Transcriptomic analysis of crustacean molting gland (Y-organ) regulation via the mTOR signaling pathway

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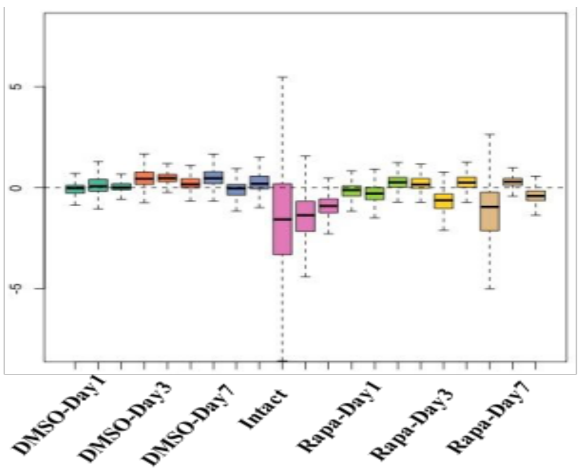
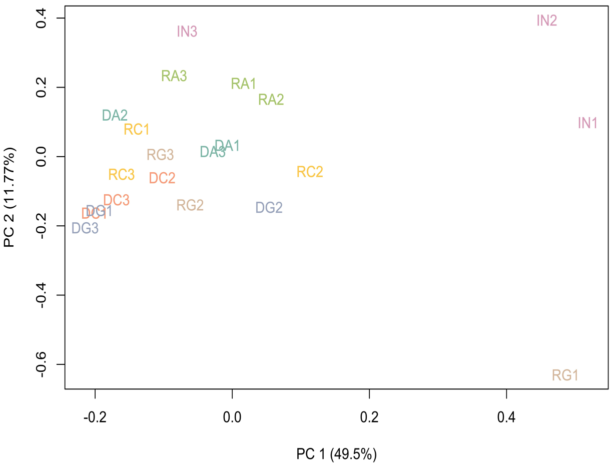
Supplementary Figures

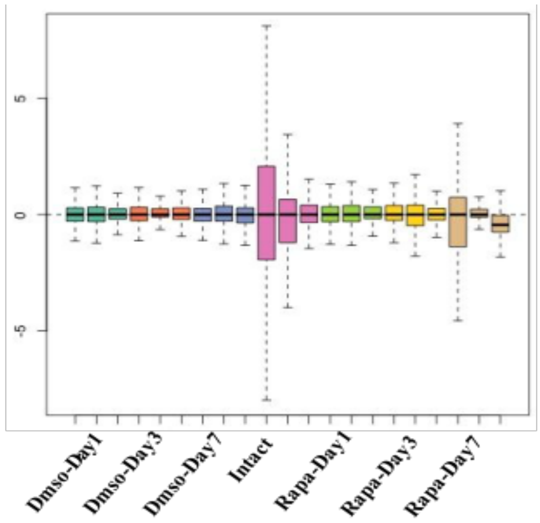
A.



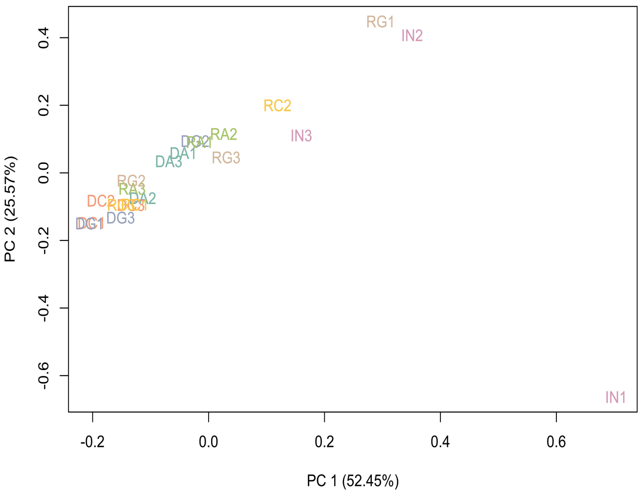
B.



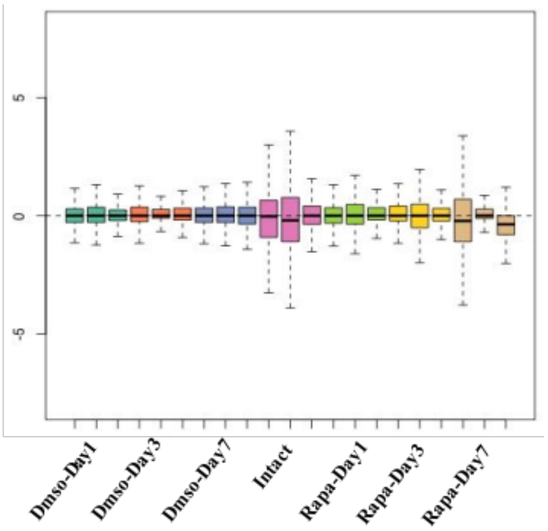
C.



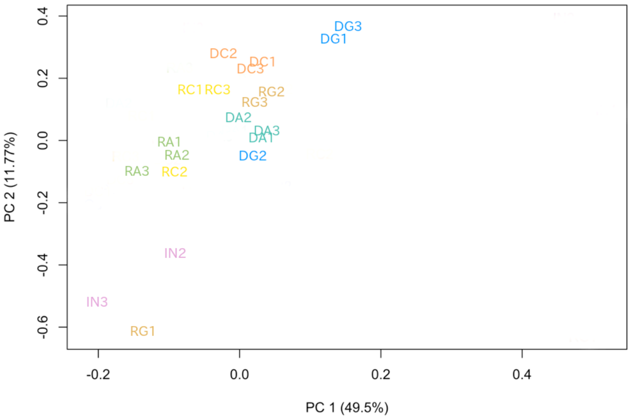
D.



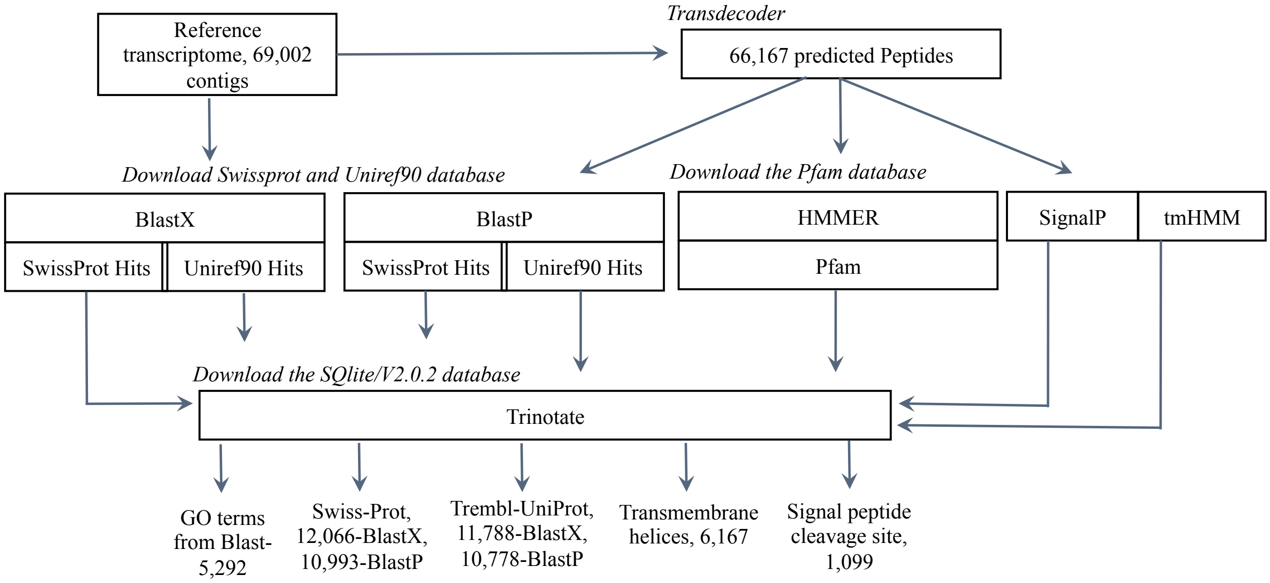
E.



