DE analysis - Day1

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Contents

Overview

- Principal Investigator: Beth Overmoyer
- Experiment: RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141
- study 6 was excluded because if low read depth in 3373-3
- https://www.bioconductor.org/packages/release/bioc/vignettes/DEGreport/inst/doc/DEGreport.html
- AnnotationHub. We use ensembl version matching bcbio pipeline - v94.
- HBC materials
- HBC materials - functional analysis
- http://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html
- this is DE for Day1 samples

```
## Setup
### Bioconductor and CRAN libraries used

library(DESeq2)
```

```
## Loading required package: S4Vectors

## Loading required package: stats4

## Loading required package: BiocGenerics

## Loading required package: parallel

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:parallel':
##
##     clusterApply, clusterApplyLB, clusterCall, clusterEvalQ,
##     clusterExport, clusterMap, parApply, parCapply, parLapply,
##     parLapplyLB, parRapply, parSapply, parSapplyLB

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##     anyDuplicated, 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:base':
##
##     expand.grid
## 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
```

```r
library(tidyverse)
```

```
## -- Attaching packages --------------------------------------- tidyverse 1.3.0 --

## v ggplot2 3.3.3     v purrr   0.3.4
## v tibble  3.1.0     v dplyr   1.0.5
## v tidyr   1.1.3     v stringr 1.4.0
## v readr   1.4.0     v forcats 0.5.1
## -- Conflicts ------------------------------------------ tidyverse_conflicts() --
## x dplyr::collapse()   masks IRanges::collapse()
## x dplyr::combine()    masks Biobase::combine(), BiocGenerics::combine()
## x dplyr::count()      masks matrixStats::count()
## x dplyr::desc()       masks IRanges::desc()
## x tidyr::expand()     masks S4Vectors::expand()
## x dplyr::filter()     masks stats::filter()
## x dplyr::first()      masks S4Vectors::first()
## x dplyr::lag()        masks stats::lag()
## x ggplot2::Position() masks BiocGenerics::Position(), base::Position()
## x purrr::reduce()     masks GenomicRanges::reduce(), IRanges::reduce()
## x dplyr::rename()     masks S4Vectors::rename()
## x dplyr::slice()      masks IRanges::slice()
```

```
library(RColorBrewer)
library(pheatmap)
library(DEGreport)
library(tximport)
library(ggplot2)
library(ggrepel)
library(knitr)
library(AnnotationHub)
```

```
## Loading required package: BiocFileCache

## Loading required package: dbplyr

##
## Attaching package: 'dbplyr'

## The following objects are masked from 'package:dplyr':
##
##     ident, sql

##
## Attaching package: 'AnnotationHub'

## The following object is masked from 'package:Biobase':
##
##     cache
```

```
library(ensembldb)
```

```
## Loading required package: GenomicFeatures

## Loading required package: AnnotationDbi

##
## Attaching package: 'AnnotationDbi'

## The following object is masked from 'package:dplyr':
##
##     select

## Loading required package: AnnotationFilter

##
## Attaching package: 'ensembldb'

## The following object is masked from 'package:dplyr':
##
```

```
##     filter
```

```
## The following object is masked from 'package:stats':
##
##     filter
```

```
ggplot2::theme_set(theme_light(base_size = 14))

opts_chunk[["set"]](
    cache = FALSE,
    dev = c("png", "pdf"),
    error = TRUE,
    highlight = TRUE,
    message = FALSE,
    prompt = FALSE,
    tidy = FALSE,
    warning = FALSE)
```

```
# Have a folder called `data`, and copy your Salmon folders here from the cluster.
## List all directories containing data
### change the pattern to something specific to your Salmon folders
samples <- list.files(path = "./data/final",
                      full.names = T,
                      pattern = "^S")

## Obtain a vector of all filenames including the path
files <- file.path(samples, "salmon", "quant.sf")
files
```

```
##  [1] "./data/final/S3154-2/salmon/quant.sf"
##  [2] "./data/final/S3169-2/salmon/quant.sf"
##  [3] "./data/final/S3188-2/salmon/quant.sf"
##  [4] "./data/final/S3190-4/salmon/quant.sf"
##  [5] "./data/final/S3193-1/salmon/quant.sf"
##  [6] "./data/final/S3194-3/salmon/quant.sf"
##  [7] "./data/final/S3220-1/salmon/quant.sf"
##  [8] "./data/final/S3234-1/salmon/quant.sf"
##  [9] "./data/final/S3291-3/salmon/quant.sf"
## [10] "./data/final/S3292-3/salmon/quant.sf"
## [11] "./data/final/S3372-1/salmon/quant.sf"
## [12] "./data/final/S3374-2/salmon/quant.sf"
## [13] "./data/final/S3404-1/salmon/quant.sf"
## [14] "./data/final/S3424-1/salmon/quant.sf"
## [15] "./data/final/S3474-3/salmon/quant.sf"
## [16] "./data/final/S3477-1/salmon/quant.sf"
## [17] "./data/final/S3563-3/salmon/quant.sf"
## [18] "./data/final/S3582-4/salmon/quant.sf"
## [19] "./data/final/S3644-1/salmon/quant.sf"
## [20] "./data/final/S3652-1/salmon/quant.sf"
## [21] "./data/final/S3688-2/salmon/quant.sf"
## [22] "./data/final/S3697-3/salmon/quant.sf"
## [23] "./data/final/S3713-1/salmon/quant.sf"
## [24] "./data/final/S3715-2/salmon/quant.sf"
## [25] "./data/final/S3723-1/salmon/quant.sf"
## [26] "./data/final/S3728-3/salmon/quant.sf"
## [27] "./data/final/S3732-1/salmon/quant.sf"
## [28] "./data/final/S3741-3/salmon/quant.sf"
## [29] "./data/final/S3816-1/salmon/quant.sf"
## [30] "./data/final/S3822-1/salmon/quant.sf"
## [31] "./data/final/S3825-1/salmon/quant.sf"
## [32] "./data/final/S3837-2/salmon/quant.sf"
## [33] "./data/final/S4047-1/salmon/quant.sf"
## [34] "./data/final/S4056-1/salmon/quant.sf"
## [35] "./data/final/S4089-1/salmon/quant.sf"
## [36] "./data/final/S4101-3/salmon/quant.sf"
## [37] "./data/final/S4136-1/salmon/quant.sf"
## [38] "./data/final/S4144-2/salmon/quant.sf"
## [39] "./data/final/S4172-1/salmon/quant.sf"
## [40] "./data/final/S4176-3/salmon/quant.sf"
## [41] "./data/final/S4237-1/salmon/quant.sf"
## [42] "./data/final/S4249-1/salmon/quant.sf"
## [43] "./data/final/S4261-1/salmon/quant.sf"
## [44] "./data/final/S4295-5/salmon/quant.sf"
```

```
## Since all quant files have the same name it is useful to have names for each element
### change the string in str_replace so the pattern matches your filenames
names(files) <- str_replace(samples, "./data/final/", "")
```

```
# Load the data and metadata
meta <- read_csv("tables/metadata_corrected.csv") %>%
  column_to_rownames(var = "samplename") %>%
  dplyr::filter(treatment == "pre") %>%
  drop_na(response)
protein_coding_genes <- read_csv("tables/ensembl_w_description.protein_coding.csv")
```

```
# Connect to AnnotationHub
ah <- AnnotationHub()

# Query AnnotationHub
hs_ens <- query(ah, c("Homo sapiens", "EnsDb"))

# Get Ensembl94 - used in bcbio
hs_ens <- hs_ens[["AH64923"]]

# Extract gene-level information
txdb <- transcripts(hs_ens,
                    return.type = "data.frame") %>%
  dplyr::select(tx_id, gene_id)

genedb <- genes(hs_ens,
                return.type = "data.frame") %>%
  dplyr::select(gene_id, gene_name, symbol)

gene_symbol <- genedb %>% dplyr::select(gene_id, symbol)

hsdb <- inner_join(txdb, genedb)
write.table(hsdb,
            file = "data/ensembl94_hg38_annotations.txt",
            sep = "\t",
            row.names = F,
            quote = F)
```

```r
# Read in  a tx2gene file with transcript identifiers in the first column and gene identifiers in the s
#wormdb <- read.table("ensembl94_WBcel235_annotations.txt", sep="\t", header=T)
tx2gene <- hsdb[, c("tx_id", "gene_id")]


# Run tximport
files <- files[rownames(meta)]
txi_file <- "data/txi.day1.RDS"
if (file.exists(txi_file)){
    txi <- readRDS(txi_file)
}else{
    txi <- tximport(files,
                    type = "salmon",
                    tx2gene = tx2gene,
                    countsFromAbundance = "lengthScaledTPM",
                    ignoreTxVersion = FALSE)
    saveRDS(txi, txi_file)
}

# Look at the counts
class(txi)
```

```
## [1] "list"
```

```r
attributes(txi)
```

```
## $names
## [1] "abundance"           "counts"              "length"
## [4] "countsFromAbundance"
```

```r
txi$counts %>% View()
```

Checking to see that the transcript to gene mapping is correct

When you have annotations that are from a different source from your reference you can run into problems (i.e lose genes). Some checks you can do before proceeding:

1. Look at the dimensions of your count matrix. Do you have ~20k genes present? `dim(txi$counts)`
2. When running `tximport()` you will get a message in your console. If you see something like `transcripts missing from tx2gene` start troubleshooting.

```r
dim(txi$counts)
```

```
## [1] 58735    20
```

Sanity check that metadata matches your expression

It is always a good idea to check if:

1. Do you have expression data for all samples listed in your metadata?
2. Are the samples in your expression data in the same order as your metadata?

```r
### Check that sample names match in both files
all(colnames(txi$counts) %in% rownames(meta))
```

```
## [1] TRUE
```

```r
# Not the same? Make them the same
### This will change depending on what names you have listed!
#paste0(meta$samplename, "_", meta$library)
#rownames(meta) <- paste0(meta$samplename, "_", meta$library)
#meta$genotype <- relevel(meta$genotype, ref="Wildtype")

### Check that sample names match in both files
all(colnames(txi$counts) %in% rownames(meta))
```

```
## [1] TRUE
```

```r
### Check that all samples are in the same order
meta <- meta[colnames(txi$counts),]
all(colnames(txi$counts) == rownames(meta))
```

```
## [1] TRUE
```

Run DESeq2

```
estimating size factors
estimating dispersions
gene-wise dispersion estimates
mean-dispersion relationship
final dispersion estimates
fitting model and testing
```

- Estimating size factors and count normalization
- Gene-wise dispersions
- Mean-dispersion(variance) relationship and the Negative Binomial Model
- Model fitting and hypothesis testing

Wald test

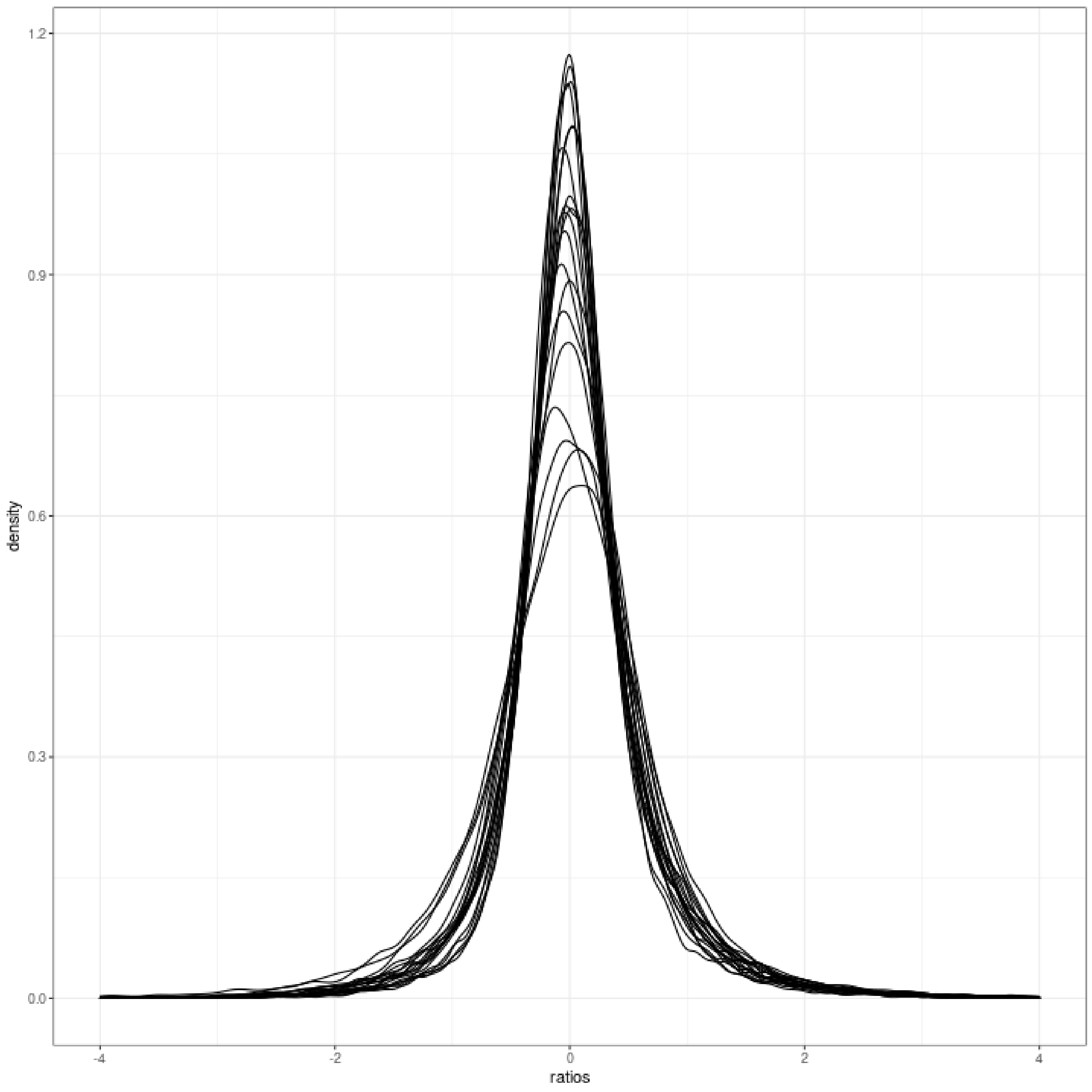
Here we subset protein coding genes.

```r
## Create DESeq2Dataset object
dds_file <- "data/dds.day1.RDS"
meta$treatment <- as.factor(meta$treatment)
meta$response <- as.factor(meta$response)
meta$er <- as.factor(meta$er)
meta$date_of <- as.factor(meta$date_of)
meta$tumor_percentage <- as.factor(meta$tumor_percentage)
meta$tumor_percentage_high <- as.factor(meta$tumor_percentage_high)

non_responders <- meta %>% dplyr::filter(study_id %in% c(2, 19)) %>% row.names()

if (file.exists(dds_file)){
    dds <- readRDS(dds_file)
}else{
    dds <- DESeqDataSetFromTximport(txi,
                                    colData = meta,
                                    design = ~response)

    #dds <- dds[,!colnames(dds) %in% non_responders]
    design(dds) <- formula(~response + er + tumor_percentage_high + date_of)
    # subset protein-coding genes
    pc_genes <- intersect(protein_coding_genes$ensembl_gene_id, row.names(dds))
    dds <- dds[pc_genes,]
    # 100 reads / 20 samples
    keep <- rowSums(counts(dds)) >= 100
    dds <- dds[keep,]

    # Run DESeq2
    dds <- DESeq(dds)
    saveRDS(dds, dds_file)
}
```

