2 Differential Gene Expression Analysis and GO Enrichment

Stefan Siebert and Bryan Teefy

08/09/2019

Differential Gene Expression

We performed differential gene expression analysis between wildtype and *hywi* knockdown animals. As a first step we generated expression estimates using RSEM/bowtie and used reads that were filtered for adapters (TruSeq3) using trimmomatic (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). We made use of the RSEM functions rsem-calculate-expression (–forward-prob 0) and rsem-generate-data-matrix to generate an expression matrix (scripts: DGE_expression.sh, DGE_count_matrix.sh). The raw count matrix is available at GEO (GSE135440).

Load the expression data / GO annotations

```
# Load the Differential Gene Expression Count Matrix

counts <- read.table("objects/Differential_Gene_Expression_Count_Matrix.txt", sep = "\t",
    check.names = FALSE, header = TRUE, row.names = 1)

# Load normalized piRNA and degradome reads and transcript characterization from
# RMD1

piRNA_Deg_counts <- read.table("objects/Annotated_piRNA_Degradome_Count_Matrix.txt",
    sep = "\t", check.names = FALSE, header = TRUE)
```

Load Required Packages

```
library(edgeR)
library(knitr)
library(xtable)
library(ggplot2)
```

Data exploration

We explore the data to get insights into the paired nature of the triplicate samples from wildtype and *hywi* knockdown tissue.

```
# We first calculate normalized counts for future use and to include them in the
# master dataframe.

# set gene IDs as rownames
rownames(counts) <- counts[, 1]
```

```r
# raw counts from all treatments
k <- counts[, c(2:7)]

# calculate normalization factors
nf_k <- calcNormFactors(k)

# calculate library sizes
ls_k <- colSums(k)

# effective library size using normalization factors
lse_k <- ls_k * nf_k

# normalization multiplier to use on counts
nm_k <- 1e+06/lse_k

# normalize counts using normalization multiplier
k <- k * nm_k

# round normalized counts
k <- round(k, digits = 0)

# restore ID column
k$ID <- rownames(k)

# reorder columns
k <- k[, c(7, 1:6)]

# combine rounded raw and normalized counts
k <- merge(counts[, c(1:7)], k, by = "ID")

# rename columns

colnames(k) <- c("ID", "WT1", "WT2", "WT3", "KD1", "KD2", "KD3", "nWT1", "nWT2",
    "nWT3", "nKD1", "nKD2", "nKD3")
```

```r
# explore replication

# define treatment groups for DGE
TR_k <- factor(c("k", "k", "k", "w", "w", "w"))

# set wild type as reference
TR_k <- relevel(TR_k, ref = "w")

# create experimental design data frame defining treatment type for each sample
d_k <- data.frame(Sample = colnames(counts[, c(5, 6, 7, 2, 3, 4)]), TR_k)

# generate DGEList object containing raw counts, the treatment type for each
# column, and the gene IDs
y <- DGEList(counts = counts[, c(5, 6, 7, 2, 3, 4)], group = d_k$TR_k, genes = counts[,
    1])

# label each column with the appropriate sample name
colnames(y) <- d_k$Sample
```