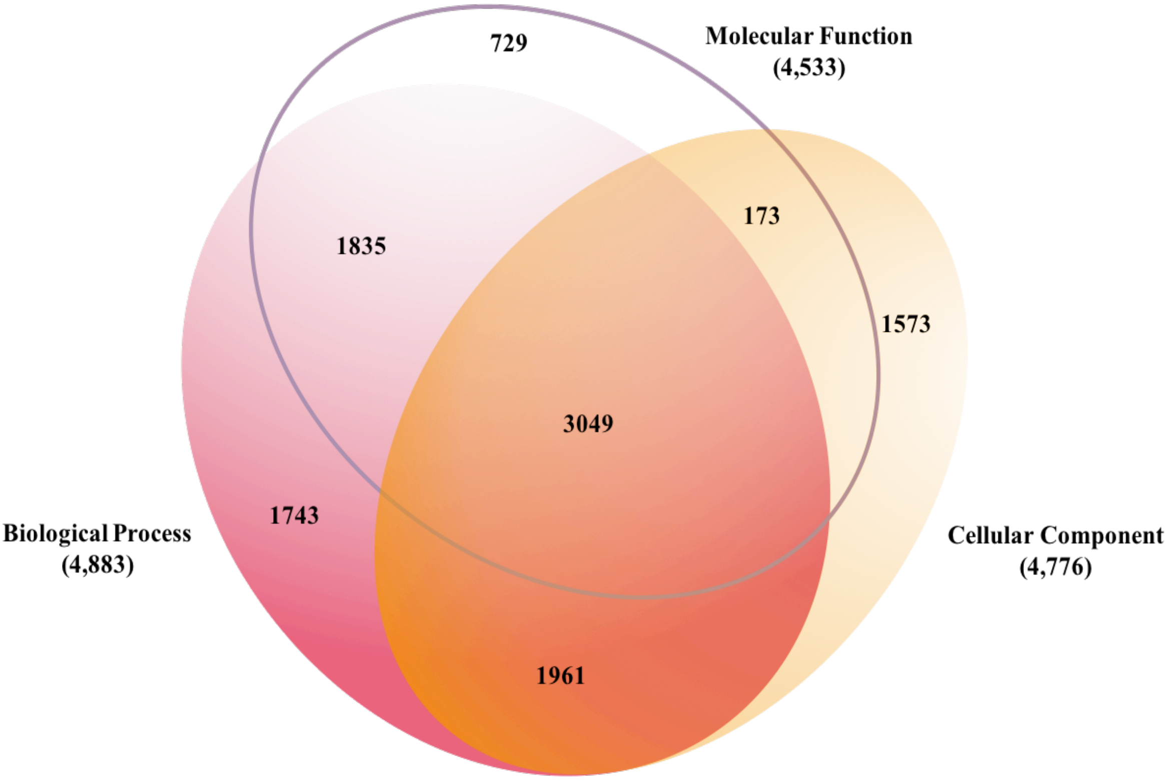
F.



Supplementary Figure 1. Removal of unwanted variation in the YO RNA-seq data set. We expect relative log expression distributions (RLE) to be centered around zero and similar to each other. (1A) The RLE boxplots prior to normalization. (The bottom and top of the box indicate, respectively, the first and third quartiles; the inside line indicates the median. (1B) Scatterplot of first two principal components for un-normalized counts (log scale, centered). Libraries do not cluster as expected according to treatment. (1C) Upper quartile normalization centers RLE around zero, but replicate interlibrary variability is high. (1D) Scatterplot of first two principal components for upper quartile normalization. (1E) Boxplots of RLE for RUVg-normalized counts. RUVg based on spike-ins leads to more uniform RLE distributions. (1F) Libraries cluster as expected by treatment; note IN1 (intact/ intermolt replicate library 1) outlier (IN1 PC1/2 value 1.473). Pearson correlation coefficients are given in table 3. Library designations are IN: intact/intermolt libraries; D: DMSO injection libraries following ESA; R: rapamycin injection libraries following ESA. All libraries had 3 technical replicates (1-3). D and R libraries were taken following day 1 (A) day 3 (C) and day 7 (G) following ESA.

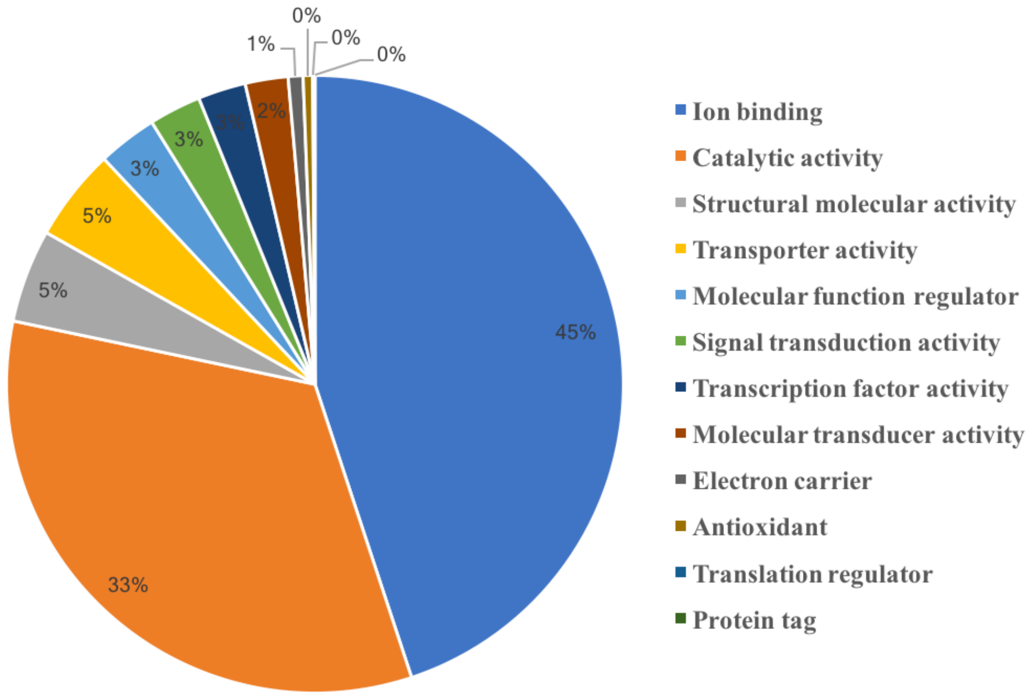

Reference transcriptome, 69,002 contigs
Transdecoder
66,167 predicted Peptides
Download Swissprot and Uniref90 database
Download the Pfam database
BlastX
SwissProt Hits
Uniref90 Hits
BlastP
SwissProt Hits
Uniref90 Hits
HMMER
Pfam
SignalP
tmHMM
Download the SQlite/V2.0.2 database
Trinotate
GO terms from Blast-5,292
Swiss-Prot, 12,066-BlastX, 10,993-BlastP
Trembl-UniProt, 11,788-BlastX, 10,778-BlastP
Transmembrane helices, 6,167
Signal peptide cleavage site, 1,099

Supplementary Figure 2. Annotation pipeline. Both nucleotide and peptide sequences obtained from the reference YO transcriptome were used for BLAST analyses against four databases. Using Trinotate, GO IDs with associated gene and domain identifiers were extracted. GO Slim Viewer was used to generate a summary of functions and GO Slim IDs (see text for details). The downloaded databases are indicated in italics; numbers represent contig assignments resulting from searches.