DEGreport QC

Size factor QC - samples 1-20

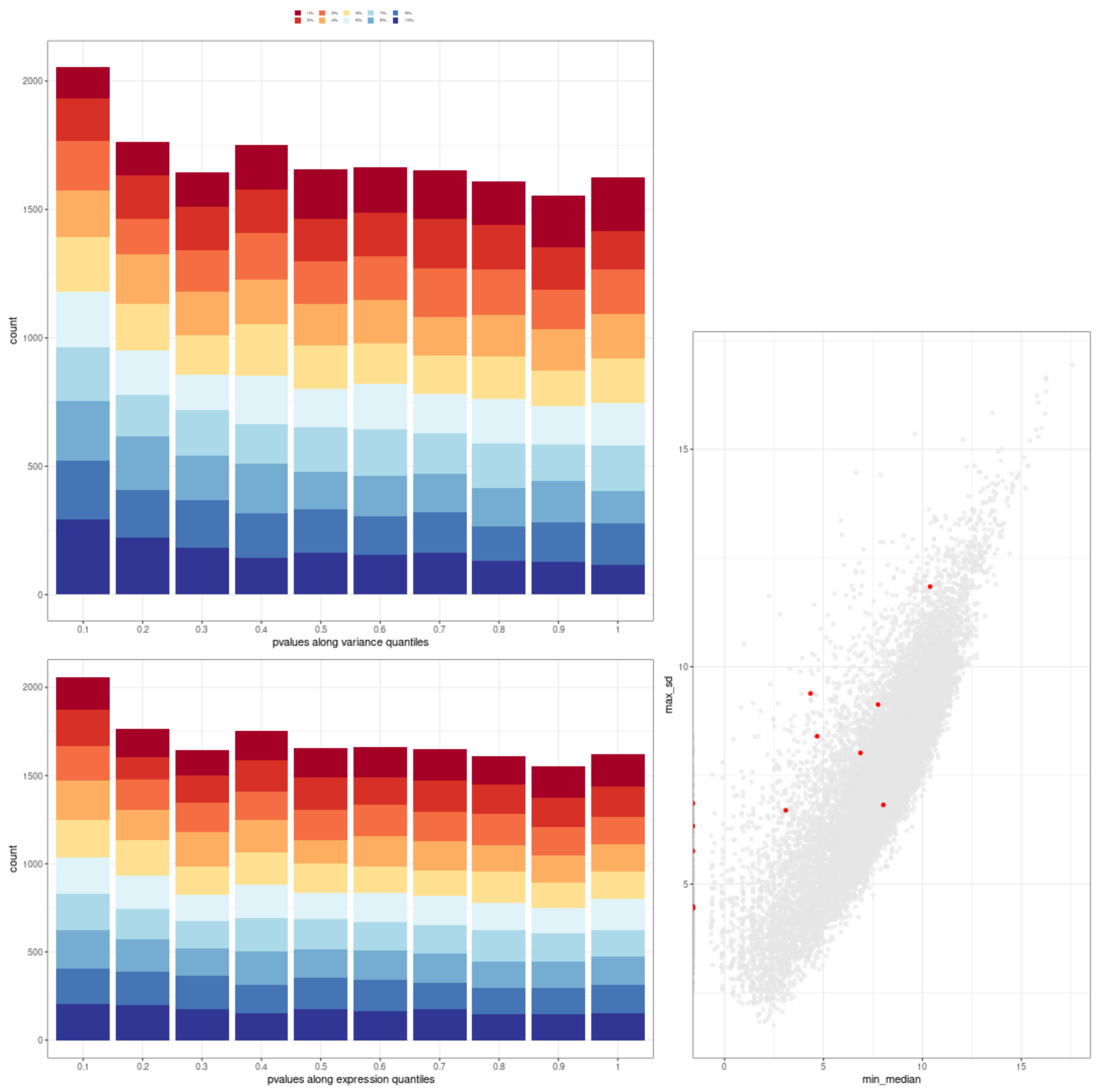
```
counts <- counts(dds, normalized = TRUE)
design <- as.data.frame(colData(dds))
degCheckFactors(counts[, 1:20])
```



Mean-Variance QC plots

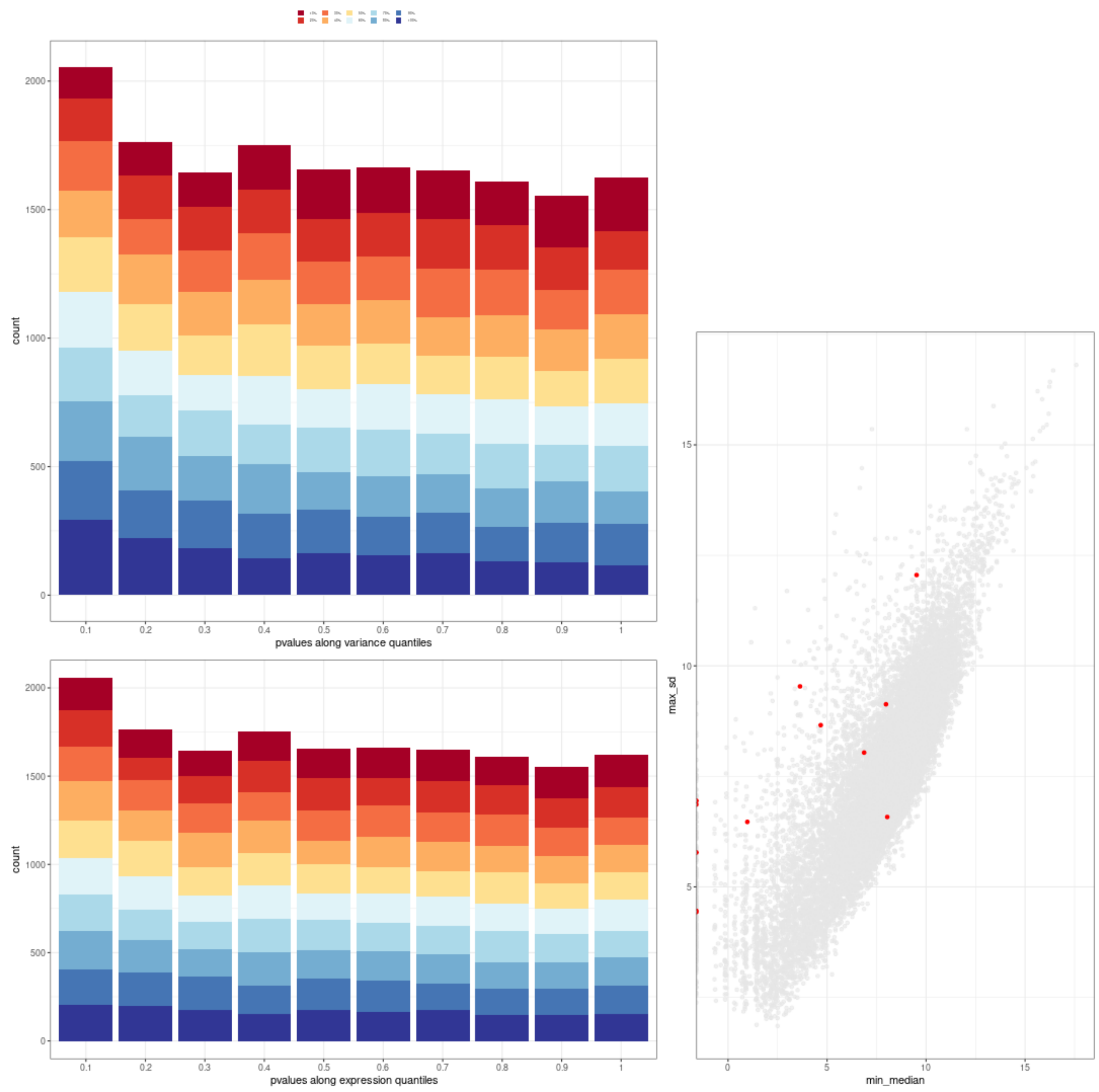
response

```
res <- results(dds)
degQC(counts, design[["response"]], pvalue = res[["pvalue"]])
```



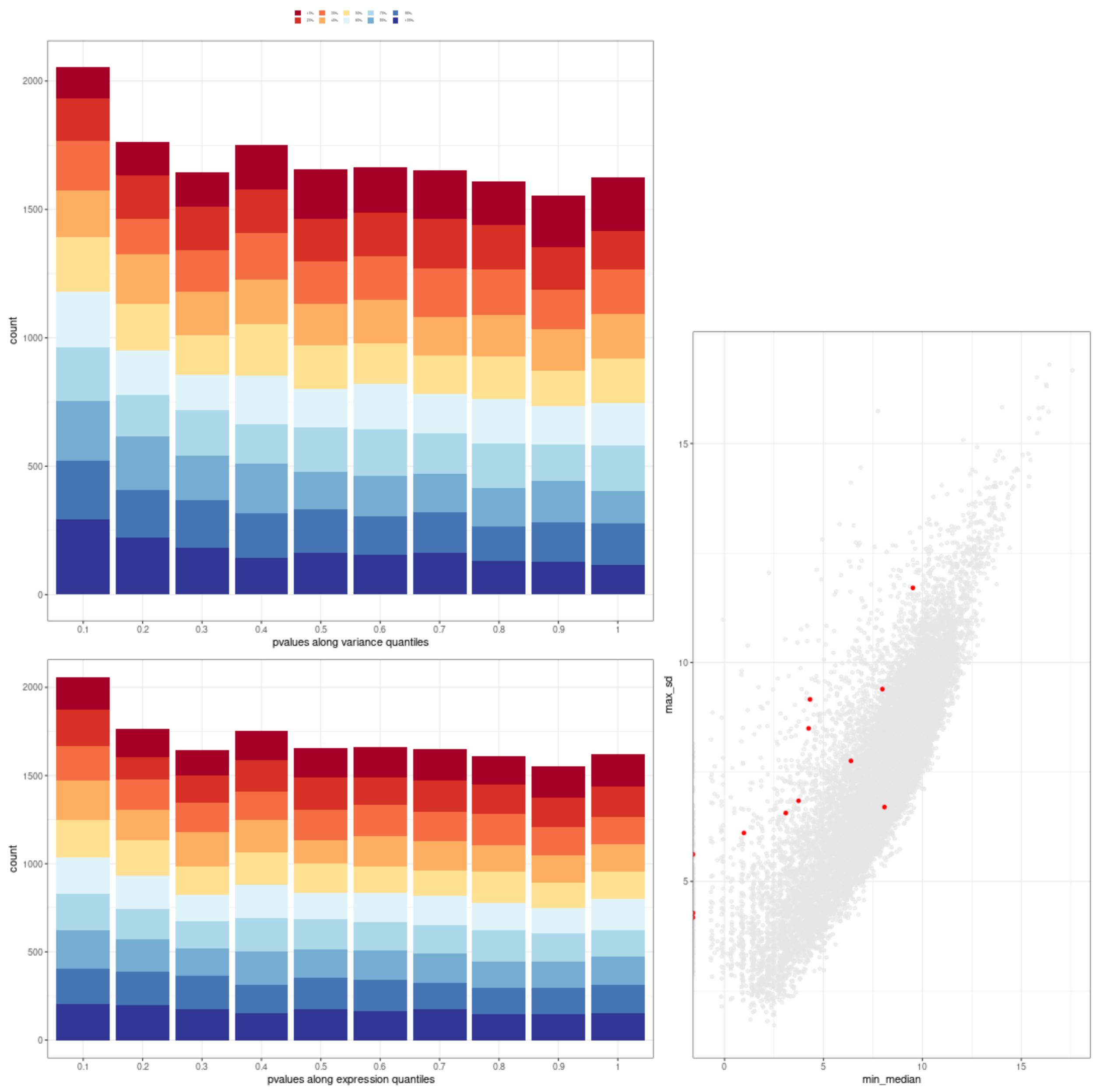
ER

```r
degQC(counts, design[["er"]], pvalue = res[["pvalue"]])
```