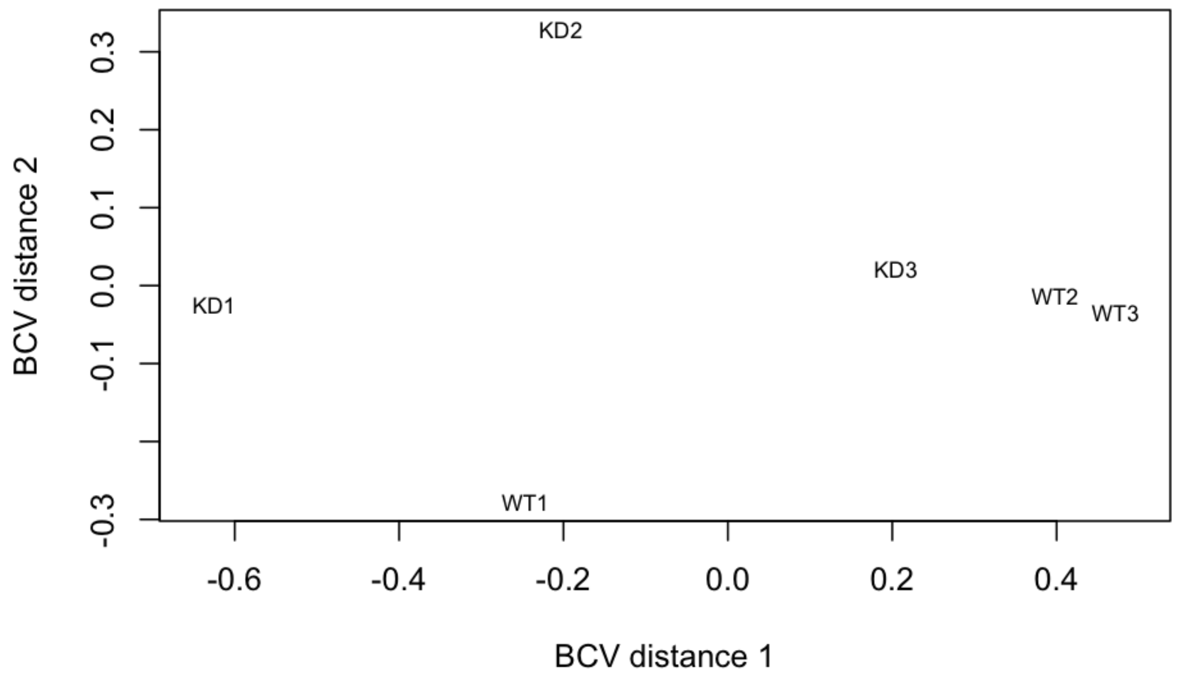
```r
# calculate library size for each sample and store within DGElist
y$samples$lib.size <- colSums(y$counts)

# exclude transcripts that do not have at least two samples with more than one
# count per million
keep <- rowSums(cpm(y) > 1) >= 2
y <- y[keep, , keep.lib.sizes = FALSE]

# number of transcripts remaining after count filtering
dim(y)
```

```
## [1] 16435     6
```

```r
# calculate normalization factors for each sample
y <- calcNormFactors(y)

# review library sizes and normalization factors
y$samples
```

```
##     group lib.size norm.factors
## KD1     k 27236412    1.0711124
## KD2     k 26955915    0.9927897
## KD3     k 27081381    1.0126300
## WT1     w 22165507    1.0181741
## WT2     w 27471090    0.9836625
## WT3     w 25597005    0.9272326
```

```r
# create a matrix defining the control and treatment groups for the analysis
# based on what is described by the TR object
design_k <- model.matrix(~TR_k)

# Estimate dispersions
y <- estimateDisp(y, design_k)

# generate MDS Plot

plotMDS(y, method = "bcv", cex = 0.7)
```



```r
# we exclude outlier replicates WT1 and KD3 from downstream analyses

# define treatment groups for DGE
TR_k <- factor(c("k", "k", "w", "w"))

# set wild type as reference
TR_k <- relevel(TR_k, ref = "w")

# create experimental design data frame defining treatment type for each sample
d_k <- data.frame(Sample = colnames(counts[, c(5, 6, 3, 4)]), TR_k)

# generate DGElist object containing raw counts, the treatment type for each
# column, and the gene annotations
y <- DGEList(counts = counts[, c(5, 6, 3, 4)], group = d_k$TR_k, genes = counts[,
    1])

# label each column with the appropriate sample name
colnames(y) <- d_k$Sample

# calculate library size for each sample and store within DGElist
y$samples$lib.size <- colSums(y$counts)

# exclude transcripts that do not have at least two samples with more than one
# count per million
keep <- rowSums(cpm(y) > 1) >= 2
y <- y[keep, , keep.lib.sizes = FALSE]
```