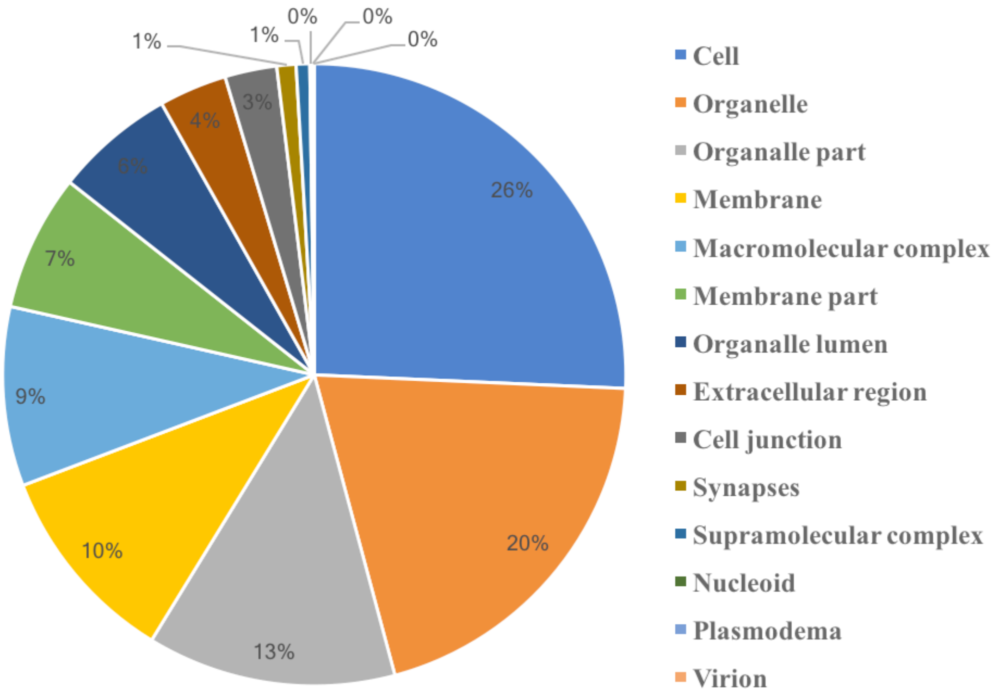


Supplementary Figure 3. Venn diagram illustrates the distribution of unique contigs annotated to one or combination of the three GO categories (Suppl. Fig. 4). Many of the contigs were assigned to two or all three categories.