tumor_percentage_high

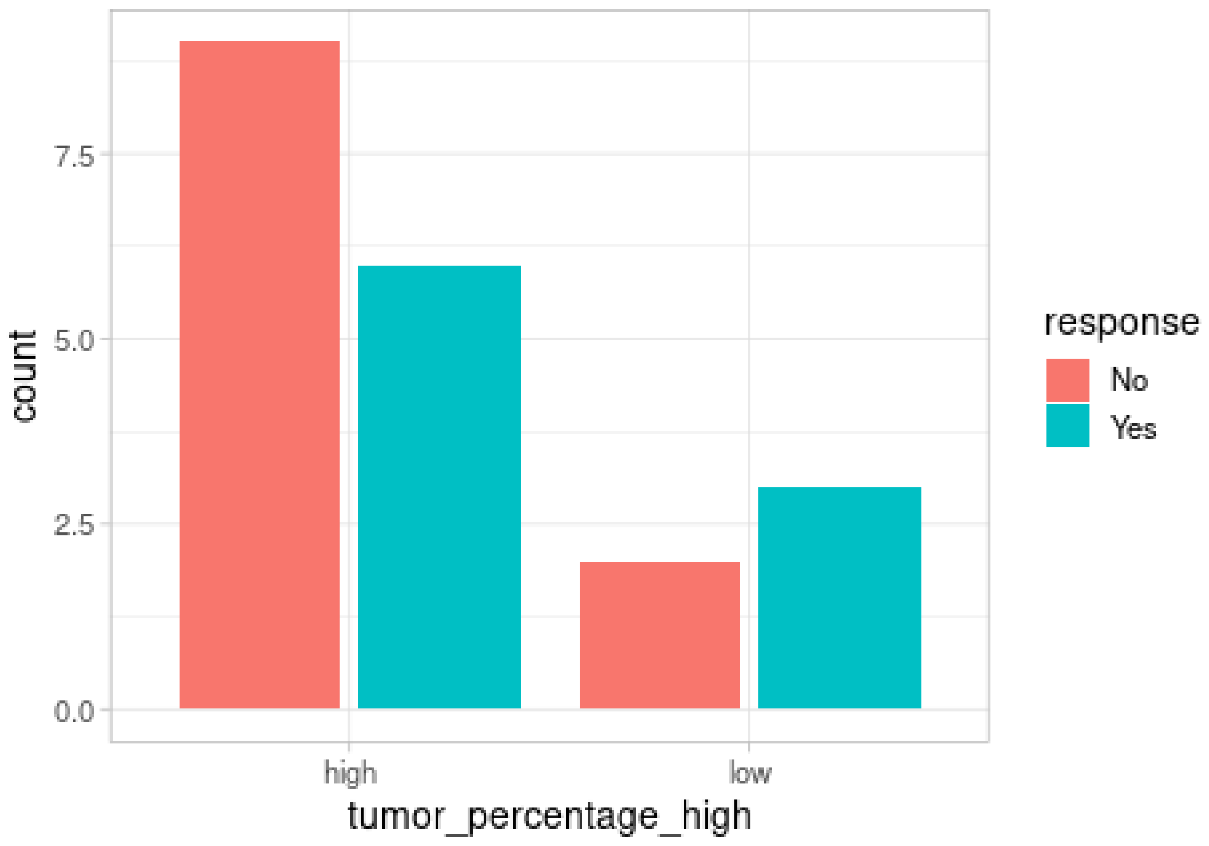
```
degQC(counts, design[["tumor_percentage_high"]], pvalue = res[["pvalue"]])
```



Covariates effect on count data

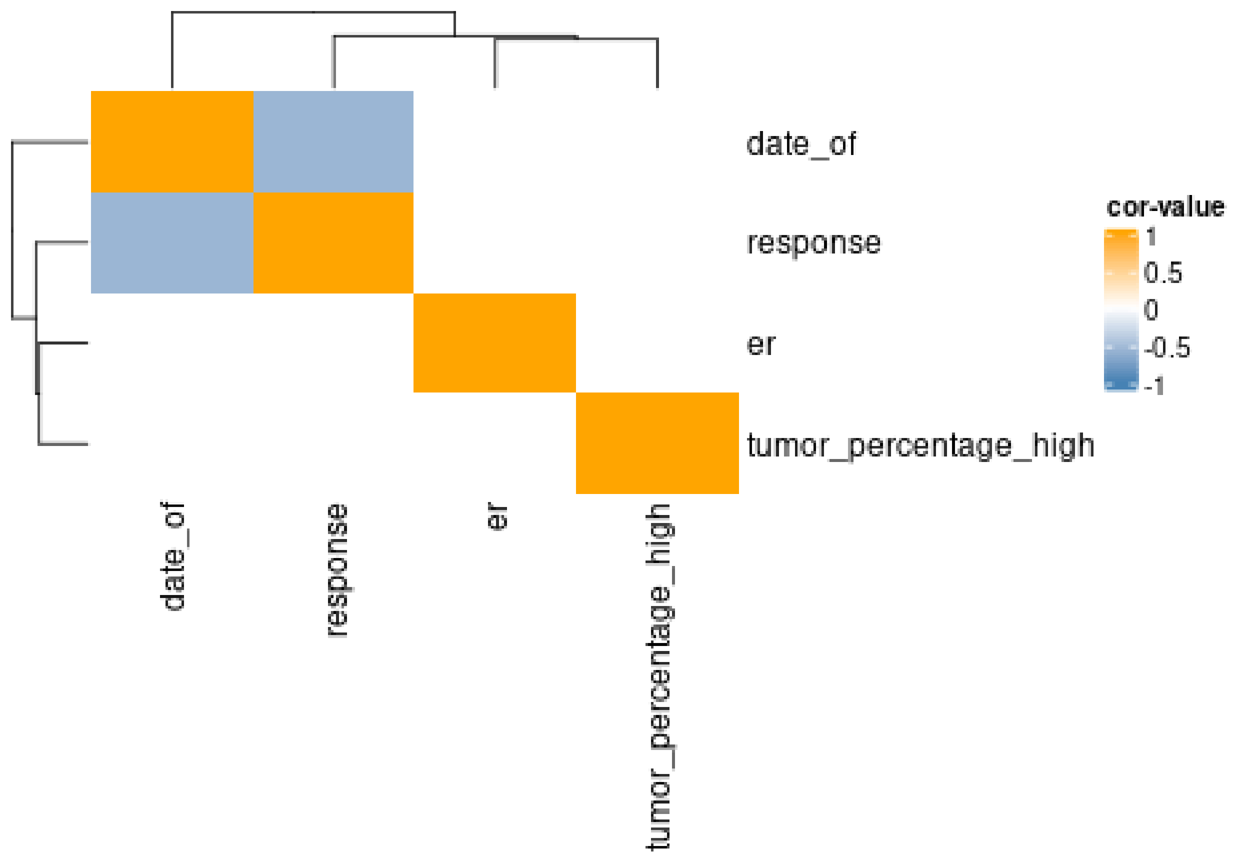
```r
mdata <- colData(dds) %>%  as.data.frame()  %>%
  dplyr::select(response, er, date_of, tumor_percentage_high)

#resCov <- degCovariates(log2(counts(dds)+0.5), mdata)
```

```r
mdata %>% ggplot(aes(tumor_percentage_high, fill = response)) + geom_bar(position = "dodge2")
```

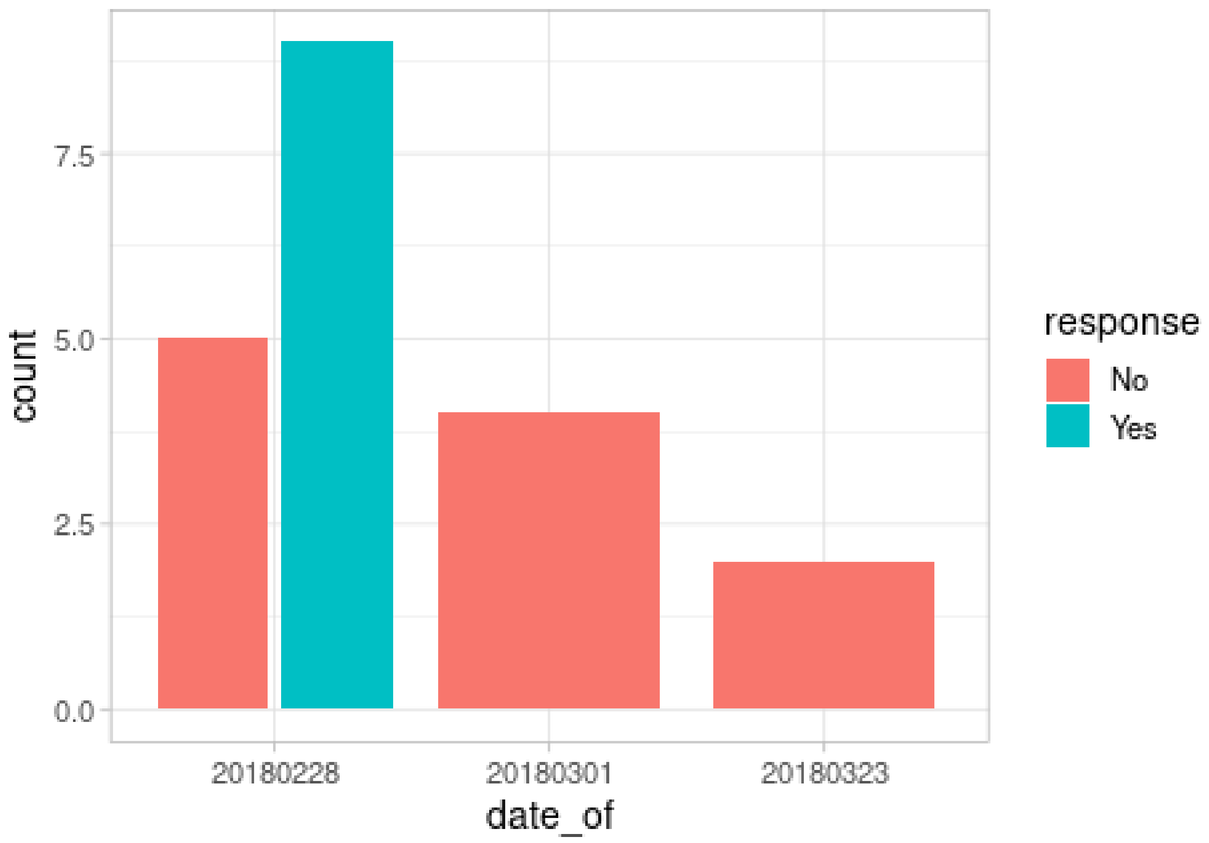


Covariates correlation with metrics

```
cor <- degCorCov(mdata)
```



```r
mdata %>% ggplot(aes(date_of, fill = response)) + geom_bar(position = "dodge2")
```



Sample-level QC analysis

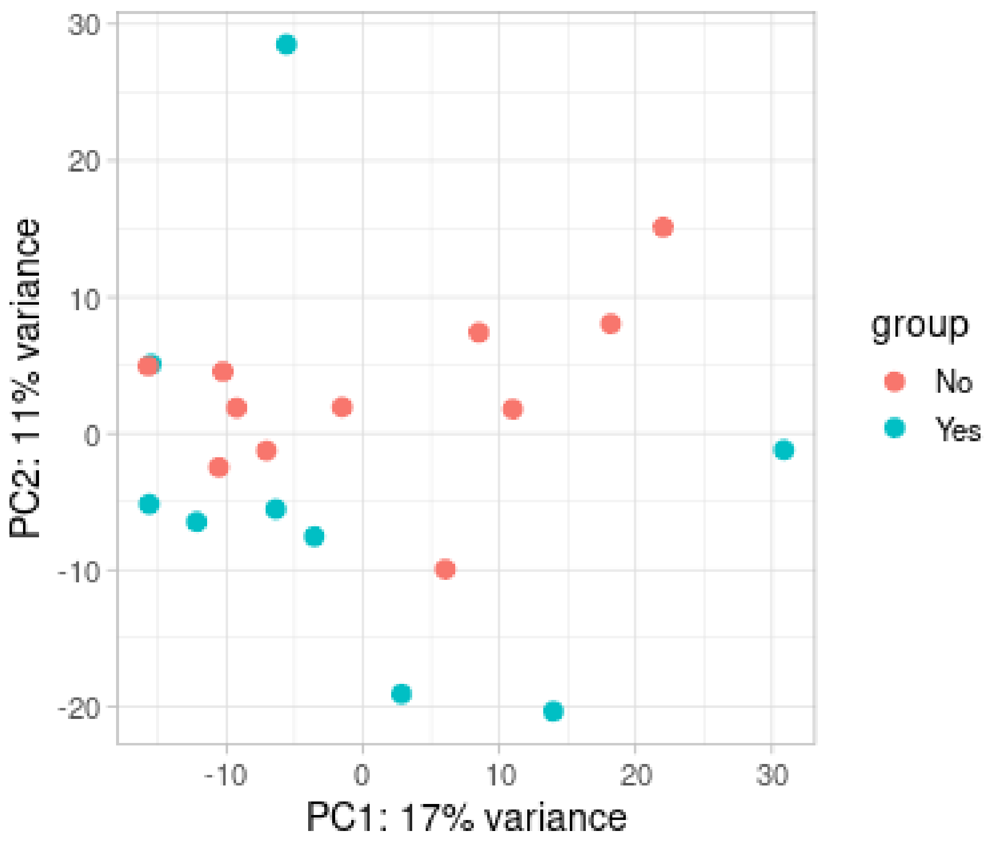
```
### Transform counts for data visualization (unsupervised analysis)
rld_file <- "data/rld.day1.RDS"
if (file.exists(rld_file)){
    rld <- readRDS(rld_file)
}else{
    rld <- rlog(dds, blind = TRUE)
    saveRDS(rld, rld_file)
}
class(rld) # what type of object is this
```

```
## [1] "DESeqTransform"
## attr(,"package")
## [1] "DESeq2"
```

```
# we also need just a matrix of transformed counts
rld_mat <- assay(rld)
```

