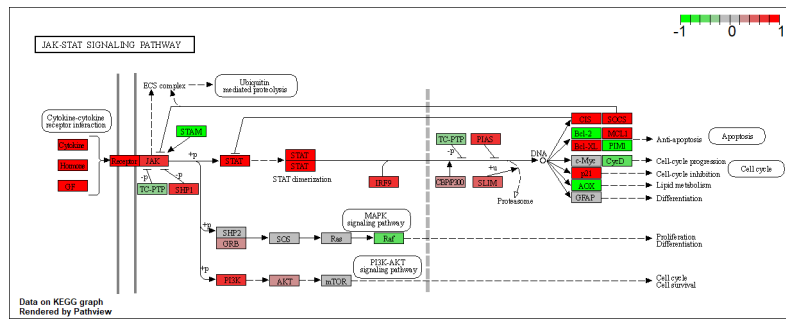
```
pathview(gene.data = foldchanges, pathway.id = keggresids_down, species = "hsa")
```

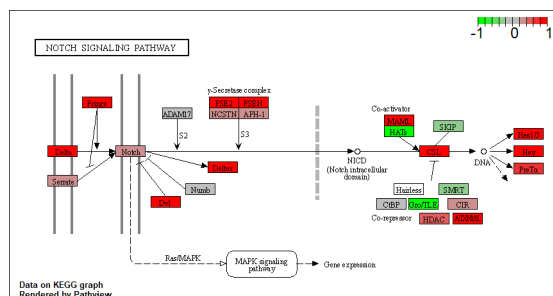
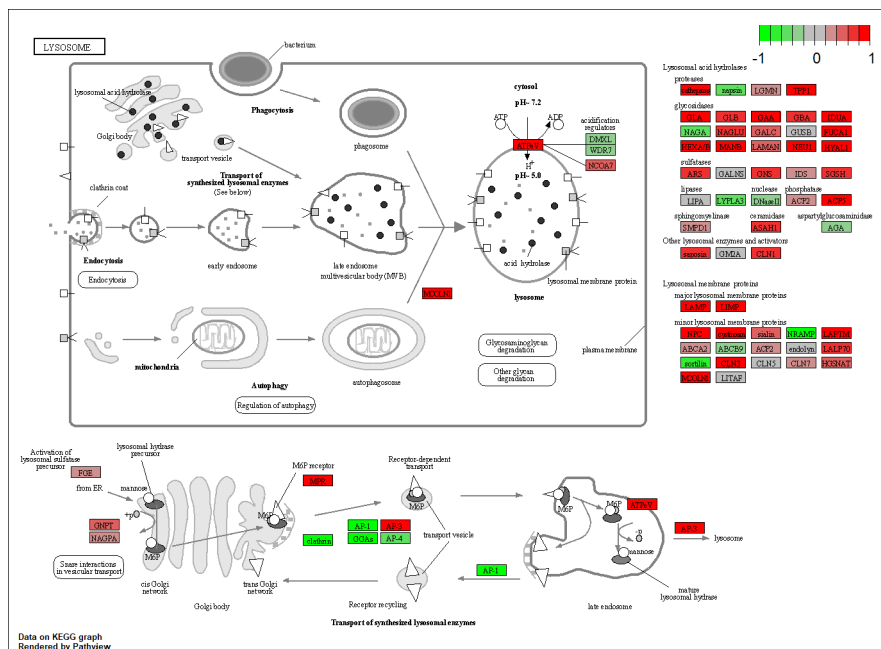
Section 3. Gene Ontology (GO)