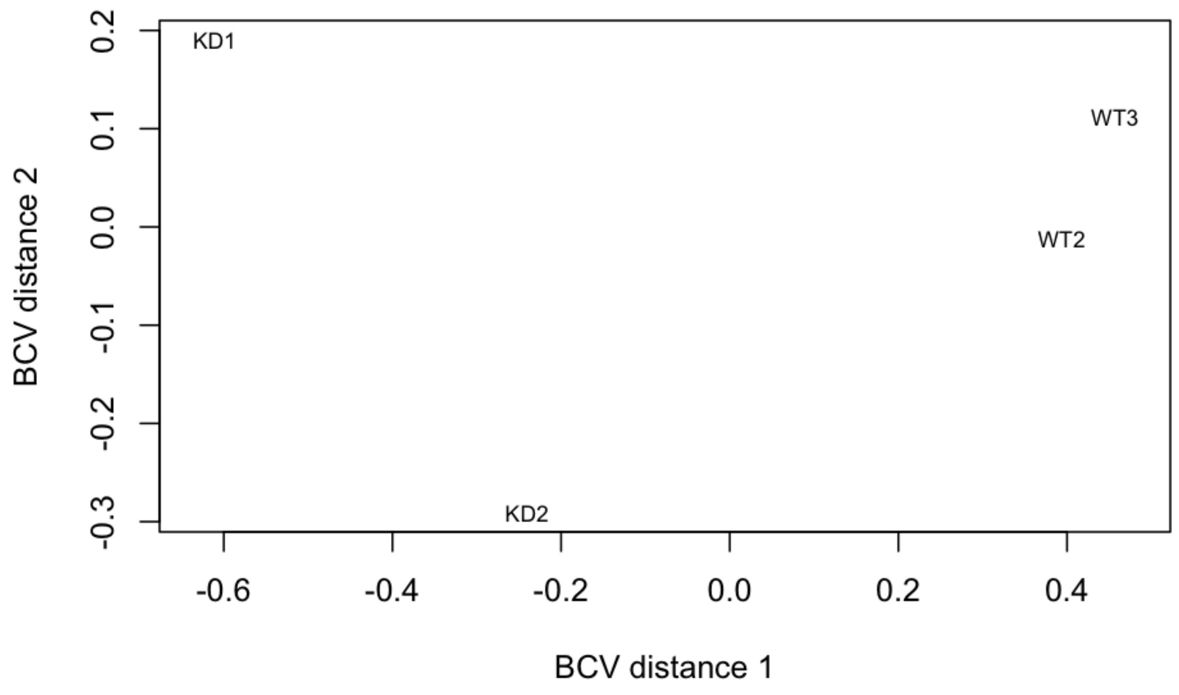
```r
# number of transcripts remaining after count filtering
dim(y)
```

```
## [1] 15924     4
```

```r
# calculate normalization factors for each sample
y <- calcNormFactors(y)

# review library sizes and normalization factors
y$samples
```

```
##     group lib.size norm.factors
## KD1     k 27217655    1.0849445
## KD2     k 26945252    0.9960237
## WT2     w 27460768    0.9922666
## WT3     w 25588479    0.9325978
```

```r
# create a matrix defining the control and treatment groups for the analysis
# based on what is described by the TR object
design_k <- model.matrix(~TR_k)

# estimate dispersions
y <- estimateDisp(y, design_k)

# generate MDS Plot
plotMDS(y, method = "bcv", cex = 0.7)
```



Differential Gene Expression (DGE) analysis - wildtype vs *hywi* knockdown.