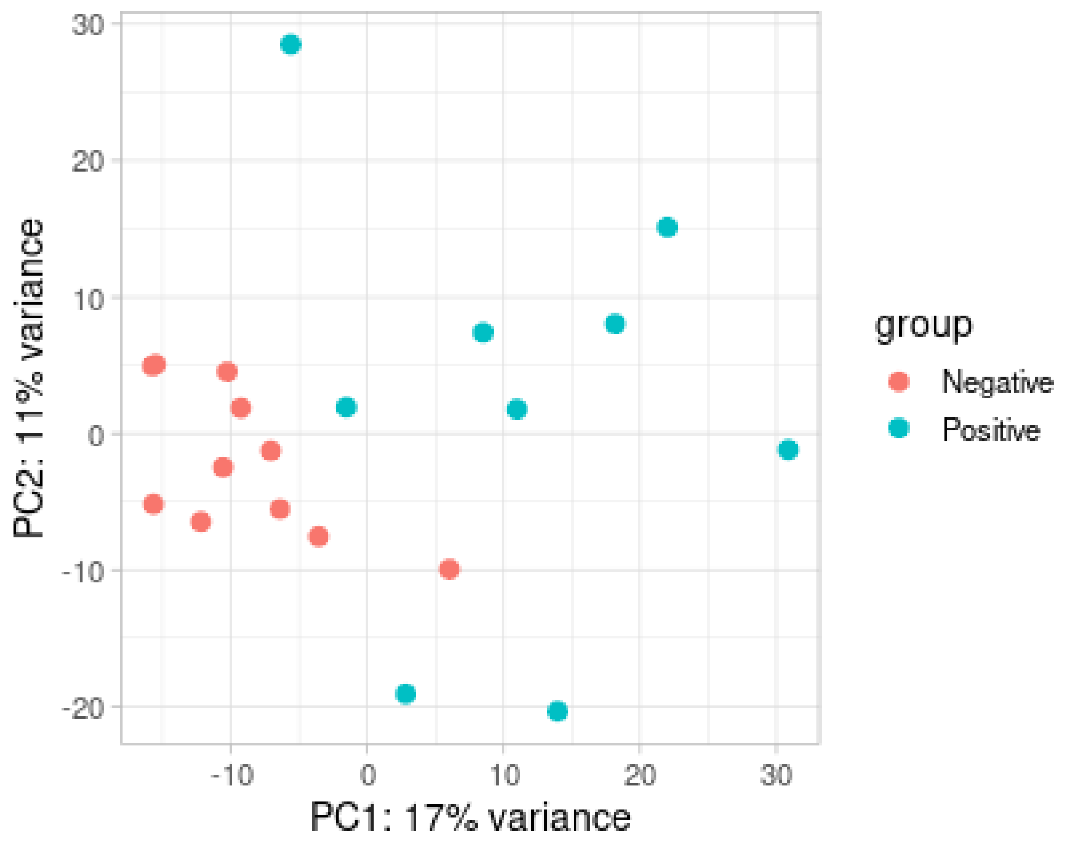
PCA - response

```
# Use the DESeq2 function
plotPCA(rld, intgroup = c("response"))
```



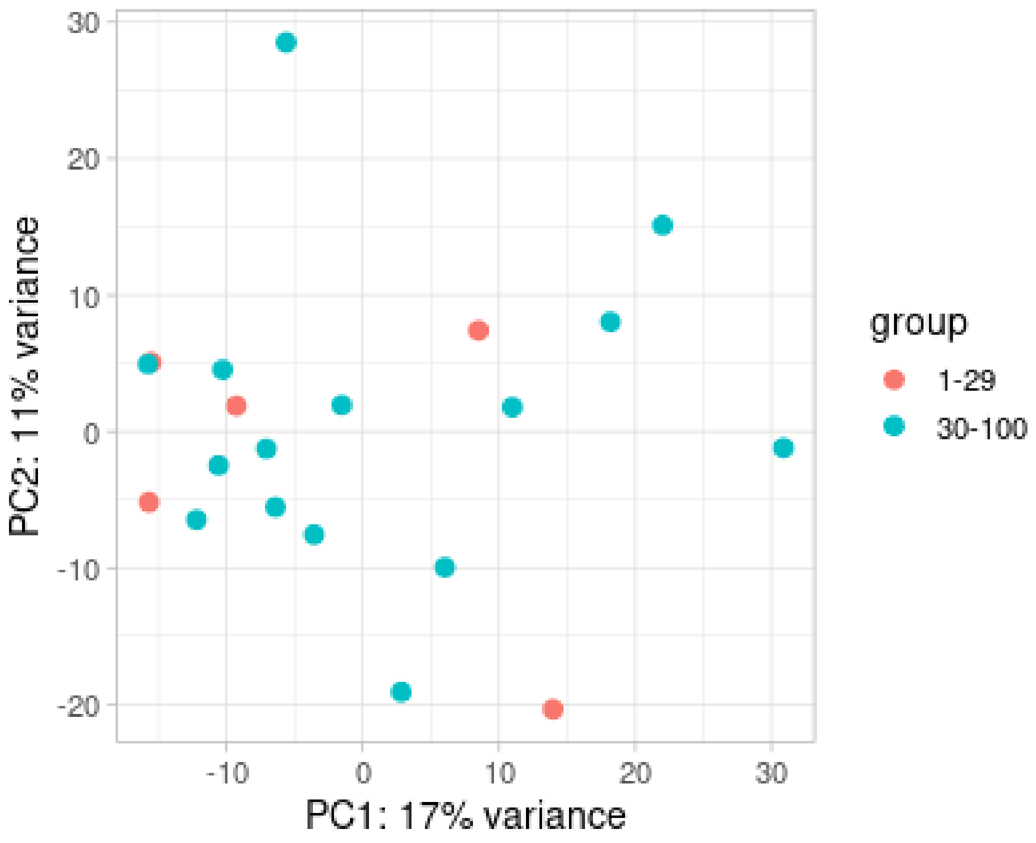
PCA - ER

```r
# Use the DESeq2 function
plotPCA(rld, intgroup = c("er"))
```



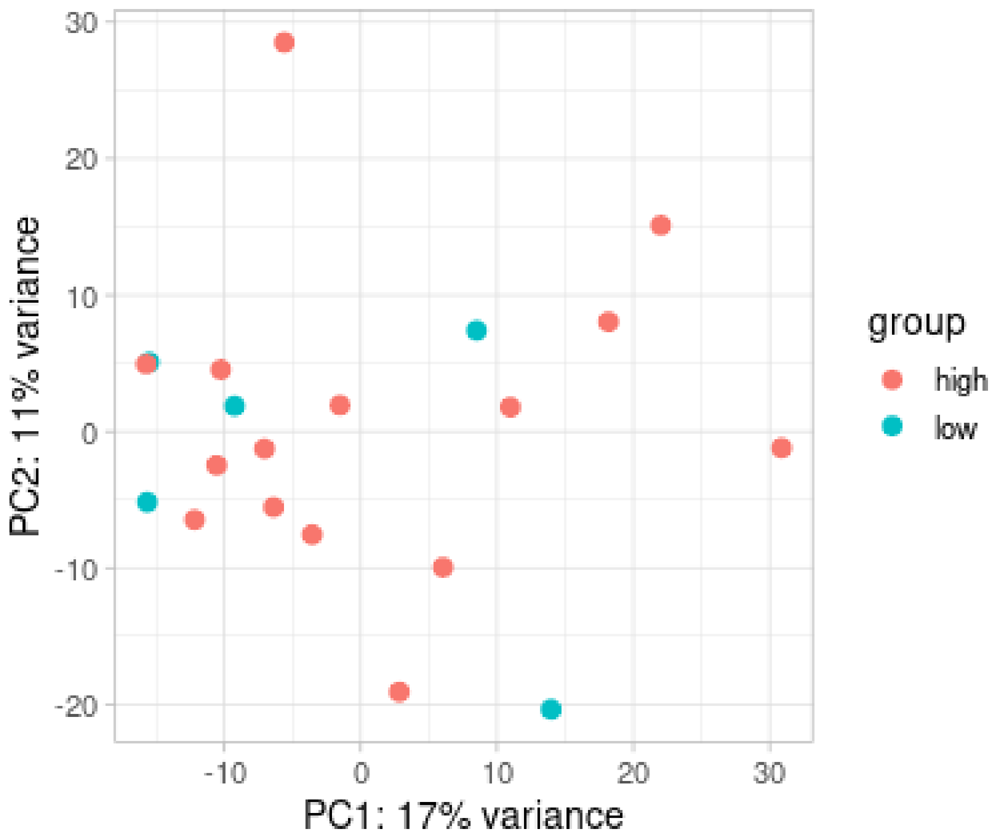
PCA - tumor_percentage

```r
# Use the DESeq2 function
plotPCA(rld, intgroup = c("tumor_percentage"))
```



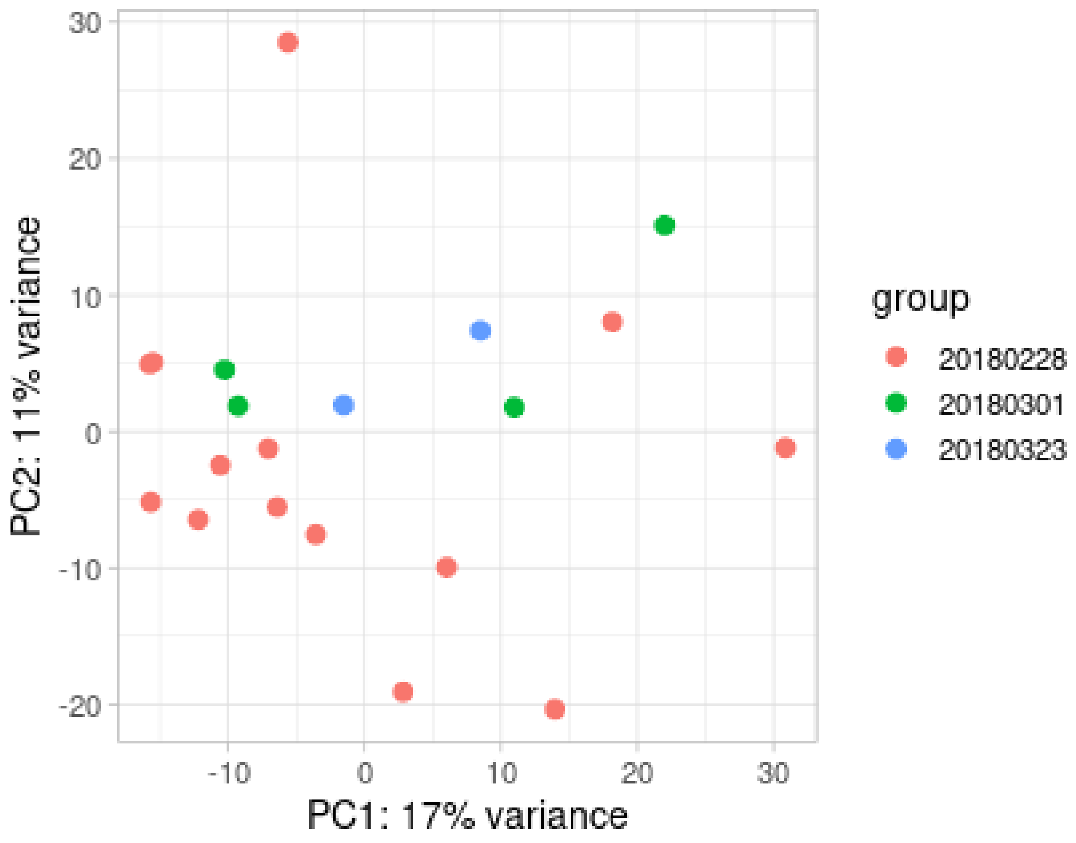
PCA - tumor_percentage_high

```
# Use the DESeq2 function
plotPCA(rld, intgroup = c("tumor_percentage_high"))
```



PCA - date_of

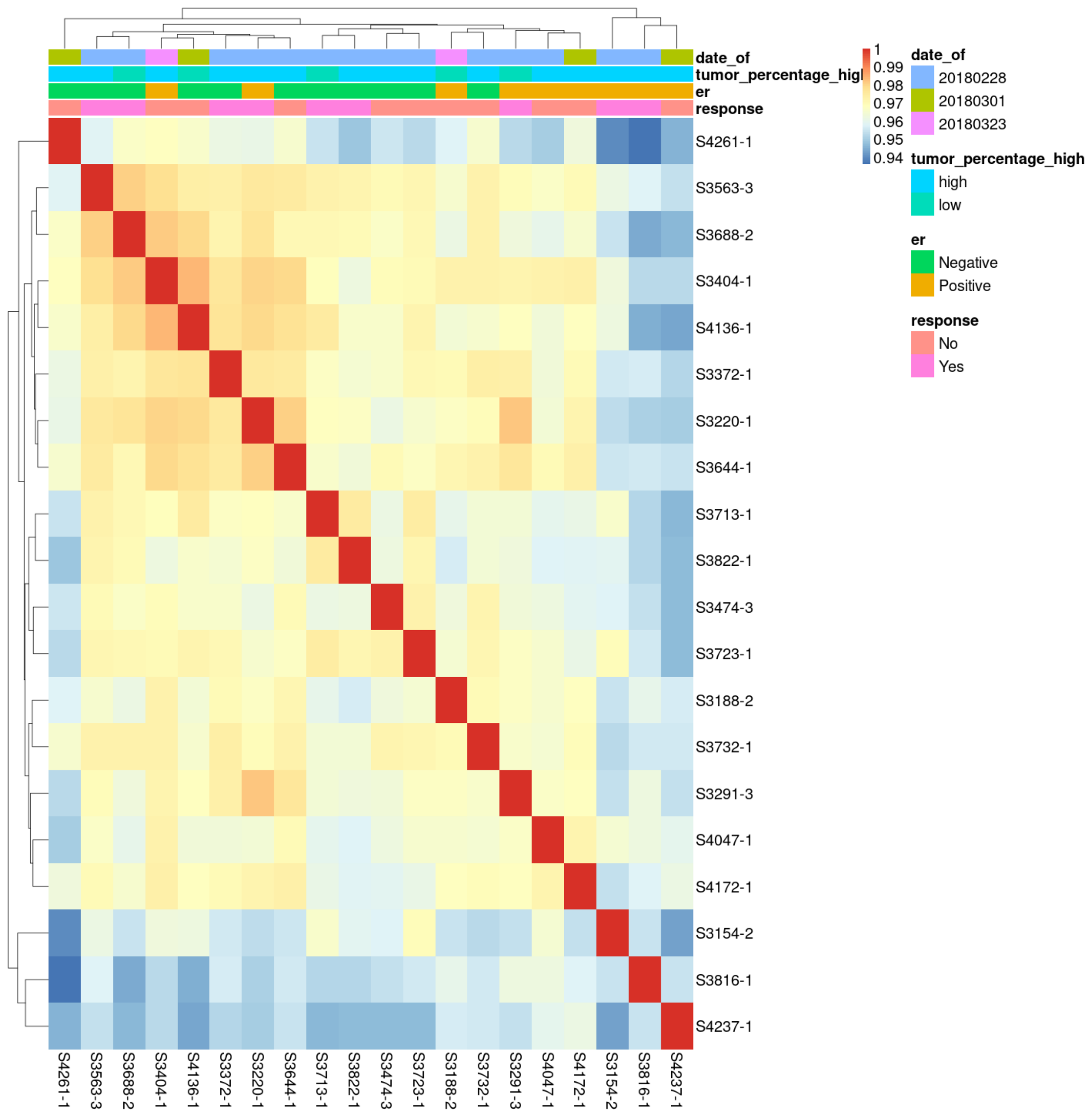
```
# Use the DESeq2 function
plotPCA(rld, intgroup = c("date_of"))
```