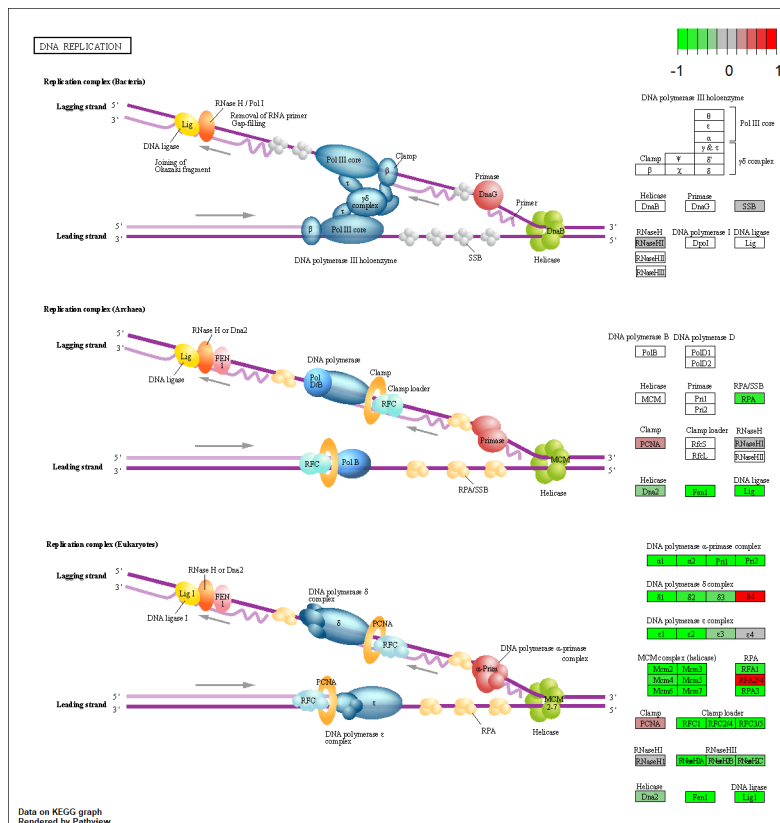
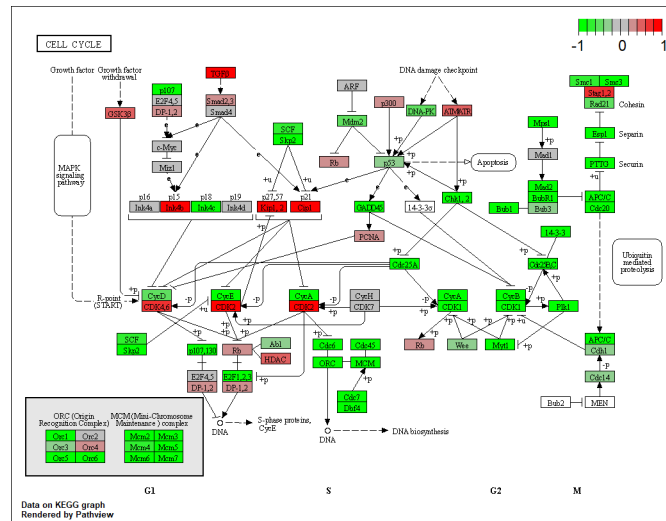
```
data(go.sets.hs)
data(go.subs.hs)

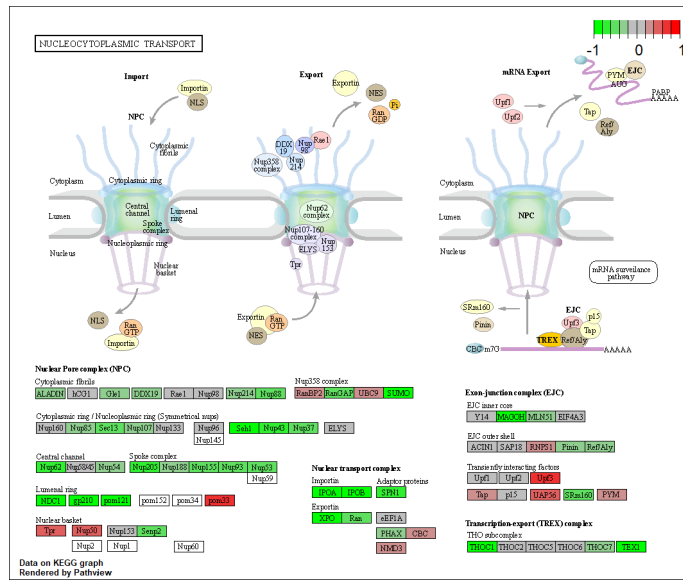
# Focus on Biological Process subset of GO
gobpsets <- go.sets.hs[go.subs.hs$BP]

gobpres <- gage(foldchanges, gsets = gobpsets, same.dir = TRUE)
```