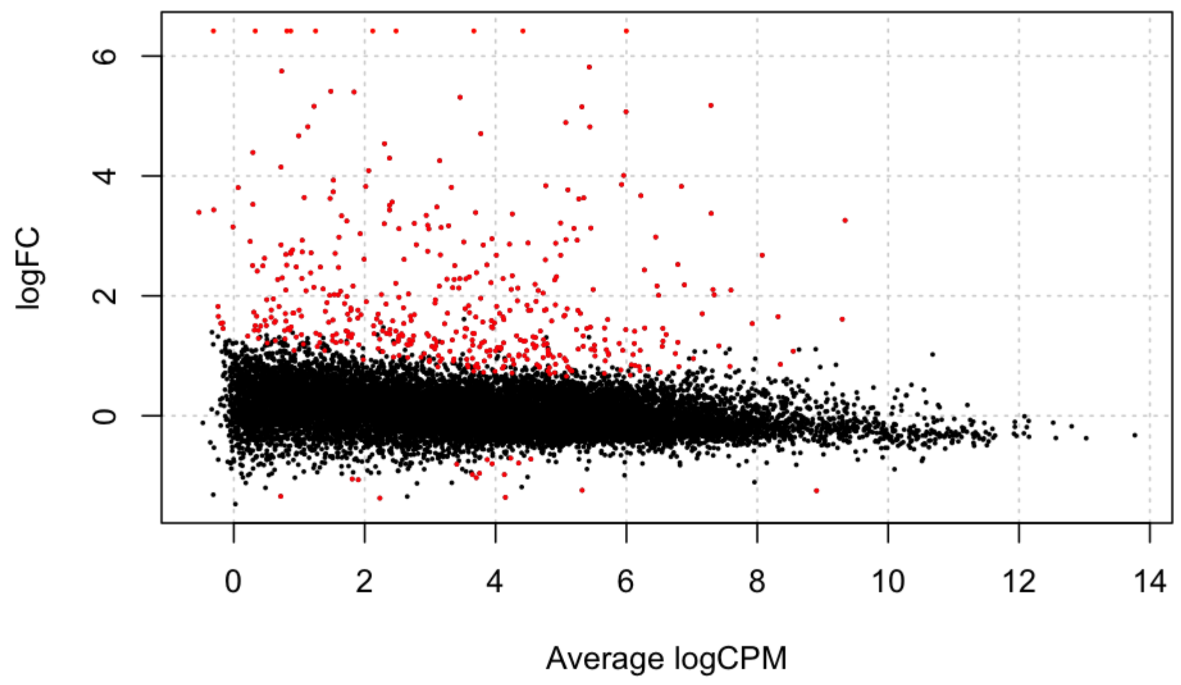
We perform DGE analysis after excluding outlier replicates WT1 and KD3.

```r
# DGE Analysis

# set p-value for cutoff
p.value = 0.05

# fit data to a negative binomial geralized log-linear model
fit_k <- glmFit(y, design_k)

# conduct a statistical test for differential gene expression based on the fit
# from
lrt_k <- glmLRT(fit_k)

# create a list of values that describes the status of each gene in the DGE test
de_k <- decideTestsDGE(lrt_k, adjust.method = "BH", p.value)

# overview of number of differentially expressed genes
summary(de_k)
```

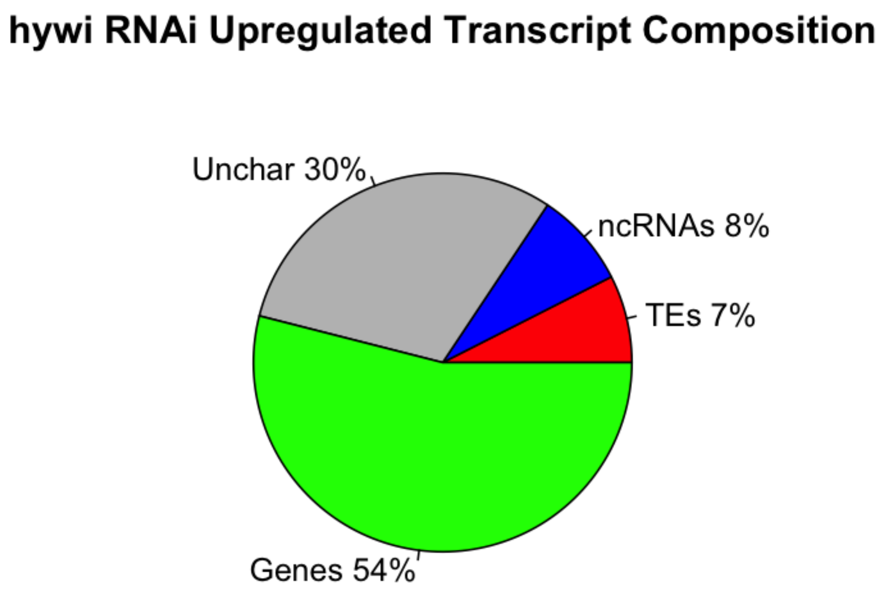
```
##        TR_kk
## Down      17
## NotSig 15466
## Up       441
```

```r
# build results table
D_k <- lrt_k$table

# restore ID column
D_k$ID <- rownames(D_k)

# add column of adjusted p values
D_k <- cbind(D_k, p.adjust(D_k$PValue, method = "BH"))
names(D_k)[names(D_k) == "p.adjust(D_k$PValue, method = \"BH\")"] = "k_Padj"

# create table summarizing rounded raw counts, normalized counts and DGE results
res_k <- merge(k, D_k, by = "ID", all = TRUE)

# call transcripts upregulated, downregulated, or unaffected

res_k$DE <- ifelse(res_k$k_Padj <= 0.05 & res_k$logFC >= 0 & !is.na(res_k$logFC &
    res_k$k_Padj), "Up", ifelse(res_k$k_Padj <= 0.05 & res_k$logFC <= 0 & !is.na(res_k$logFC &
    res_k$k_Padj), "Down", "None"))

# merge with the master dataframe

piRNA_Deg_counts <- merge(piRNA_Deg_counts, res_k, by = "ID")

# generate volcano plot

detags <- rownames(y)[as.logical(de_k)]
```

```r
plotSmear(lrt_k, de.tags = detags, cex = 0.2, cex.lab = 1, cex.axis = 1)
```



```r
# generate plot of upregulated transcripts expression fold change

Upreg_Types <- c(sum(piRNA_Deg_counts$DE == "Up" & piRNA_Deg_counts$Transcript_Class ==
    "TE"), sum(piRNA_Deg_counts$DE == "Up" & piRNA_Deg_counts$Transcript_Class ==
    "ncRNA"), sum(piRNA_Deg_counts$DE == "Up" & piRNA_Deg_counts$Transcript_Class ==
    "Unchar"), sum(piRNA_Deg_counts$DE == "Up" & piRNA_Deg_counts$Transcript_Class ==
    "Gene"))

lbls <- c("TEs", "ncRNAs", "Unchar", "Genes")
colors = c("red", "blue", "gray", "green")
pct <- round(Upreg_Types/sum(Upreg_Types) * 100)
lbls <- paste(lbls, pct)  # add percents to labels
lbls <- paste(lbls, "%", sep = "")  # ad % to labels
pie(Upreg_Types, labels = lbls, col = colors, main = "hywi RNAi Upregulated Transcript Composition")
```