Inter-correlation analysis

Without study_id

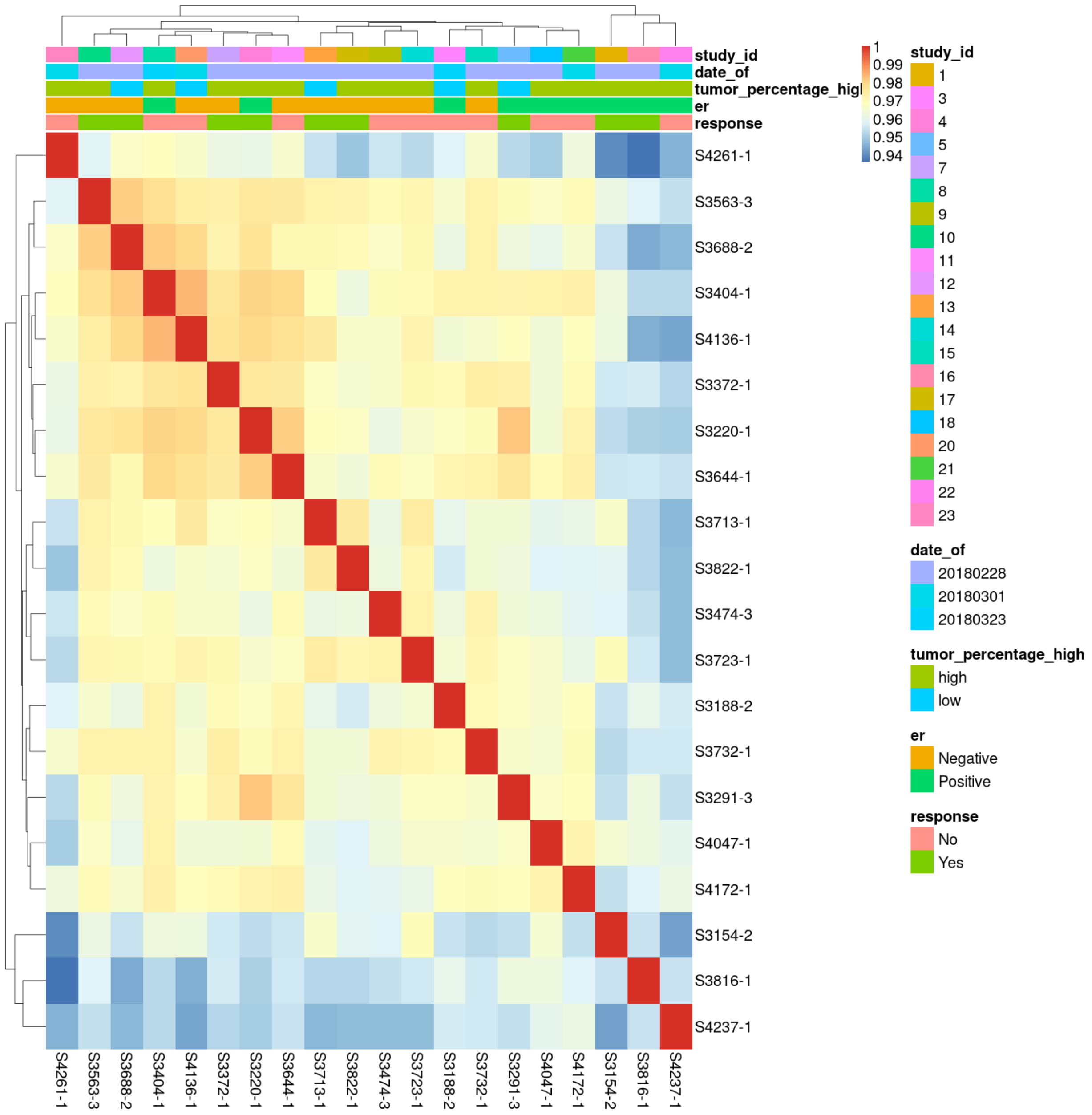
```
# Correlation matrix
rld_cor <- cor(rld_mat)

meta$study_id <- as.factor(meta$study_id)
# Create annotation file for samples
annotation <- meta[, c("response", "er", "tumor_percentage_high", "date_of")]

# Change colors
heat.colors <- brewer.pal(6, "Blues")

# Plot heatmap
pheatmap(rld_cor,
         annotation = annotation,
         border = NA,
         fontsize = 20)
```



With study_id

```
# Correlation matrix
rld_cor <- cor(rld_mat)

meta$study_id <- as.factor(meta$study_id)
# Create annotation file for samples
annotation <- meta[, c("response", "er", "tumor_percentage_high", "date_of", "study_id")]

# Change colors
heat.colors <- brewer.pal(6, "Blues")

# Plot heatmap
```

```
pheatmap(rld_cor,
         annotation = annotation,
         border = NA,
         fontsize = 20)
```



Response Yes vs No for Day 1- see Table9

```
# Get results for rescue vs wt
contrast <- c("response", "Yes", "No")
resResponse <- results(dds, contrast = contrast, alpha = 0.05)
length(which(resResponse$padj < 0.05))
```

```
## [1] 15
```

```
# Add annotations
resResponse_tb <- resResponse %>%
  data.frame() %>%
  rownames_to_column(var = "gene") %>%
  as_tibble() %>%
  left_join(gene_symbol, by = c("gene" = "gene_id"))

resResponse_tb_significant <- dplyr::filter(resResponse_tb, padj < 0.05)

samples_no <- meta %>% dplyr::filter(response == "No") %>% row.names()

counts_no <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_no)) %>%
                rowSums() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")
colnames(counts_no) <- c("ensembl_gene_id", "no_expression_mean_tpm")

samples_yes <- meta %>% dplyr::filter(response == "Yes") %>% row.names()

counts_yes <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_yes)) %>%
                rowSums() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")

colnames(counts_yes) <- c("ensembl_gene_id", "yes_expression_mean_tpm")

counts_yes <-counts_yes %>%
            left_join(counts_no,
                      by = c("ensembl_gene_id" = "ensembl_gene_id"))

resResponse_tb_significant <- resResponse_tb_significant %>%
          left_join(counts_yes, by = c("gene" = "ensembl_gene_id"))


write_csv(resResponse_tb_significant,
          "tables/T9.DE_response_day1.csv")

# Separate into up and down-regulated gene sets
sigResponse_up <- rownames(resResponse)[which(resResponse$padj < 0.01 & resResponse$log2FoldChange > 0)
sigResponse_down <- rownames(resResponse)[which(resResponse$padj < 0.01 & resResponse$log2FoldChange <
```

ER : Positive vs Negative for Day1 - Table 10

```
contrast <- c("er", "Positive", "Negative")
resER <- results(dds, contrast = contrast, alpha = 0.05)
length(which(resER$padj < 0.05))
```

```
## [1] 571
```

```r
# Add annotations
resER_tb <- resER %>%
  data.frame() %>%
  rownames_to_column(var = "gene") %>%
  as_tibble() %>%
  left_join(gene_symbol, by = c("gene" = "gene_id"))

resER_tb_significant <- dplyr::filter(resER_tb, padj < 0.05)

samples_pos <- meta %>% dplyr::filter(er == "Positive") %>% row.names()

counts_pos <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_pos)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")
colnames(counts_pos) <- c("ensembl_gene_id", "Positive_expression_mean_tpm")

samples_neg <- meta %>% dplyr::filter(er == "Negative") %>% row.names()

counts_neg <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_neg)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")

colnames(counts_neg) <- c("ensembl_gene_id", "Negative_expression_mean_tpm")

counts_pos <-counts_pos %>%
            left_join(counts_neg,
                      by = c("ensembl_gene_id" = "ensembl_gene_id"))

resER_tb_significant <- resER_tb_significant %>%
          left_join(counts_pos, by = c("gene" = "ensembl_gene_id"))


write_csv(resER_tb_significant,
          "tables/T10.DE_ER.day1.csv")

# Separate into up and down-regulated gene sets
sigER_up <- rownames(resER)[which(resER$padj < 0.01 & resER$log2FoldChange > 0)]
sigER_down <- rownames(resER)[which(resER$padj < 0.01 & resER$log2FoldChange < 0)]
```

tumor_percentage_high : High vs Low for Day1- Table 11

```r
contrast <- c("tumor_percentage_high", "high", "low")
resTP <- results(dds, contrast = contrast, alpha = 0.05)
length(which(resTP$padj < 0.05))
```

```
## [1] 7
```

```r
# Add annotations
resTP_tb <- resTP %>%
  data.frame() %>%
  rownames_to_column(var = "gene") %>%
  as_tibble() %>%
  left_join(gene_symbol, by = c("gene" = "gene_id"))

resTP_tb_significant <- dplyr::filter(resTP_tb, padj < 0.05)

samples_high <- meta %>% dplyr::filter(tumor_percentage_high == "high") %>% row.names()

counts_high <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_high)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")
colnames(counts_high) <- c("ensembl_gene_id", "High_expression_mean_tpm")

samples_low <- meta %>% dplyr::filter(tumor_percentage_high == "low") %>% row.names()

counts_low <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_low)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")

colnames(counts_low) <- c("ensembl_gene_id", "Low_expression_mean_tpm")

counts_high <-counts_high %>%
            left_join(counts_low,
                      by = c("ensembl_gene_id" = "ensembl_gene_id"))

resTP_tb_significant <- resTP_tb_significant %>%
          left_join(counts_high, by = c("gene" = "ensembl_gene_id"))


write_csv(resTP_tb_significant,
          "tables/T11.DE_tumor_percentage_high.day1.csv")

# Separate into up and down-regulated gene sets
sigTP_up <- rownames(resTP)[which(resTP$padj < 0.01 & resTP$log2FoldChange > 0)]
sigTP_down <- rownames(resTP)[which(resTP$padj < 0.01 & resTP$log2FoldChange < 0)]
```

date_of: 20180323 vs 20180228 - for Day1: Table 12

```r
contrast <- c("date_of", "20180323", "20180228")
resDO <- results(dds, contrast = contrast, alpha = 0.05)
length(which(resDO$padj < 0.05))
```

```
## [1] 30
```

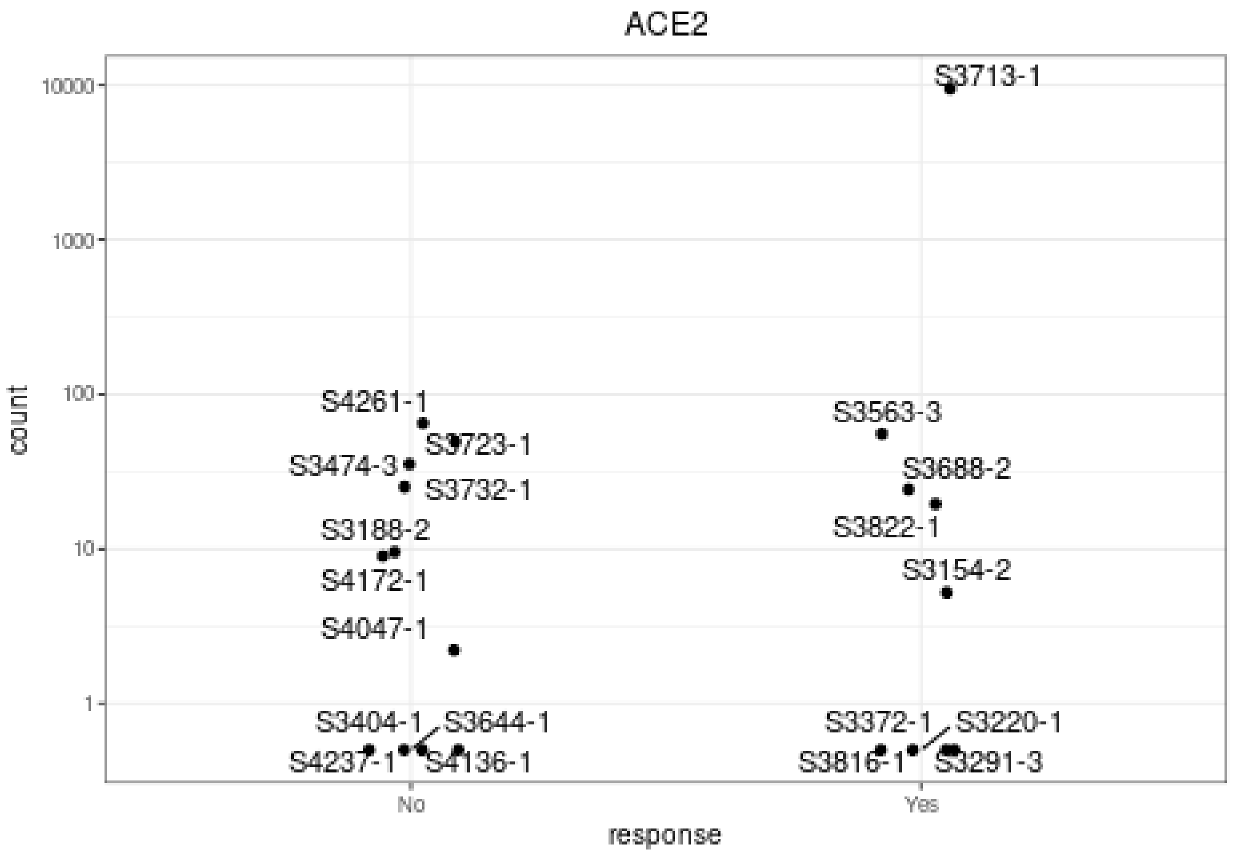
```r
# Add annotations
resDO_tb <- resDO %>%
  data.frame() %>%
  rownames_to_column(var = "gene") %>%
  as_tibble() %>%
  left_join(gene_symbol, by = c("gene" = "gene_id"))

resDO_tb_significant <- dplyr::filter(resDO_tb, padj < 0.05)

samples_23 <- meta %>% dplyr::filter(date_of == "20180323") %>% row.names()

counts_23 <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_23)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")
colnames(counts_23) <- c("ensembl_gene_id", "20180323_expression_mean_tpm")

samples_28 <- meta %>% dplyr::filter(date_of == "20180228") %>% row.names()

counts_28 <- txi$abundance %>%
                as.data.frame() %>%
                dplyr::select(any_of(samples_28)) %>%
                rowMeans() %>%
                as.data.frame() %>%
                rownames_to_column(var = "ensembl_gene_id")

colnames(counts_28) <- c("ensembl_gene_id", "20180228_expression_mean_tpm")

counts_23 <-counts_23 %>%
            left_join(counts_28,
                      by = c("ensembl_gene_id" = "ensembl_gene_id"))

resDO_tb_significant <- resDO_tb_significant %>%
          left_join(counts_23, by = c("gene" = "ensembl_gene_id"))


write_csv(resDO_tb_significant,
          "tables/T12.DE_date_of.day1.csv")

# Separate into up and down-regulated gene sets
sigDO_up <- rownames(resDO)[which(resDO$padj < 0.01 & resDO$log2FoldChange > 0)]
sigDO_down <- rownames(resDO)[which(resDO$padj < 0.01 & resDO$log2FoldChange < 0)]
```