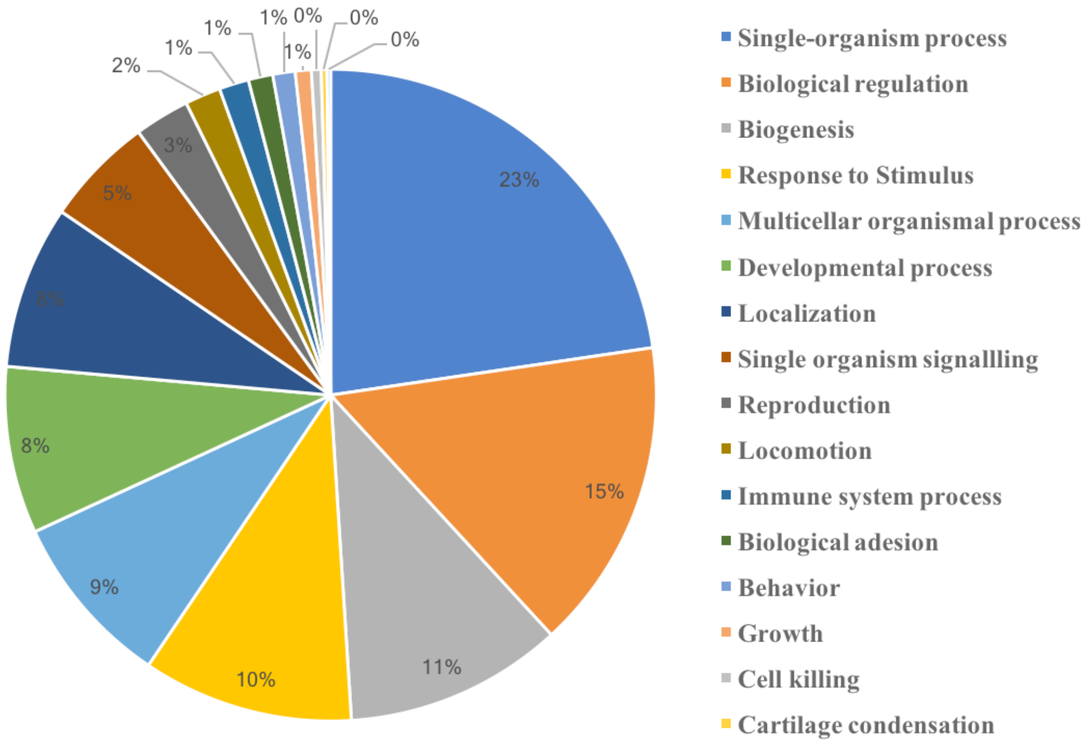
A. Molecular Function



B. Cellular Component

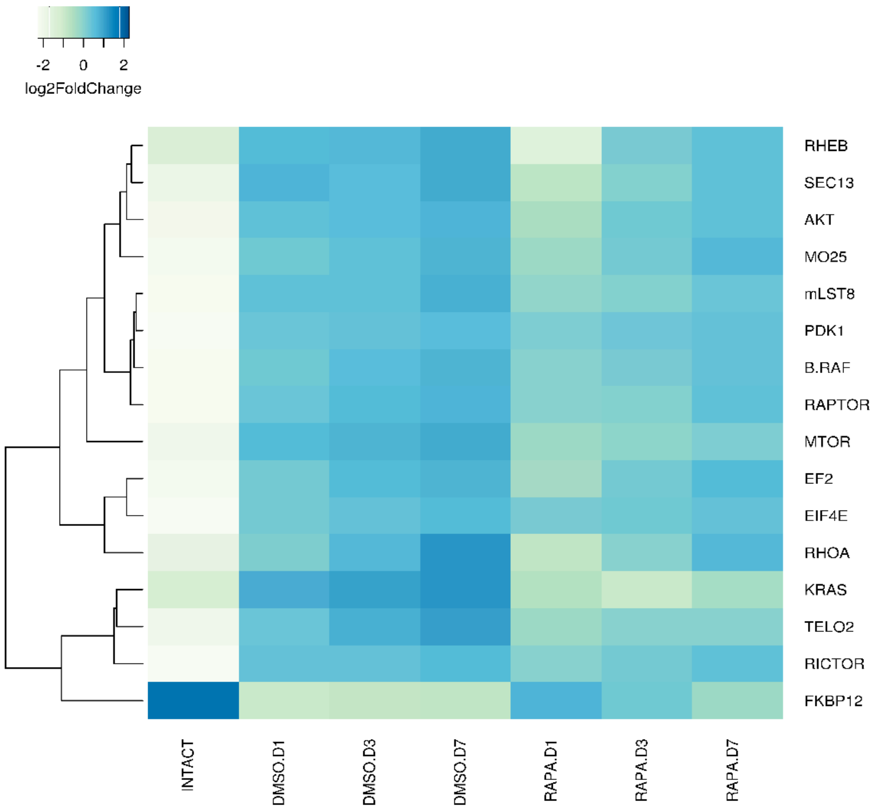


C. Biological Processes

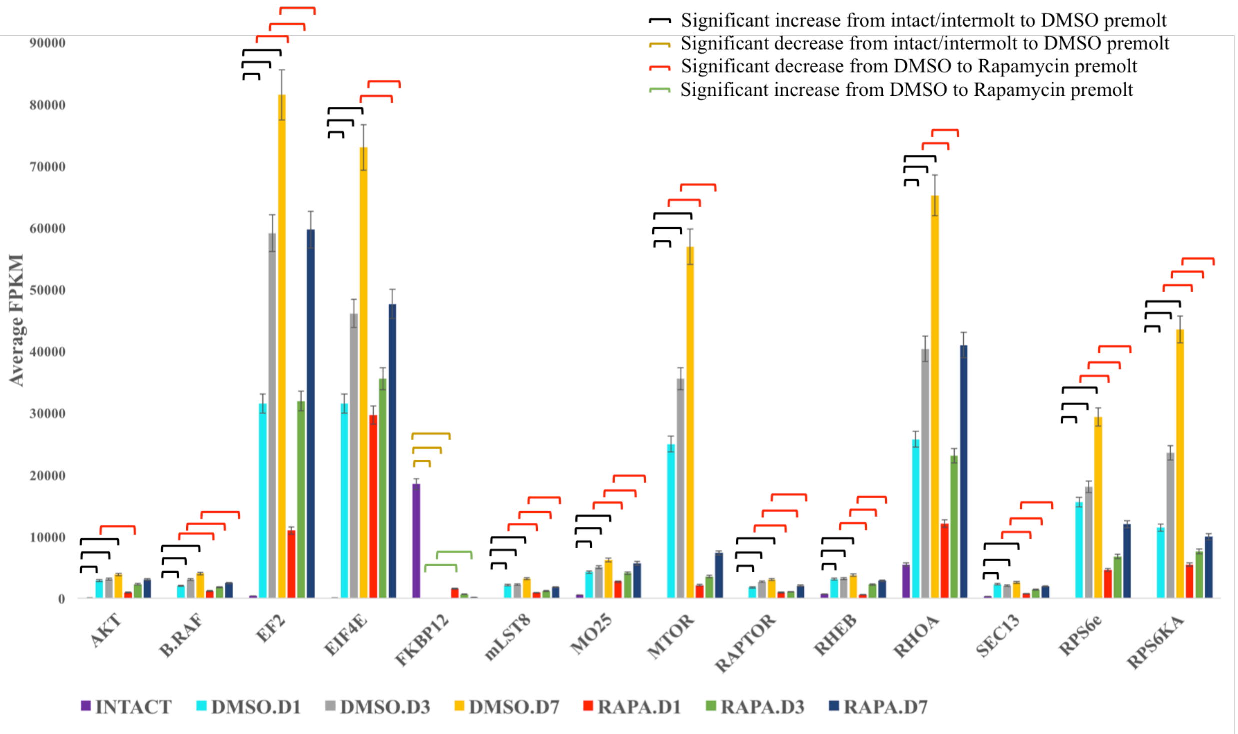


Supplementary Figure 4. Number of reference transcriptome contigs distributed among second-tier GO terms. The sections show the distribution of second-tier GO terms associated with three parent terms: molecular function (A), cellular component (B), and biological process (C). The sections rank the terms from the highest to lowest number of contigs in each of the second-tier GO categories.

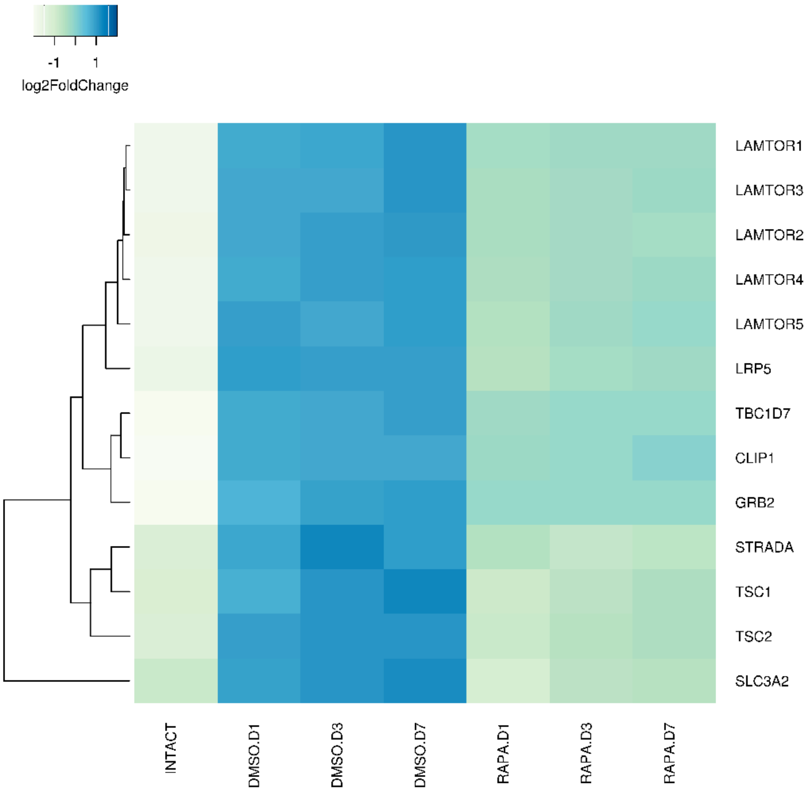
A. Heat map showing the log2fold change of the FPKM values of the DEGs representing the mTOR signaling pathway