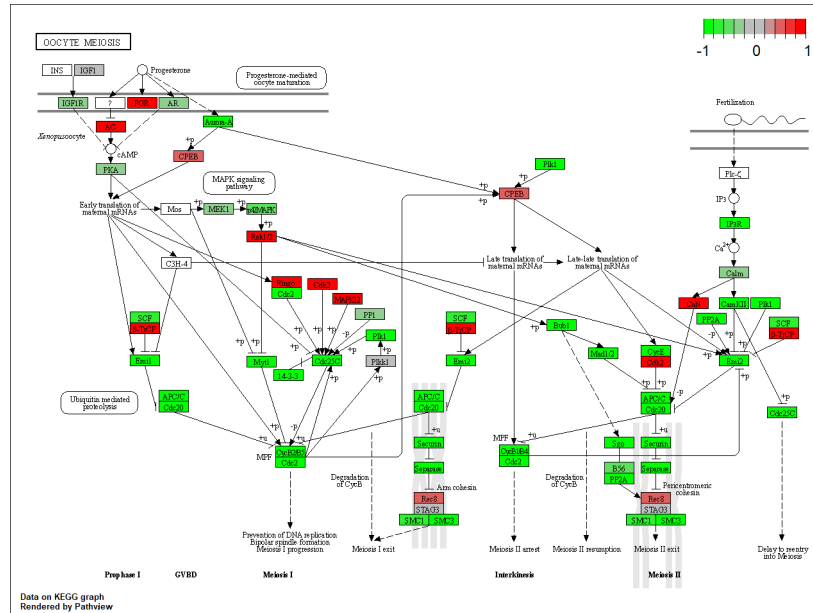


Figure 12: hsa04114

```
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	2.195494e-04	3.530241	2.195494e-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	0.1951953	113	8.519724e-05
G0:0002009	morphogenesis of an epithelium	0.1951953	339	1.396681e-04
G0:0048729	tissue morphogenesis	0.1951953	424	1.432451e-04
G0:0007610	behavior	0.2243795	427	2.195494e-04
G0:0060562	epithelial tube morphogenesis	0.3711390	257	5.932837e-04
G0:0035295	tube development	0.3711390	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	5.841698e-12	376	1.536227e-15
G0:0000280	nuclear division	5.841698e-12	352	4.286961e-15
G0:0007067	mitosis	5.841698e-12	352	4.286961e-15
G0:0000087	M phase of mitotic cell cycle	1.195672e-11	362	1.169934e-14
G0:0007059	chromosome segregation	1.658603e-08	142	2.028624e-11
G0:0000236	mitotic prometaphase	1.178402e-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	3.530241	3.530241
G0:0060562	epithelial tube morphogenesis	3.261376	3.261376
G0:0035295	tube development	3.253665	3.253665

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: 8147"
```

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

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?

Ans: The pathway has the most significant “Entities p-value” is the “Endosomal/Vacuolar pathway.” No, the most significant pathways listed does not match your previous KEGG results. In my opinion, factors

that could cause differences between the two methods are due to their differences in the collection of biological information data

Section 5. GO online (OPTIONAL)

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?

Ans: The pathway has the most significant “Entities p-value” is the “detection of chemical stimulus involved in sensory perception.” No, the most significant pathways listed does not match your previous KEGG results. In my opinion, factors that could cause differences between the two methods are due to their differences in the collection of biological information data