Visualization

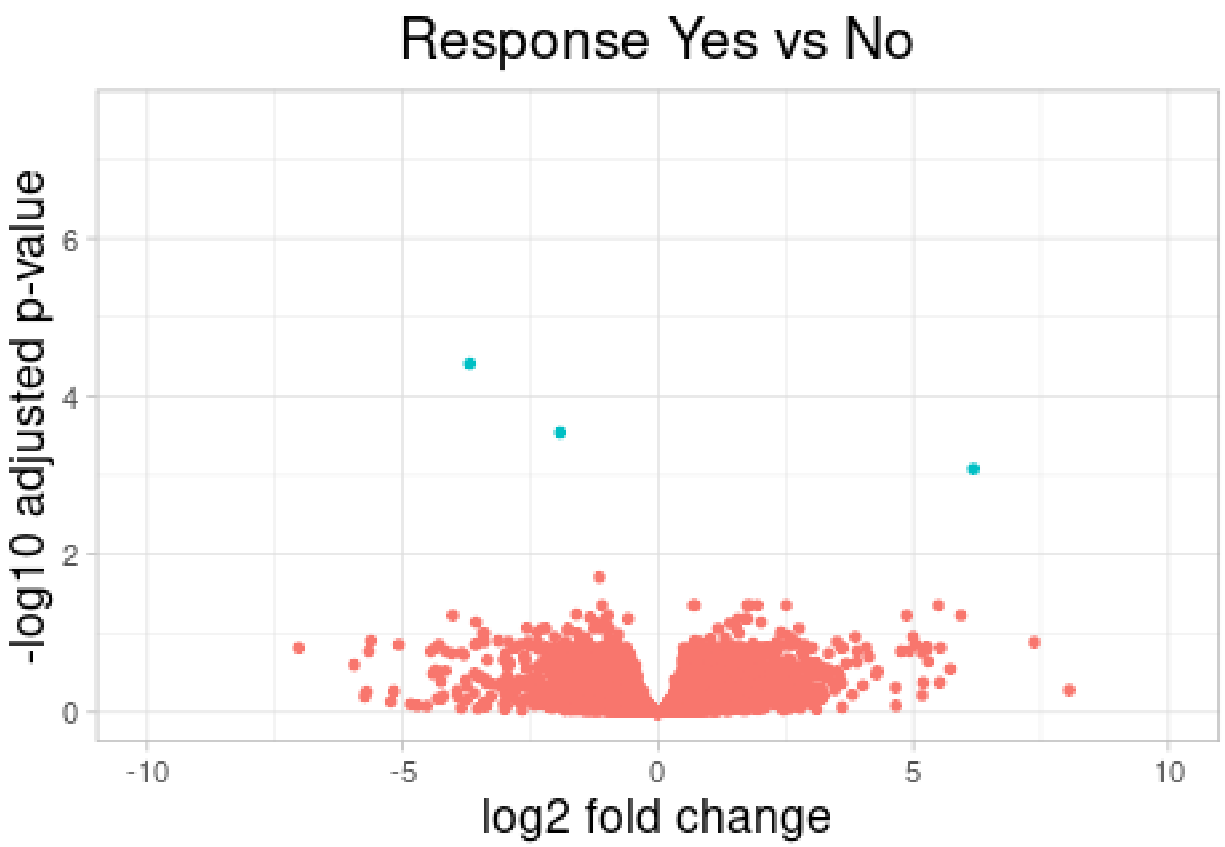
Gene example

```r
d <- plotCounts(dds,
                gene = "ENSG00000130234",
                intgroup = "response",
                returnData = TRUE)
```

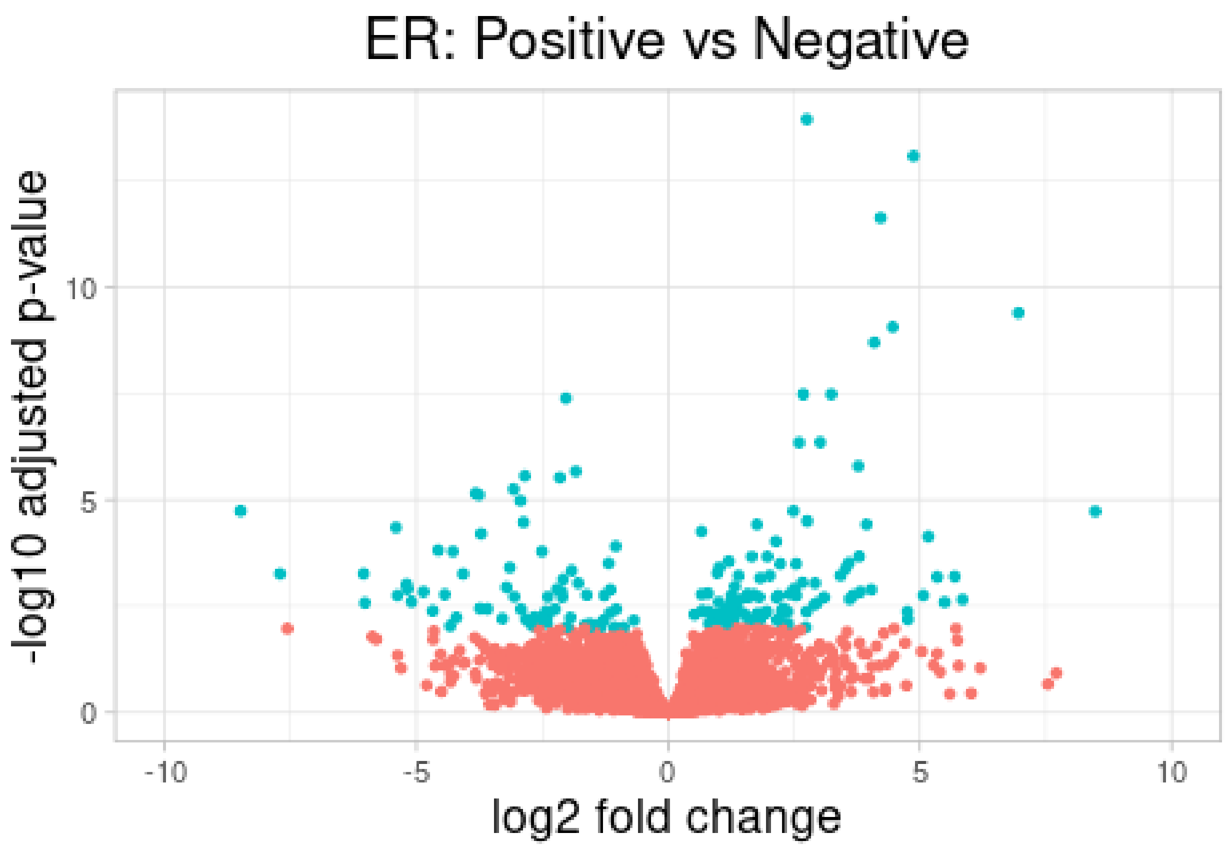
```
ggplot(d, aes(x = response, y = count)) +
     geom_point(position = position_jitter(w = 0.1, h = 0)) +
     geom_text_repel(aes(label = rownames(d))) +
     theme_bw(base_size = 10) +
     ggtitle("ACE2") +
     theme(plot.title = element_text(hjust = 0.5)) +
     scale_y_log10()
```



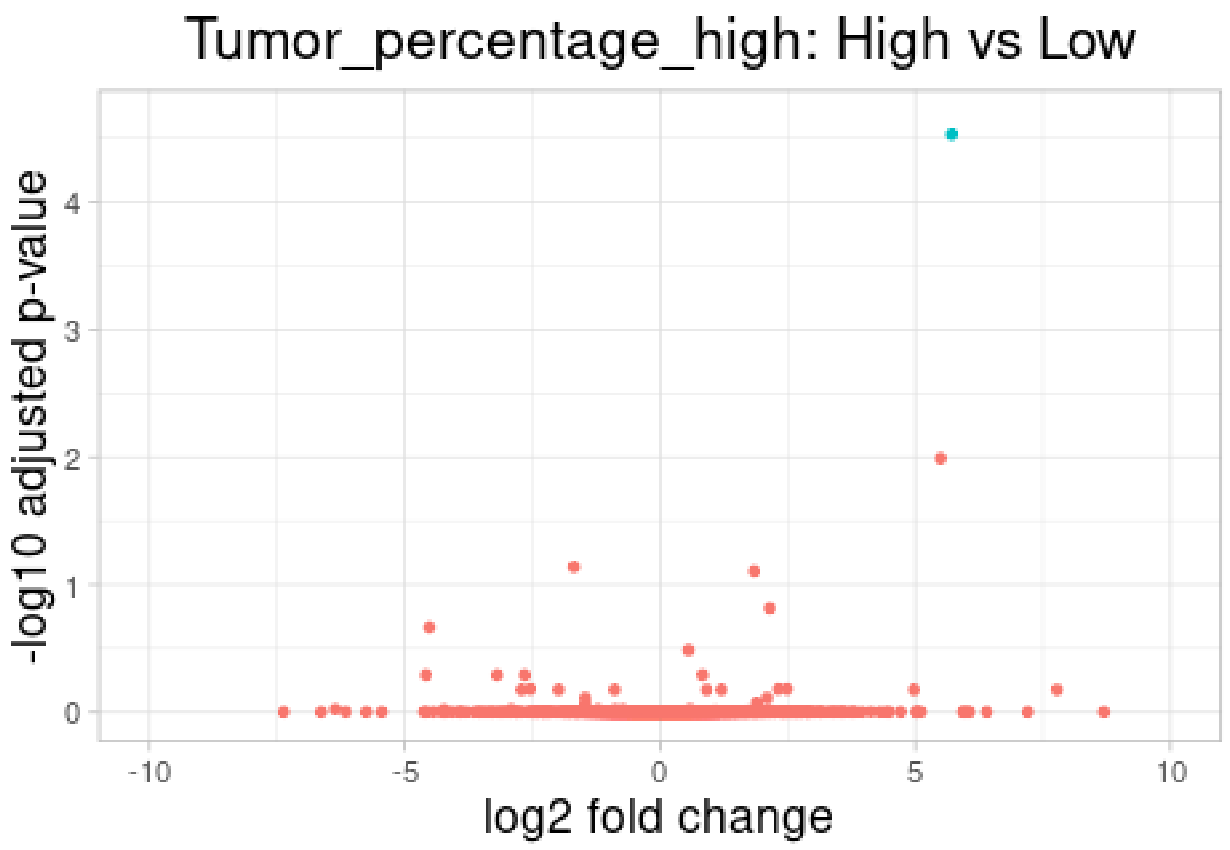
```
# Add a column for significant genes
resResponse_tb <- resResponse_tb %>% mutate(threshold = padj < 0.01)

ggplot(resResponse_tb) +
  geom_point(aes(x = log2FoldChange, y = -log10(padj), colour = threshold)) +
  ggtitle("Response Yes vs No") +
  xlab("log2 fold change") +
  ylab("-log10 adjusted p-value") +
  scale_x_continuous(limits = c(-10,10)) +
  scale_y_continuous(limits = c(0, 7.5))+
  theme(legend.position = "none",
        plot.title = element_text(size = rel(1.5), hjust = 0.5),
        axis.title = element_text(size = rel(1.25)))
```