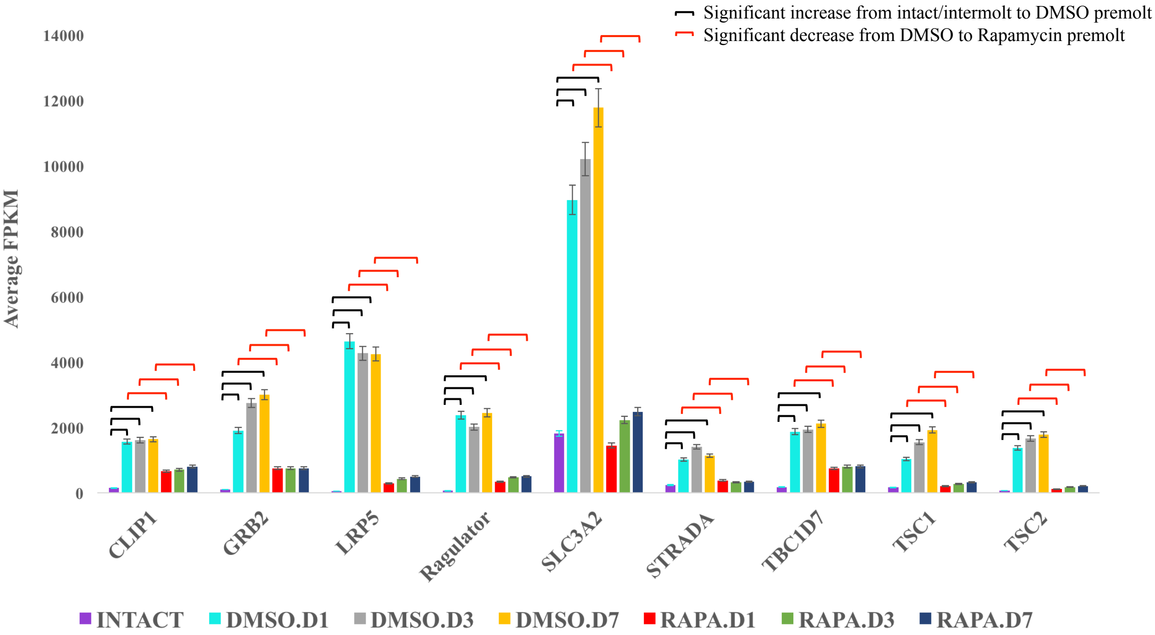


B. Changes in mRNA abundance for mTOR signaling pathway genes

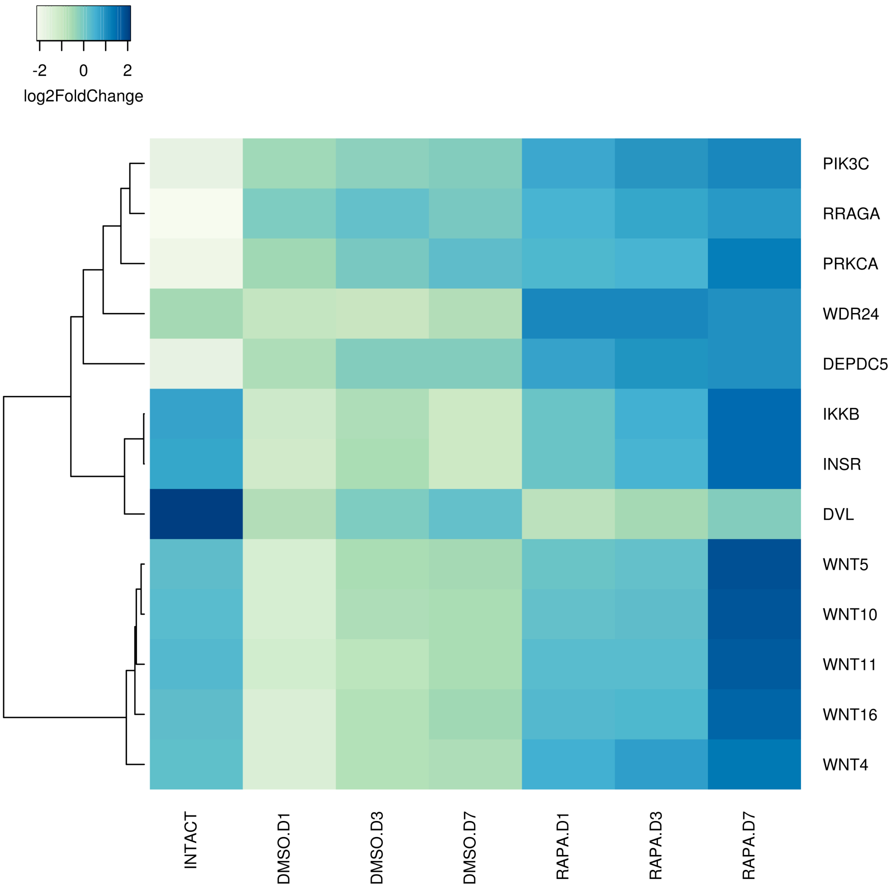


C. Heat map showing the log2fold change of the FPKM values of the DEGs representing the AMPK signaling pathway

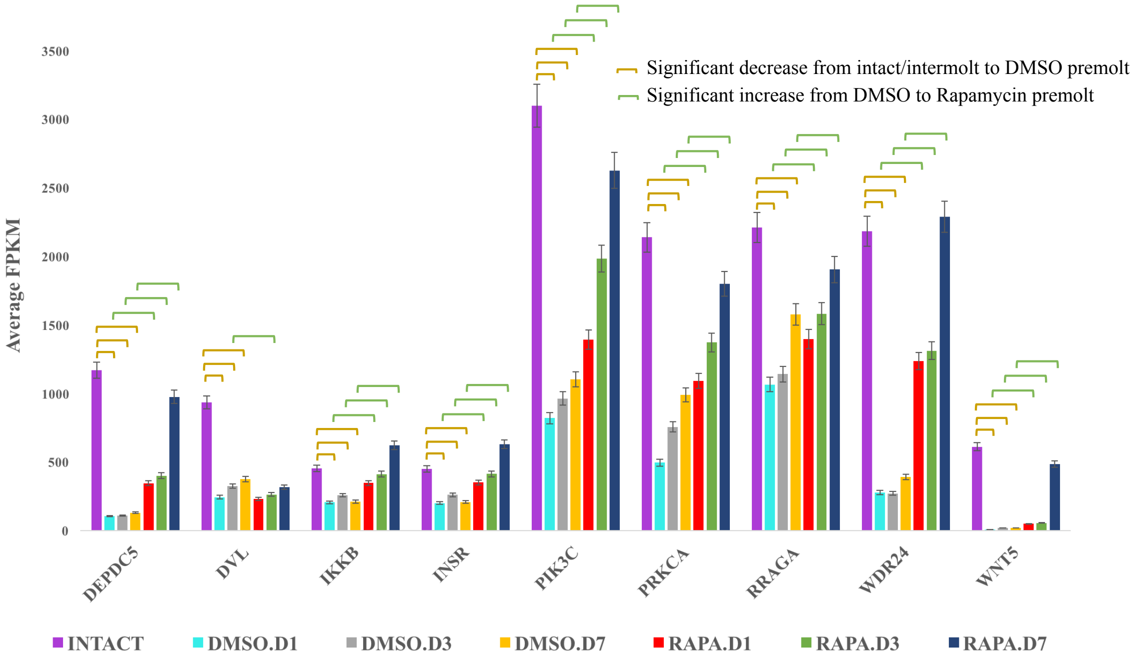




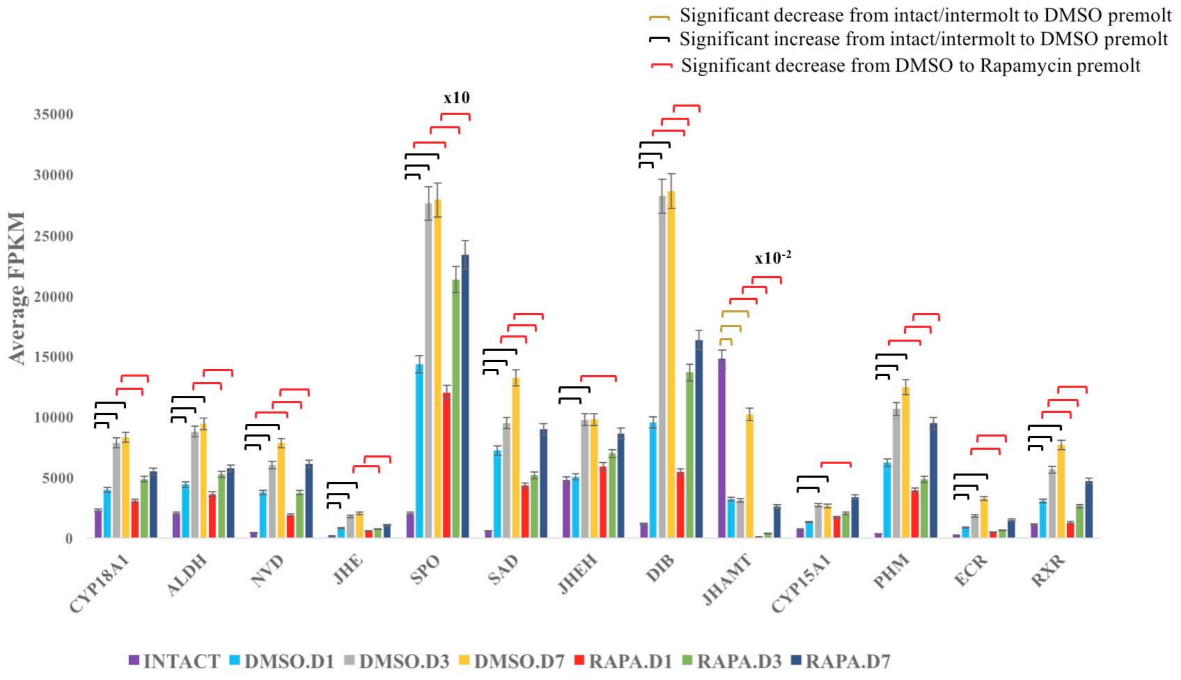
E. Heat map showing the log2fold change of the FPKM values of the DEGs representing the Wnt signaling pathway



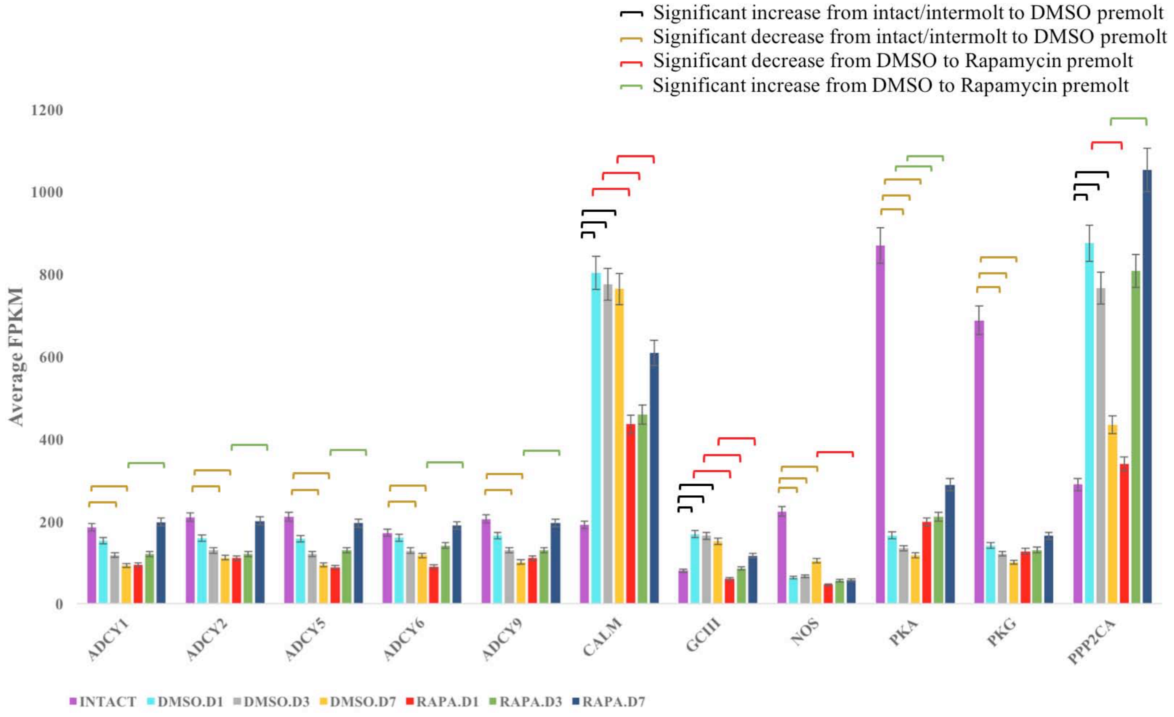
F. Changes in mRNA abundance for Wnt signaling pathway genes.