##Somatic Hywi piRNA Mapping Ordering

To infer direct targets of Hywi, *hywi* RNAi upregulated transcripts can be described as high and low Hywi piRNA mapping transcripts. “High-mapping”" transcripts are likely to be direct Hywi targets while “low-mapping” transcripts are unlikely to be direct Hywi targets. High Mapping transcripts were defined as those transcripts that were in the top 20% of read counts per kilobase million after combining Colch Hywi Sense and Colch Hywi Antisense reads.

To infer which transcripts were most likely to be involved in the ping-pong cycle in somatic stem cells, transcripts were ordered by Colch Hywi Antisense reads per kilobase million. Transcripts that fall within the top 5% in this category were considered to be putative ping-pong transcripts.

To infer which transcripts may be “primary-processed” by Hywi in somatic stem cells, transcripts were ordered by Colch Hywi Sense reads per kilobase million. Transcripts that fall within the top 5% in this category were considered to be putative primary-processed transcripts.

```r
# generate RPM (Read counts Per kilobase Million) data for Colch Hywi
# Sense/Antisense piRNAs (i.e. summed Colch Hywi Sense/Antisense piRNA read
# counts mapped per kb of transcript per million piRNA read counts)

piRNA_Deg_counts$Somatic_Hywi_Perc <- (piRNA_Deg_counts$Colch_Hywi_AS_kb + piRNA_Deg_counts$Colch_Hywi_S
    piRNA_Deg_counts$Colch_Hywi_S_kb)/1e+06)

piRNA_Deg_counts$Somatic_Hywi_Perc <- ecdf(piRNA_Deg_counts$Somatic_Hywi_Perc)(piRNA_Deg_counts$Somatic_

# repeat for only Colch Hywi Antisense piRNAs

piRNA_Deg_counts$Somatic_Hywi_Perc_AS <- (piRNA_Deg_counts$Colch_Hywi_AS_kb)/(sum(piRNA_Deg_counts$Colch
    piRNA_Deg_counts$Colch_Hywi_S_kb)/1e+06)
```

```
piRNA_Deg_counts$Somatic_Hywi_Perc_AS <- ecdf(piRNA_Deg_counts$Somatic_Hywi_Perc_AS)(piRNA_Deg_counts$So

# repeat for only Colch Hywi Sense piRNAs

piRNA_Deg_counts$Somatic_Hywi_Perc_S <- (piRNA_Deg_counts$Colch_Hywi_S_kb)/(sum(piRNA_Deg_counts$Colch_H
    piRNA_Deg_counts$Colch_Hywi_S_kb)/1e+06)

piRNA_Deg_counts$Somatic_Hywi_Perc_S <- ecdf(piRNA_Deg_counts$Somatic_Hywi_Perc_S)(piRNA_Deg_counts$Soma

# write table that summarizes data write.table(piRNA_Deg_counts, file =
# 'Annotated_piRNA_Degradome_DGE_Count_Matrix.txt')
```

GO-Term Enrichment Analysis

GO-term enrichment analysis was performed on upregulated transcripts against the entire transcriptome to investigate a functional response to somatic *hywi* knockdown.