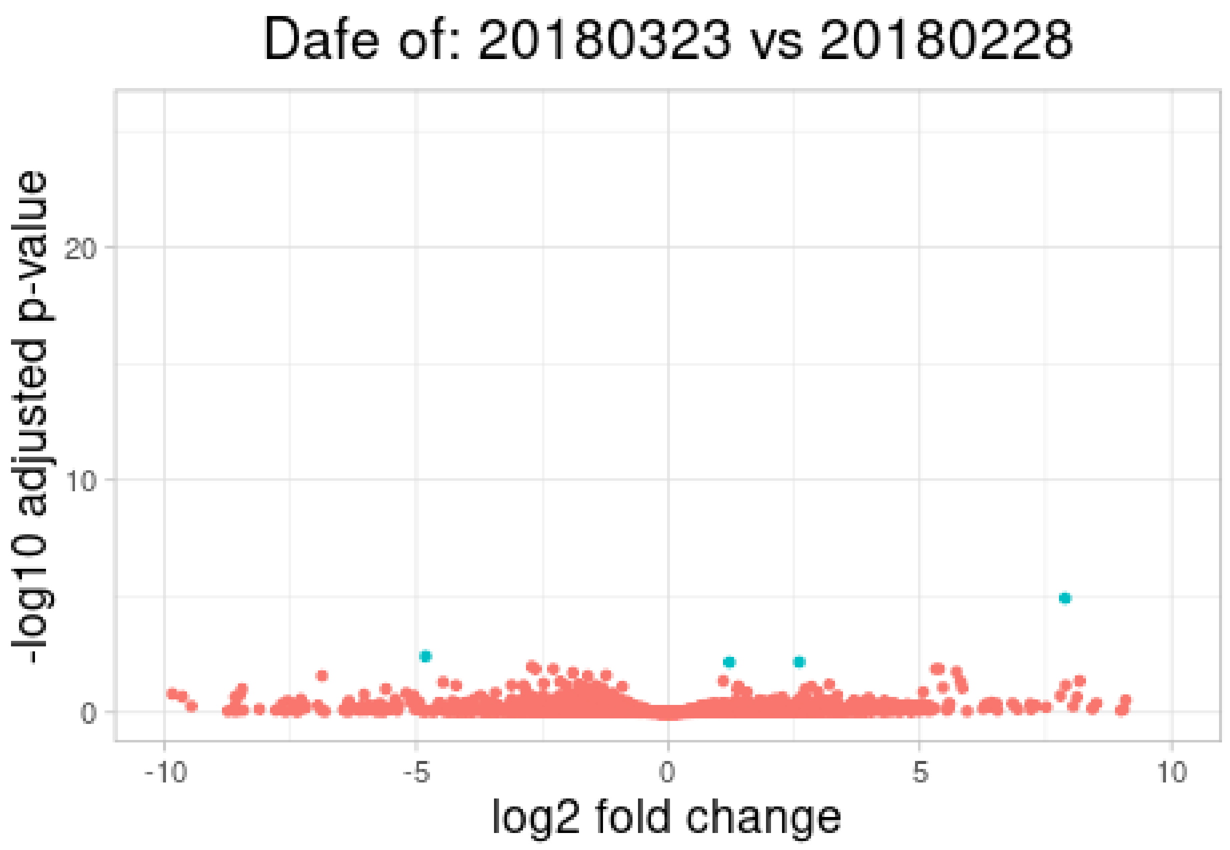
```r
# Add a column for significant genes
resER_tb <- resER_tb %>% mutate(threshold = padj < 0.01)

ggplot(resER_tb) +
  geom_point(aes(x = log2FoldChange, y = -log10(padj), colour = threshold)) +
  ggtitle("ER: Positive vs Negative") +
  xlab("log2 fold change") +
  ylab("-log10 adjusted p-value") +
  scale_x_continuous(limits = c(-10,10)) +
  theme(legend.position = "none",
        plot.title = element_text(size = rel(1.5), hjust = 0.5),
        axis.title = element_text(size = rel(1.25)))
```



```
# Add a column for significant genes
resTP_tb <- resTP_tb %>% mutate(threshold = padj < 0.01)

ggplot(resTP_tb) +
  geom_point(aes(x = log2FoldChange, y = -log10(padj), colour = threshold)) +
  ggtitle("Tumor_percentage_high: High vs Low") +
  xlab("log2 fold change") +
  ylab("-log10 adjusted p-value") +
  scale_x_continuous(limits = c(-10,10)) +
  theme(legend.position = "none",
        plot.title = element_text(size = rel(1.5), hjust = 0.5),
        axis.title = element_text(size = rel(1.25)))
```

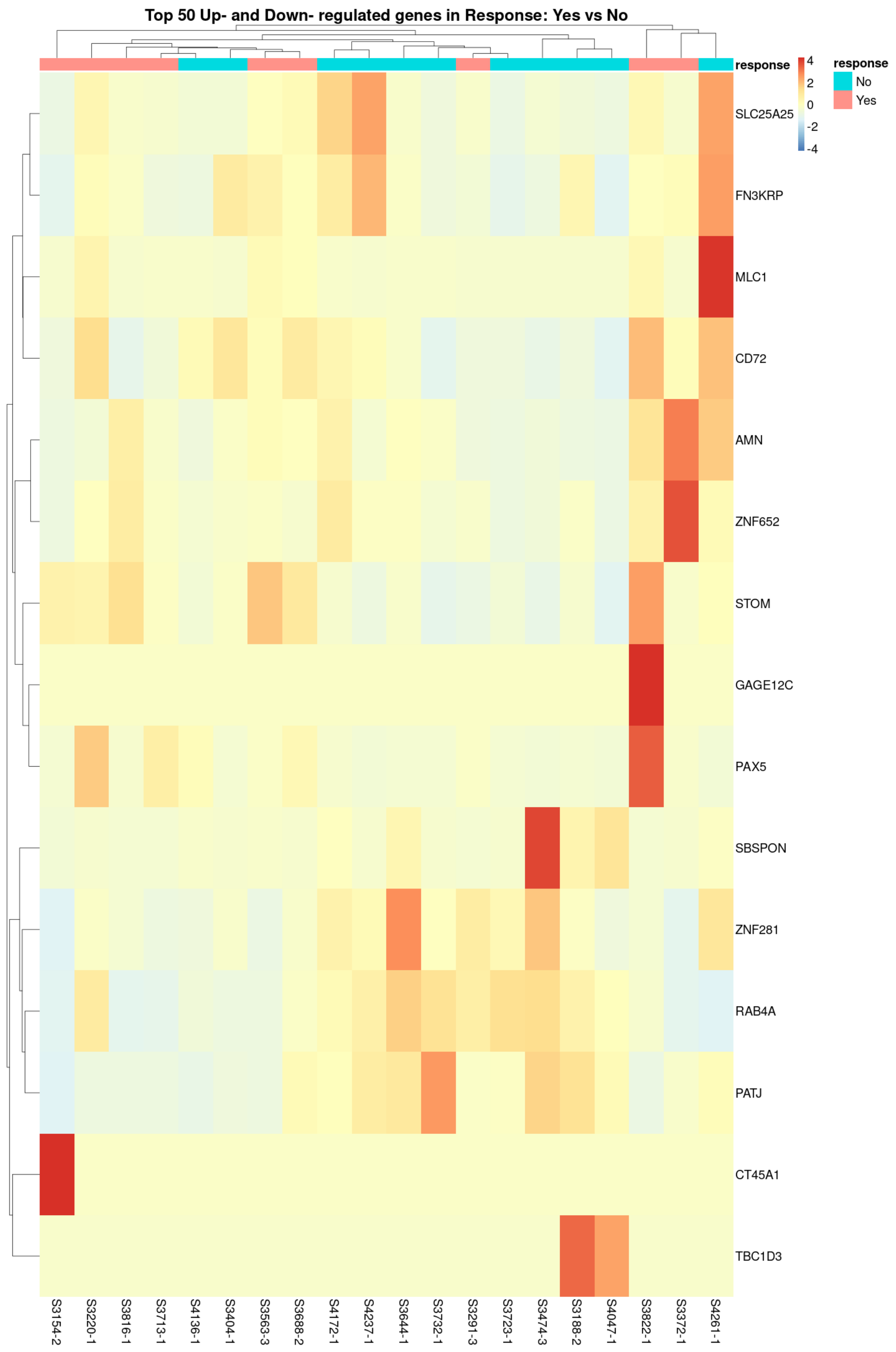


```r
# Add a column for significant genes
resDO_tb <- resDO_tb %>% mutate(threshold = padj < 0.01)

ggplot(resDO_tb) +
  geom_point(aes(x = log2FoldChange, y = -log10(padj), colour = threshold)) +
  ggtitle("Dafe of: 20180323 vs 20180228") +
  xlab("log2 fold change") +
  ylab("-log10 adjusted p-value") +
  scale_x_continuous(limits = c(-10,10)) +
  theme(legend.position = "none",
        plot.title = element_text(size = rel(1.5), hjust = 0.5),
        axis.title = element_text(size = rel(1.25)))
```



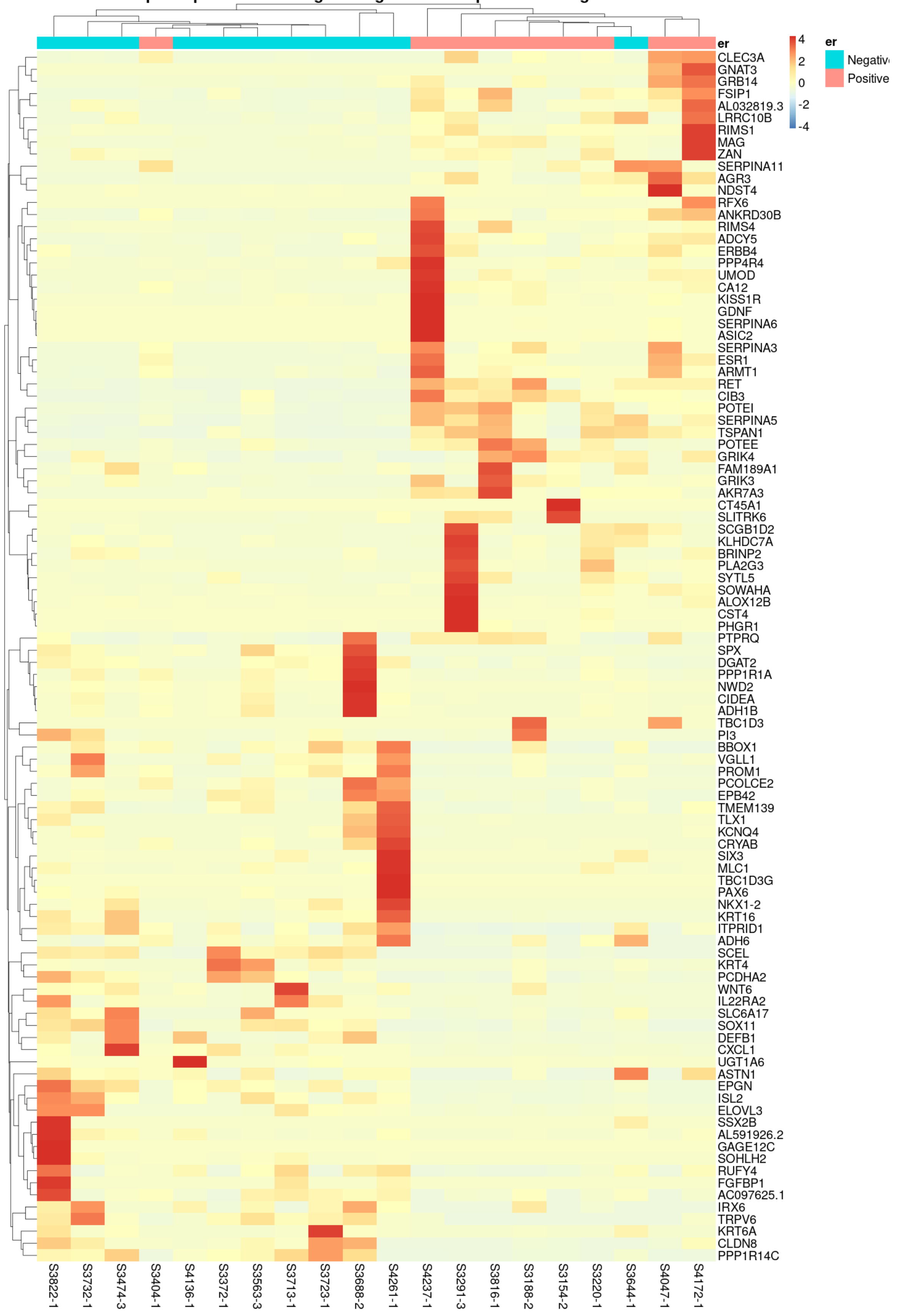
# Heatmaps

```
# Create a matrix of normalized expression
sig_up <- resResponse_tb_significant %>% arrange(-log2FoldChange) %>% head(50) %>% pull(gene)
sig_down <- resResponse_tb_significant %>% arrange(log2FoldChange) %>% head(50) %>% pull(gene)
sig <- c(sig_up, sig_down)

row_annotation <- gene_symbol %>%
                    as_tibble() %>%
                    dplyr::filter(gene_id %in% sig)

plotmat <- txi$abundance[c(sig_up, sig_down),] %>% as.data.frame() %>%
          rownames_to_column(var = "ensembl_gene_id") %>%
          left_join(gene_symbol, by = c("ensembl_gene_id" = "gene_id")) %>%
          drop_na(symbol)

plotmat$ensembl_gene_id <- NULL

plotmat <- plotmat %>% column_to_rownames(var = "symbol") %>% as.matrix()

# Color palette
heat.colors <- brewer.pal(6, "YlOrRd")

# Plot heatmap
pheatmap(plotmat,
         scale = "row",
         show_rownames = TRUE,
         border = FALSE,
         annotation = meta[, c("response"), drop = FALSE],
         main = "Top 50 Up- and Down- regulated genes in Response: Yes vs No",
         fontsize = 20)
```