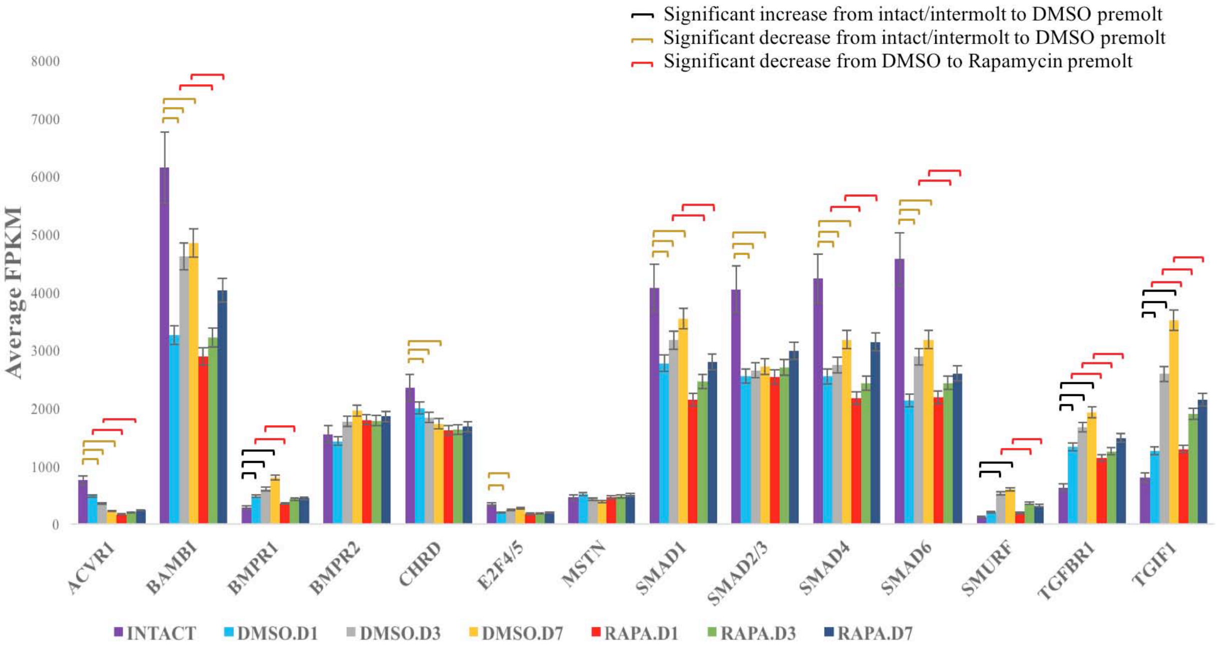
G. Changes in mRNA abundance for insect hormone biosynthetic pathway genes



H. Changes in mRNA abundance for Molt Inhibiting Hormone pathway genes



I. Changes in mRNA abundance for TGFβ pathway genes



Supplementary Figure 5. Graphical representation of expression levels of selected components from four signaling pathways: mTOR/WNT/AMPK/MIH/TGFβ (A) Heat map for mTOR DEGs; (B) Histogram identifying significant differences in average FPKM values across libraries for mTOR pathway genes; (C) Heat map for AMPK DEGs; (D) Histogram identifying significant differences in average FPKM values across libraries for AMPK pathway genes; (E) Heat map for Wnt DEGs; (F) Histogram identifying significant differences in average FPKM values across libraries for Wnt pathway genes; (G) Histogram identifying significant differences in average FPKM values across libraries for insect hormone biosynthetic pathway genes (note scale change for SPO [10x increase] and JHAMT [100x decrease]); (H) Histogram identifying significant differences in average FPKM values across libraries for Molt Inhibiting Hormone pathway genes; (I) Histogram identifying significant differences in average FPKM values across libraries for TGFβ pathway genes. The differentially expressed genes were identified via ANOVA. Statistical significance was detected at 0.05 p-value. A total of 464 genes were selected from these four pathways, of which 106 were differentially expressed across the molt cycle.

	Treatment	**Time**
Sample 1	IN	0d
Sample 2	IN	0d
Sample 3	DMSO	1d
Sample 4	DMSO	1d
Sample 5	DMSO	1d
Sample 6	Rapamycin	1d
Sample 7	Rapamycin	1d
Sample 8	Rapamycin	1d
Sample 9	DMSO	3d
Sample 10	DMSO	3d
Sample 11	DMSO	3d
Sample 12	Rapamycin	3d
Sample 13	Rapamycin	3d
Sample 14	Rapamycin	3d
Sample 15	DMSO	7d
Sample 16	DMSO	7d
Sample 17	DMSO	7d
Sample 18	Rapamycin	7d
Sample 19	Rapamycin	7d
Sample 20	Rapamycin	7d

Supplementary Table 1: Design matrix for the generalized linear (GLM) fit model used for DEG analysis (see text, section 4.4). note IN1 (intact/ intermolt replicate library 1)