GO-term enrichment analysis was performed using goatools v0.6.10 using the script: goatools_GO_enrichment.pl

```
# load GO annotation results

GO_table <- read.table("objects/GO_upreg_trans_full_ref.txt", header = T, sep = "\t")

# take enriched biological processes

GO_table_sub <- subset(GO_table, p_bonferroni <= 0.05 & NS == "BP")

# include GO accession number, GO term, ratio in study, ratio in population,
# bonferroni corrected p-value, and transcript IDs

GO_table_sub <- GO_table_sub[, c(1, 4:6, 10, 14)]
colnames(GO_table_sub) <- c("GO", "GO_Term", "Study", "Pop", "p_val", "ID")

# print table

kable(GO_table_sub, format = "markdown", padding = 100)
```

GO	GO_Term	Study	Pop	p_val	ID
. . . GO: 0006952	defense response	26/441	753/38747	0.0142	t11117aep, t12198aep, t16424aep, t17178aep, t17750aep, t21013aep, t21682aep, t22133aep, t24687aep, t32280aep, t33020aep, t34385aep, t34424aep, t34475aep, t35573aep, t35608aep, t35837aep, t38672aep, t38673aep, t5914aep, t6387aep, t7326aep, t7388aep, t8582aep, t8645aep, t8946aep
. . . .GO: 0051899	membrane depolarization	7/441	46/38747	0.0170	t17803aep, t18338aep, t24938aep, t25020aep, t526aep, t527aep, t9936aep

GO	GO_Term	Study	Pop	p_val	ID
.......GO: 2000051	negative regulation of non-canonical Wnt signaling pathway	4/441	8/38747	0.0221	t29674aep, t30176aep, t33022aep, t8142aep
....GO: 0048440	carpel development	3/441	3/38747	0.0290	t35573aep, t38672aep, t38673aep
...GO: 0045087	innate immune response	14/441	258/38747	0.0370	t11117aep, t17178aep, t22133aep, t24687aep, t34385aep, t34424aep, t35573aep, t35608aep, t35837aep, t38672aep, t38673aep, t6387aep, t7326aep, t8946aep
.......GO: 0031050	dsRNA processing	5/441	19/38747	0.0377	t30770aep, t35488aep, t38672aep, t38673aep, t5914aep
......GO: 0042108	positive regulation of cytokine biosynthetic process	5/441	20/38747	0.0498	t21682aep, t22133aep, t24687aep, t34424aep, t8645aep

Software versions

This document was computed on Fri Aug 09 19:36:19 2019 with the following R package versions.

```
R version 3.5.3 (2019-03-11)
Platform: x86_64-apple-darwin15.6.0 (64-bit)
Running under: macOS Mojave 10.14.5

Matrix products: default
BLAS: /Library/Frameworks/R.framework/Versions/3.5/Resources/lib/libRblas.0.dylib
LAPACK: /Library/Frameworks/R.framework/Versions/3.5/Resources/lib/libRlapack.dylib

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

attached base packages:
[1] stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] ggplot2_3.2.0 xtable_1.8-3  edgeR_3.22.5  limma_3.38.3  knitr_1.22

loaded via a namespace (and not attached):
 [1] Rcpp_1.0.1       magrittr_1.5     splines_3.5.3    tidyselect_0.2.5
 [5] munsell_0.5.0    colorspace_1.4-1 lattice_0.20-38  R6_2.4.0
 [9] rlang_0.4.0      highr_0.7        dplyr_0.8.3      stringr_1.4.0
[13] tools_3.5.3      grid_3.5.3       gtable_0.3.0     xfun_0.5
[17] withr_2.1.2      htmltools_0.3.6  assertthat_0.2.1 yaml_2.2.0
[21] lazyeval_0.2.2   digest_0.6.20    tibble_2.1.3     crayon_1.3.4
[25] purrr_0.3.2      formatR_1.7      glue_1.3.1       evaluate_0.13
[29] rmarkdown_1.12   stringi_1.4.3    compiler_3.5.3   pillar_1.4.2
[33] scales_1.0.0     locfit_1.5-9.1   pkgconfig_2.0.2
```