Supplementary Scripts

Protocols and command line

All computational jobs were performed on a local departmental computer unless otherwise mentioned in text, section 4.6.

```
### Quality check using fastQC ###
fastqc ./*.fq.gz


### De novo Assembly using Trinity ###

Trinity --seqType fq --max_memory 180G \
--left ESA_forward_paired.fq.gz \
--right ESA_reverse_paired.fq.gz \
--CPU 12 --output ESA_Trinity.fasta --full_cleanup

### Running cdHIT on the trinity output for clustering the files ###

time cd-hit -T 12 -i ESA_Trinity.fasta -o ESA_99-Cdhit.fasta -c 0.99 -n 5 -M 30000 -d 0 -p 1

### Assembly quality check assesment ###

/opt/trinity/2.0.6/gcc/util/TrinityStats.pl ESA_reference.fasta

### Bowtie2 build indexer ###

bowtie2-build ESA_reference_09_20_16.fasta ESA_ref.bt2

### Running bowtie 2 ###

bowtie2 -p 12 --time -x ESA_ref.bt2 -1 ESA_FR_paired.fq.gz -2 ESA_RV_reverse.fq.gz -S ESA.sam.gz

### Converting sam fle into bam files by running Samtools ###

samtools view -b -S -o ESA.bam ESA.sam.gz

### Running express to get the count matrix ###

./express --rf-stranded -o ESA_reference_09_20_16.fasta ESA.bam

### Running transdecoder ###
```

```
/opt/transdecoder/2.0.1/gcc/TransDecoder.LongOrfs -t
/media/sharmishtha/LimbBudSharmi/LB-NGS/assembled_LB/LB_ref98_CD-06-01.fasta

### Downloaded SwissProt and pfam Database on May 11 2016 ###

# SwissProt Download SwissProt here: https:
//data.broadinstitute.org/Trinity/Trinotate_v3_RESOURCES/uniprot_sprot.pep.gz
gunzip uniprot_sprot.pep.gz
makeblastdb -in uniprot_sprot.pep -dbtype prot

# Pfam domains Download Pfam-A here: https:
//data.broadinstitute.org/Trinity/Trinotate_v3_RESOURCES/Pfam-A.hmm.gz
gunzip Pfam-A.hmm.gz
hmmpress Pfam-A.hmm

### Downloaded Uniref90 Database on Sep 28 2016 ###

wget
https://data.broadinstitute.org/Trinity/Trinotate_v2.0_RESOURCES/uniprot_uniref90.trinotate_
v2.0.pep.gz
gzip -d uniprot_uniref90.trinotate_v2.0.pep.gz # unzipping the the database
makeblastdb -in uniprot_uniref90.trinotate_v2.0.pep.gz -parse_seqids -dbtype prot # make the
database

### Blasting against Swissprot database ###

blastx -query ESA_reference_09_20_16.fasta -db uniprot_sprot.pep -num_threads 12 -
max_target_seqs 1 -outfmt 6 -out ESA_SwPr_BlstX.outfmt6

blastp -query ESA_ref_092016.pep -db uniprot_sprot.pep -num_threads 12 -max_target_seqs 1
-outfmt 6 -out ESA_SwPr_BlstP.outfmt6

### Running blast against Uniref90 on Sep 28 2016 ###

blastx -query ESA_reference_09_20_16.fasta -db uniprot_uniref90.trinotate_v2.0.pep -
num_threads 12 -max_target_seqs 1 -outfmt 6 -out ESA_Uniref90_BlstX.outfmt6

blastp -query ESA_ref_092016.pep -db uniprot_uniref90.trinotate_v2.0.pep -num_threads 12 -
max_target_seqs 1 -outfmt 6 -out ESA_Uniref90_BlastP.outfmt6

### Running HMMER to identify protein domains ###

hmmscan --cpu 12 --domtblout ESA_PFAM.out Pfam-A.hmm ESA_ref_092016.pep >
```

```
ESA_PFAM.log

### Running SignalP  ###

signalp -f short -n ESA_signalP.out ESA_ref_092016.pep

### Running tmHMM ###

tmhmm --short ESA_ref_092016.pep > ESA_tmhmm.out


### Running transdecoder ###
TransDecoder.LongOrfs -t ESA_reference.fasta

### Making the gene trans map for Trinotate ###

/opt/trinity/2.0.6/gcc/util/support_scripts/get_Trinity_gene_to_trans_map.pl
ESA_reference_09_20_16.fasta >> ESA_reference_09_20_16.fasta.gene_trans_map

### Running Trinotate ###

Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite init --gene_trans_map
ESA_reference_09_20_16.fasta.gene_trans_map --transcript_fasta
ESA_reference_09_20_16.fasta --transdecoder_pep ESA_ref_092016.pep

Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_swissprot_blastp
ESA_SwPr_BlstP.outfmt6
Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_trembl_blastp
ESA_Uniref90_BlastP.outfmt6
Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_pfam ESA_PFAM.out
Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_tmhmm
ESA_tmhmm.out
Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_signalp ESA_signalP.out

Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_swissprot_blastx
ESA_SwPr_BlstX.outfmt6
Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite LOAD_trembl_blastx
ESA_Uniref90_BlstX.outfmt6

Trinotate Trinotate.sprot_uniref90.20150131.boilerplate.sqlite report -E 1e-5 --pfam_cutoff
DNC > ESA_annotation_report_31Oct2016.xls

### RUVg, Filtering and EdgeR ###
```

```
source("https://bioconductor.org/biocLite.R")
biocLite("RUVSeq")
biocLite("zebrafishRNASeq")
biocLite("edgeR")

library(edgeR)
library(RUVSeq)

### Filtering the data ###

setwd("~/Google Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq")
Ruvg_count_matrix <- read.csv("~/Google Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq/Ruvg_count_matrix.csv", row.names=1)
View(Ruvg_count_matrix)
keep <- rowSums(cpm(Ruvg_count_matrix)>1) >= 3
Ruvg_count_matrix <- Ruvg_count_matrix[keep,]
filter <- apply(Ruvg_count_matrix, 1, function(x) length(x[x>5])>=2)
# arranging the file for further analysis
filtered <- Ruvg_count_matrix[filter,]
write.csv(filtered,file="~/Google Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq/EdgeRRUVgcount_matrix.csv")

## try http:// if https:// URLs are not supported
source("https://bioconductor.org/biocLite.R")
biocLite("RUVSeq")
biocLite("zebrafishRNASeq")
biocLite("edgeR")

library(edgeR)
library(RUVSeq)

### Filter the data ########
setwd("~/Google Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq")
Ruvg_count_matrix <- read.csv("~/Google Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq/Ruvg_count_matrix.csv", row.names=1)
View(Ruvg_count_matrix)
keep <- rowSums(cpm(Ruvg_count_matrix)>1) >= 3
Ruvg_count_matrix <- Ruvg_count_matrix[keep,]
head(Ruvg_count_matrix) #shows the first few lines of the dataset
tail(Ruvg_count_matrix) # shows the last few lines of the dataset

### read in data from csv file
### while importing the database select heading=Yes and Rownames=Use first column
filter <- apply(Ruvg_count_matrix, 1, function(x) length(x[x>5])>=2)
```

```
# arranging the file for further analysis
filtered <- Ruvg_count_matrix[filter,] # puts the filtered dataset in the filtered filename for
further use
write.csv(filtered,file="~/Google
Drive/PDF/NGS/ESA_Bowtie2_xprs/RUVSeq/EdgeRRUVgcount_matrix.csv")
genes <- rownames(filtered)[grep("c", rownames(filtered))] #tells the number of transcripts in
the filtered data without the ERCC spikeins

spikes <- rownames(filtered)[grep("ERCC", rownames(filtered))] #tells you the number of
transcripts for ERCC spikeins in the filtered data

#x <- as.factor(rep(c("DA","DC","DG","IN","RA","RC","RG"), each=3)) # picks out the rows
defined according to the experimental and control
groups = c("DA","DA", "DA", "DC","DC" ,"DC", "DG","DG", "DG", "IN",
"IN","RA","RA","RA", "RC", "RC", "RC","RG", "RG","RG") # picks out the rows defined

x <- as.factor (groups)
x
set <- newSeqExpressionSet(as.matrix(filtered),
                    phenoData = data.frame(x, row.names=colnames(filtered)))
set

library(RColorBrewer)
colors <- brewer.pal(7, "Set2")
tiff ("no_norm_Final.tiff", height = 6, width = 8,
    units = 'in', res = 1000, compression = 'lzw')
plotRLE(set, outline=FALSE, ylim=c(-8, 8), col=colors[x])
dev.off()
tiff ("no_norm_Final.tiff", height = 6, width = 8,
    units = 'in', res = 1000, compression = 'lzw')
plotPCA(set, col=colors[x], cex=1)
dev.off()


set1 <- betweenLaneNormalization(set, which="upper")
tiff ("norm2_Final.tiff", height = 6, width = 8,
    units = 'in', res = 1000, compression = 'lzw')
plotRLE(set1, outline=FALSE, ylim=c(-8, 8), col=colors[x])
dev.off()
tiff ("norm2_final.tiff", height = 6, width = 8,
    units = 'in', res = 1000, compression = 'lzw')
plotPCA(set1, col=colors[x], cex=1)
dev.off()
```

```
set2 <- RUVg(set1, spikes, k=1)
pData(set2)
tiff ("ERCC_Norm2_Final.tiff", height = 6, width = 8,
     units = 'in', res = 1000, compression = 'lzw')
plotRLE(set2, outline=FALSE, ylim=c(-8, 8), col=colors[x])
dev.off()
tiff ("ERCC_Norm2_Final.tiff", height = 6, width = 8,
     units = 'in', res = 1000, compression = 'lzw')
plotPCA(set2, cex=1, col=colors[x])
dev.off()

# saving the set2 file from RUVg as a .csv for further EdgeR analysis
write.csv(set2@assayData$normalizedCounts, "~/Google
Drive/PDF/NGS/Trinotate/EdgeR/FilteredNormalizedRUVg_ESA.csv")


#########################
### EdgeR
#########################

biocLite("NBPSeq")
biocLite("edgeR")
library(NBPSeq)
library(edgeR)
setwd("~/Google Drive/PDF/NGS/Trinotate/EdgeR")
CountMatrix <- read.csv("~/Google
Drive/PDF/NGS/Trinotate/EdgeR/FilteredNormalizedRUVg_ESA.csv",
stringsAsFactors=FALSE)
targets <- read.csv("~/Google Drive/PDF/NGS/Trinotate/EdgeR/GroupESA_EdgeR.csv")
Group <- factor(paste(targets$Treat,targets$Time,sep="."))
cbind(targets,Group=Group)
design <- model.matrix(~0+Group)
colnames(design) <- levels(Group)

### Making the count matrix with treatment and time
### IN corresponds to I (intact/intermolt)


ModifiedCountMatrix <- DGEList(counts=CountMatrix[,2:21],
group=Group,genes=CountMatrix[,1:2])
ModifiedCountMatrix
DispersedModifiedCountMatrix <- estimateDisp(ModifiedCountMatrix, design)
fit <- glmFit(DispersedModifiedCountMatrix, design)
my.contrasts <- makeContrasts(DMSO.1dvsDMSO.3d = (DMSO.1d-IN.0d)-(DMSO.3d-IN.0d),
                    DMSO.3dvsDMSO.7d = (DMSO.3d-IN.0d)-(DMSO.7d-IN.0d),
                    DMSO.1dvsDMSO.7d = (DMSO.1d-IN.0d)-(DMSO.7d-IN.0d),
                    Rapamycin.1dvsRapamycin.3d = (Rapamycin.1d-IN.0d)-(Rapamycin.3d-
IN.0d),
                    Rapamycin.1dvsRapamycin.7d = (Rapamycin.1d-IN.0d)-(Rapamycin.7d-
IN.0d),
                    Rapamycin.3dvsRapamycin.7d = (Rapamycin.3d-IN.0d)-(Rapamycin.7d-
IN.0d),
                    DMSO.1dvsRapamycin.1d = (DMSO.1d-IN.0d)-(Rapamycin.1d-IN.0d),
                    DMSO.3dvsRapamycin.3d = (DMSO.3d-IN.0d)-(Rapamycin.3d-IN.0d),
                    DMSO.7dvsRapamycin.7d = (DMSO.7d-IN.0d)-(Rapamycin.7d-IN.0d),
                    levels=design)



lrt.DAvsRA <- glmLRT(fit, contrast=my.contrasts[,"DMSO.1dvsRapamycin.1d"])
topTags(lrt.DAvsRA)
ArrangedlrtDAvsRA <- topTags(lrt.DAvsRA, n=35696)
Gene <- ArrangedlrtDAvsRA[[1]]$X
log2FoldChange <- ArrangedlrtDAvsRA[[1]]$logFC
pvalue <- ArrangedlrtDAvsRA[[1]]$PValue
padj <- ArrangedlrtDAvsRA[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDAvsRA<-subset(res,padj<0.05)
DiffExpGenesDAvsRA<-significantresDAvsRA$Gene

lrt.DCvsRC <- glmLRT(fit, contrast=my.contrasts[,"DMSO.3dvsRapamycin.3d"])
topTags(lrt.DCvsRC)
ArrangedlrtDCvsRC <- topTags(lrt.DCvsRC, n=35696)
Gene <- ArrangedlrtDCvsRC[[1]]$X
log2FoldChange <- ArrangedlrtDCvsRC[[1]]$logFC
pvalue <- ArrangedlrtDCvsRC[[1]]$PValue
padj <- ArrangedlrtDCvsRC[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDCvsRC<-subset(res,padj<0.05)
DiffExpGenesDCvsRC<-significantresDCvsRC$Gene

lrt.DGvsRG <- glmLRT(fit, contrast=my.contrasts[,"DMSO.7dvsRapamycin.7d"])
topTags(lrt.DGvsRG)
ArrangedlrtDGvsRG <- topTags(lrt.DGvsRG, n=35696)
Gene <- ArrangedlrtDGvsRG[[1]]$X
log2FoldChange <- ArrangedlrtDGvsRG[[1]]$logFC
pvalue <- ArrangedlrtDGvsRG[[1]]$PValue
padj <- ArrangedlrtDGvsRG[[1]]$FDR
```

```
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDGvsRG<-subset(res,padj<0.05)
DiffExpGenesDGvsRG<-significantresDGvsRG$Gene

lrt.DAvsDC <- glmLRT(fit, contrast=my.contrasts[,"DMSO.1dvsDMSO.3d"])
topTags(lrt.DAvsDC)
ArrangedlrtDAvsDC <- topTags(lrt.DAvsDC, n=35696)
Gene <- ArrangedlrtDAvsDC[[1]]$X
log2FoldChange <- ArrangedlrtDAvsDC[[1]]$logFC
pvalue <- ArrangedlrtDAvsDC[[1]]$PValue
padj <- ArrangedlrtDAvsDC[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDAvsDC<-subset(res,padj<0.05)
DiffExpGenesDAvsDC<-significantresDAvsDC$Gene

lrt.DAvsDG <- glmLRT(fit, contrast=my.contrasts[,"DMSO.1dvsDMSO.7d"])
topTags(lrt.DAvsDG)
ArrangedlrtDAvsDG <- topTags(lrt.DAvsDG, n=35696)
ArrangedlrtDAvsDG
Gene <- ArrangedlrtDAvsDG[[1]]$X
log2FoldChange <- ArrangedlrtDAvsDG[[1]]$logFC
pvalue <- ArrangedlrtDAvsDG[[1]]$PValue
padj <- ArrangedlrtDAvsDG[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDAvsDG<-subset(res,padj<0.05)
DiffExpGenesDAvsDG<-significantresDAvsDG$Gene

lrt.DCvsDG <- glmLRT(fit, contrast=my.contrasts[,"DMSO.3dvsDMSO.7d"])
topTags(lrt.DCvsDG)
ArrangedlrtDCvsDG <- topTags(lrt.DCvsDG, n=35696)
Gene <- ArrangedlrtDAvsDC[[1]]$X
log2FoldChange <- ArrangedlrtDCvsDG[[1]]$logFC
pvalue <- ArrangedlrtDCvsDG[[1]]$PValue
padj <- ArrangedlrtDCvsDG[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresDCvsDG<-subset(res,padj<0.05)
DiffExpGenesDCvsDG<-significantresDCvsDG$Gene



lrt.RAvsRC <- glmLRT(fit, contrast=my.contrasts[,"Rapamycin.1dvsRapamycin.3d"])
topTags(lrt.RAvsRC)
ArrangedlrtRAvsRC <- topTags(lrt.RAvsRC, n=35696)
Gene <- ArrangedlrtRAvsRC[[1]]$X
```

```
log2FoldChange <- ArrangedlrtRAvsRC[[1]]$logFC
pvalue <- ArrangedlrtRAvsRC[[1]]$PValue
padj <- ArrangedlrtRAvsRC[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresRAvsRC<-subset(res,padj<0.05)
DiffExpGenesRAvsRC<-significantresRAvsRC$Gene


lrt.RAvsRG <- glmLRT(fit, contrast=my.contrasts[,"Rapamycin.1dvsRapamycin.7d"])
topTags(lrt.RAvsRG)
ArrangedlrtRAvsRG <- topTags(lrt.RAvsRG, n=35696)
Gene <- ArrangedlrtRAvsRG[[1]]$X
log2FoldChange <- ArrangedlrtRAvsRG[[1]]$logFC
pvalue <- ArrangedlrtRAvsRG[[1]]$PValue
padj <- ArrangedlrtRAvsRG[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresRAvsRG<-subset(res,padj<0.05)
DiffExpGenesRAvsRG<-significantresRAvsRG$Gene


lrt.RCvsRG <- glmLRT(fit, contrast=my.contrasts[,"Rapamycin.3dvsRapamycin.7d"])
topTags(lrt.RCvsRG)
ArrangedlrtRCvsRG <- topTags(lrt.RCvsRG, n=35696)
Gene <- ArrangedlrtRCvsRG[[1]]$X
log2FoldChange <- ArrangedlrtRCvsRG[[1]]$logFC
pvalue <- ArrangedlrtRCvsRG[[1]]$PValue
padj <- ArrangedlrtRCvsRG[[1]]$FDR
res <- data.frame(Gene,log2FoldChange,pvalue,padj)
significantresRCvsRG<-subset(res,padj<0.05)
DiffExpGenesRCvsRG<-significantresRCvsRG$Gene

### Gives you the differentially expressed genes in the whole dataset ###

DiffExpGenesAll<-
c(as.character(DiffExpGenesRAvsRC),as.character(DiffExpGenesRAvsRG),as.character(DiffExpGenesRCvsRG),as.character(DiffExpGenesDAvsDC),as.character(DiffExpGenesDAvsDG),as.character(DiffExpGenesDCvsDG),as.character(DiffExpGenesDAvsRA),as.character(DiffExpGenesDCvsRC),as.character(DiffExpGenesDGvsRG))

DiffExpGenesAll<-data.frame(unique(DiffExpGenesAll))
head(DiffExpGenesAll)
write.csv(DiffExpGenesAll,file="~/Google Drive/PDF/NGS/Trinotate/EdgeR/DiffExpGenesAll.csv")
```

```
# Make a basic volcano plot
with(res, plot(log2FoldChange, -log10(pvalue), col = "grey", pch=20, main="DMSO-Day7 vs
Rapamycin-Day7", xlim=c(-12,12),ylim=c(0,12)))
# Add colored points: red if padj<0.5, orange of log2FC>1, green if both)
with(subset(res, padj<.50  & log2FoldChange>3), points(log2FoldChange, -log10(pvalue),
pch=20, col="green"))
with(subset(res, padj<.50  & log2FoldChange<(-3)), points(log2FoldChange, -log10(pvalue),
pch=20, col="red"))
```