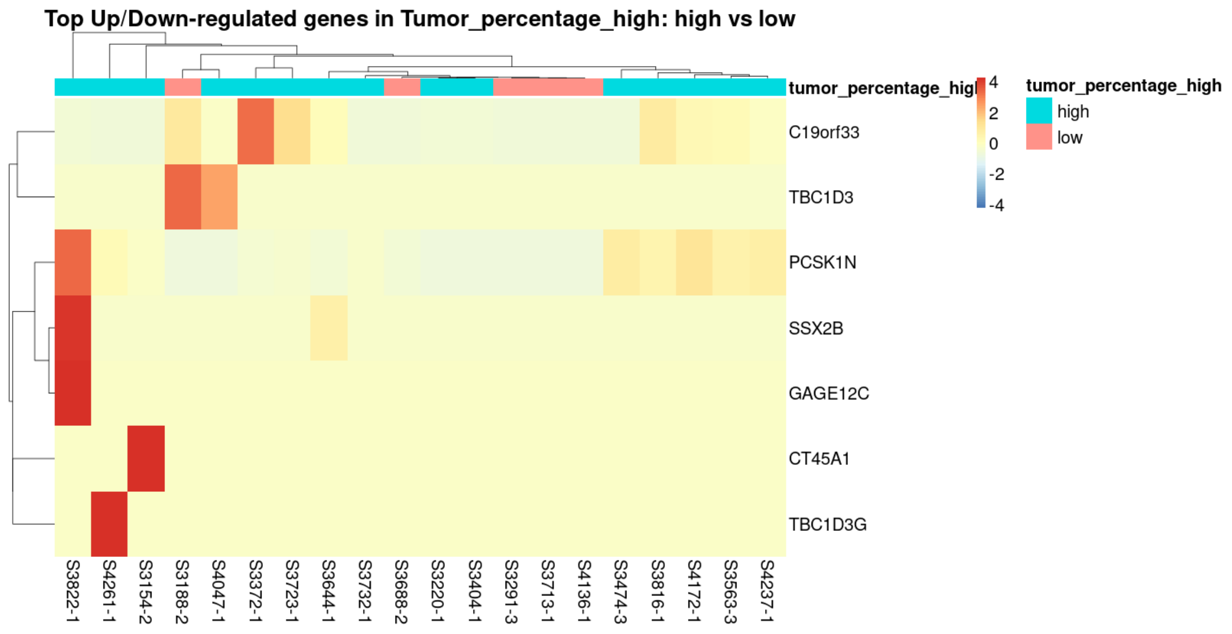

Top 50 Up- and Down- regulated genes in Response: Yes vs No
response
4
2
0
-2
-4
response
No
Yes
SLC25A25
FN3KRP
MLC1
CD72
AMN
ZNF652
STOM
GAGE12C
PAX5
SBSPON
ZNF281
RAB4A
PATJ
CT45A1
TBC1D3
S3154-2
S3220-1
S3816-1
S3713-1
S4136-1
S3404-1
S3563-3
S3688-2
S4172-1
S4237-1
S3644-1
S3732-1
S3291-3
S3723-1
S3474-3
S3188-2
S4047-1
S3822-1
S3372-1
S4261-1

```r
# Create a matrix of normalized expression
sig_up <- resER_tb_significant %>% arrange(-log2FoldChange) %>% head(50) %>% pull(gene)
sig_down <- resER_tb_significant %>% arrange(log2FoldChange) %>% head(50) %>% pull(gene)
sig <- c(sig_up, sig_down)

row_annotation <- gene_symbol %>%
                    as_tibble() %>%
                    dplyr::filter(gene_id %in% sig)

plotmat <- txi$abundance[c(sig_up, sig_down),] %>% as.data.frame() %>%
          rownames_to_column(var = "ensembl_gene_id") %>%
          left_join(gene_symbol, by = c("ensembl_gene_id" = "gene_id")) %>%
          drop_na(symbol)

plotmat$ensembl_gene_id <- NULL

plotmat <- plotmat %>% column_to_rownames(var = "symbol") %>% as.matrix()

# Color palette
heat.colors <- brewer.pal(6, "YlOrRd")

# Plot heatmap
pheatmap(plotmat,
         scale = "row",
         show_rownames = TRUE,
         border = FALSE,
         annotation = meta[, c("er"), drop = FALSE],
         main = "Top 50 Up- and Down- regulated genes in ER: positive vs negative",
         fontsize = 20)
```

Top 50 Up- and Down- regulated genes in ER: positive vs negative

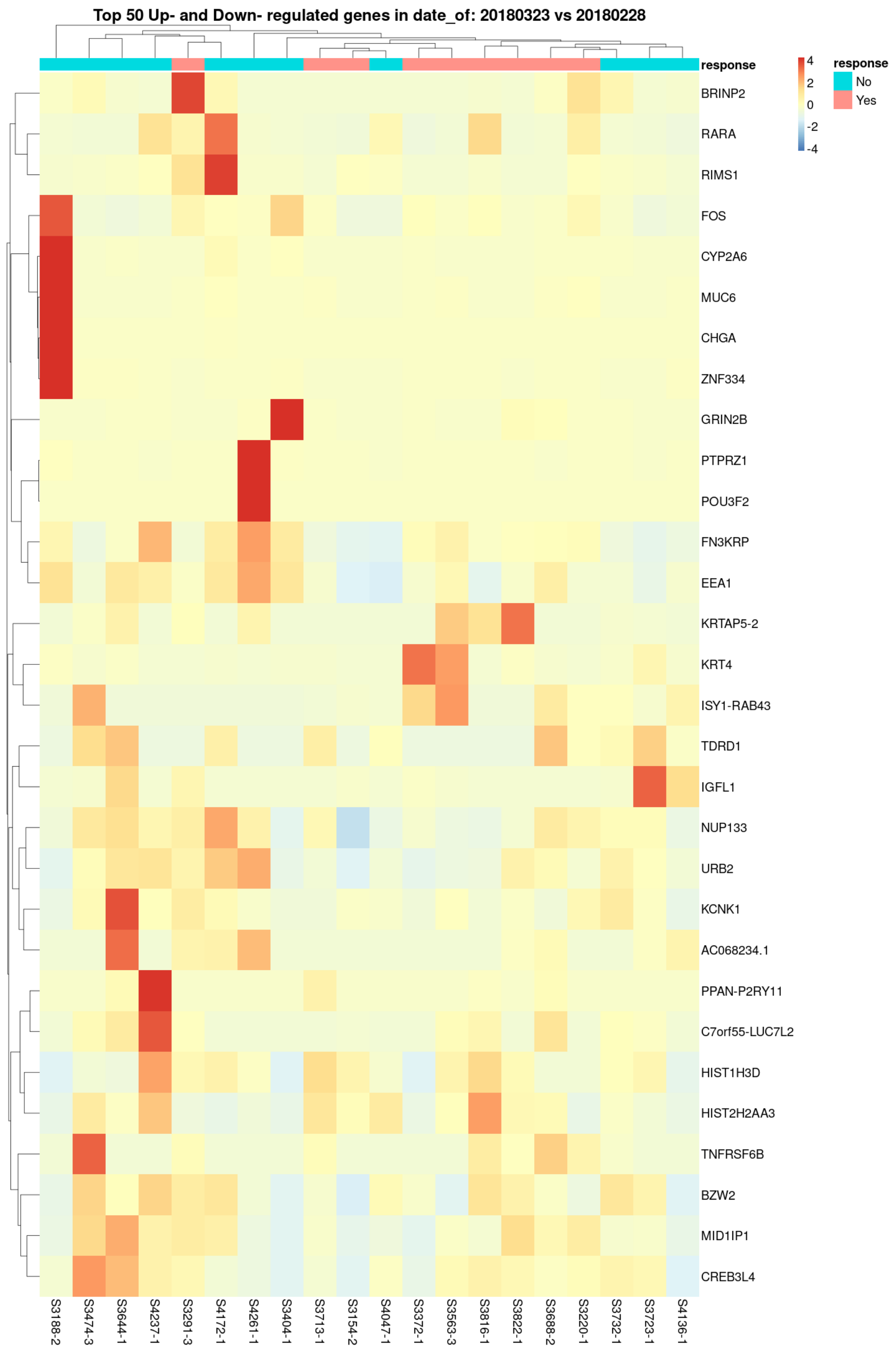


```r
# Create a matrix of normalized expression
sig_up <- resTP_tb_significant %>% arrange(-log2FoldChange) %>% head(50) %>% pull(gene)
sig_down <- resTP_tb_significant %>% arrange(log2FoldChange) %>% head(50) %>% pull(gene)
sig <- c(sig_up, sig_down)

row_annotation <- gene_symbol %>%
                    as_tibble() %>%
                    dplyr::filter(gene_id %in% sig)

plotmat <- txi$abundance[c(sig_up, sig_down),] %>% as.data.frame() %>%
          rownames_to_column(var = "ensembl_gene_id") %>%
          left_join(gene_symbol, by = c("ensembl_gene_id" = "gene_id")) %>%
          drop_na(symbol)

plotmat$ensembl_gene_id <- NULL

plotmat <- plotmat %>% column_to_rownames(var = "symbol") %>% as.matrix()

# Color palette
heat.colors <- brewer.pal(6, "YlOrRd")

# Plot heatmap
pheatmap(plotmat,
         scale = "row",
         show_rownames = TRUE,
         border = FALSE,
         annotation = meta[, c("tumor_percentage_high"), drop = FALSE],
         main = "Top Up/Down-regulated genes in Tumor_percentage_high: high vs low",
         fontsize = 20)
```



```r
# Create a matrix of normalized expression
sig_up <- resDO_tb_significant %>% arrange(-log2FoldChange) %>% head(50) %>% pull(gene)
sig_down <- resDO_tb_significant %>% arrange(log2FoldChange) %>% head(50) %>% pull(gene)
sig <- c(sig_up, sig_down)
```

```r
row_annotation <- gene_symbol %>%
                    as_tibble() %>%
                    dplyr::filter(gene_id %in% sig)

plotmat <- txi$abundance[c(sig_up, sig_down),] %>% as.data.frame() %>%
          rownames_to_column(var = "ensembl_gene_id") %>%
          left_join(gene_symbol, by = c("ensembl_gene_id" = "gene_id")) %>%
          drop_na(symbol)

plotmat$ensembl_gene_id <- NULL

plotmat <- plotmat %>% column_to_rownames(var = "symbol") %>% as.matrix()

# Color palette
heat.colors <- brewer.pal(6, "YlOrRd")

# Plot heatmap
pheatmap(plotmat,
         scale = "row",
         show_rownames = TRUE,
         border = FALSE,
         annotation = meta[, c("response"), drop = FALSE],
         main = "Top 50 Up- and Down- regulated genes in date_of: 20180323 vs 20180228",
         fontsize = 20)
```


Top 50 Up- and Down- regulated genes in date_of: 20180323 vs 20180228
response
BRINP2
RARA
RIMS1
FOS
CYP2A6
MUC6
CHGA
ZNF334
GRIN2B
PTPRZ1
POU3F2
FN3KRP
EEA1
KRTAP5-2
KRT4
ISY1-RAB43
TDRD1
IGFL1
NUP133
URB2
KCNK1
AC068234.1
PPAN-P2RY11
C7orf55-LUC7L2
HIST1H3D
HIST2H2AA3
TNFRSF6B
BZW2
MID1IP1
CREB3L4
S3188-2
S3474-3
S3644-1
S4237-1
S3291-3
S4172-1
S4261-1
S3404-1
S3713-1
S3154-2
S4047-1
S3372-1
S3563-3
S3816-1
S3822-1
S3688-2
S3220-1
S3732-1
S3723-1
S4136-1
4
2
0
-2
-4
response
No
Yes

Functional analysis

Biological Process (BP)

```
bg_genes <- rownames(resResponse)

## Run GO enrichment analysis
compGO <- enrichGO(gene = sigResponse_up,
                   universe = bg_genes,
                   keyType = "ENSEMBL",
                   OrgDb = "org.Hs.eg.db",
                   ont = "BP",
                   qvalueCutoff  = 0.05,
                   pAdjustMethod = "BH",
                   readable = TRUE)
```

```
## Error in enrichGO(gene = sigResponse_up, universe = bg_genes, keyType = "ENSEMBL", : could not find
```

```
#dotplot(compGO,
#        showCategory = 20,
#        title = "GO (Biological Process) Enrichment \n Analysis for UP in Responders)",
#        label_format = 20,
#        font.size = 10)
# image pdf 12 x 12

## Output results from GO analysis to a table
print("UP")
```

```
## [1] "UP"
```

```
results_up <- data.frame(compGO@result) %>% dplyr::filter(p.adjust < 0.05)
```

```
## Error in data.frame(compGO@result): object 'compGO' not found
```

```
nrow(results_up)
```

```
## Error in h(simpleError(msg, call)): error in evaluating the argument 'x' in selecting a method for f
```

```
write_csv(results_up, "tables/T21.day8.GO_BP_UP.csv")
```

```
## Error in is.data.frame(x): object 'results_up' not found
```

```
compGO <- enrichGO(gene = sigResponse_down,
                   universe = bg_genes,
                   keyType = "ENSEMBL",
                   OrgDb = "org.Hs.eg.db",
                   ont = "BP",
                   qvalueCutoff  = 0.05,
                   pAdjustMethod = "BH",
                   readable = TRUE)
```

```
## Error in enrichGO(gene = sigResponse_down, universe = bg_genes, keyType = "ENSEMBL", : could not fin
```

```
results_down <- data.frame(compGO@result) %>% dplyr::filter(p.adjust < 0.05)
```

```
## Error in data.frame(compGO@result): object 'compGO' not found
```

```
print("Down")
```

```
## [1] "Down"
```

```r
nrow(results_down)
```

```
## Error in h(simpleError(msg, call)): error in evaluating the argument 'x' in selecting a method for f
```

R session

```r
sessionInfo()
```

```
## R version 4.0.3 (2020-10-10)
## Platform: x86_64-redhat-linux-gnu (64-bit)
## Running under: Fedora 32 (Workstation Edition)
##
## Matrix products: default
## BLAS/LAPACK: /usr/lib64/libopenblas-r0.3.12.so
##
## locale:
##  [1] LC_CTYPE=en_CA.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=en_CA.UTF-8        LC_COLLATE=en_CA.UTF-8
##  [5] LC_MONETARY=en_CA.UTF-8    LC_MESSAGES=en_CA.UTF-8
##  [7] LC_PAPER=en_CA.UTF-8       LC_NAME=C
##  [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_CA.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] parallel  stats4    stats     graphics  grDevices utils     datasets
## [8] methods   base
##
## other attached packages:
##  [1] ensembldb_2.14.0            AnnotationFilter_1.14.0
##  [3] GenomicFeatures_1.42.1      AnnotationDbi_1.52.0
##  [5] AnnotationHub_2.22.0        BiocFileCache_1.14.0
##  [7] dbplyr_2.1.0                knitr_1.30
##  [9] ggrepel_0.9.1               tximport_1.18.0
## [11] DEGreport_1.26.0            pheatmap_1.0.12
## [13] RColorBrewer_1.1-2          forcats_0.5.1
## [15] stringr_1.4.0               dplyr_1.0.5
## [17] purrr_0.3.4                 readr_1.4.0
## [19] tidyr_1.1.3                 tibble_3.1.0
## [21] ggplot2_3.3.3               tidyverse_1.3.0
## [23] DESeq2_1.30.1               SummarizedExperiment_1.20.0
## [25] Biobase_2.50.0              MatrixGenerics_1.2.1
## [27] matrixStats_0.58.0          GenomicRanges_1.42.0
## [29] GenomeInfoDb_1.26.2         IRanges_2.24.1
## [31] S4Vectors_0.28.1            BiocGenerics_0.36.0
##
## loaded via a namespace (and not attached):
##   [1] readxl_1.3.1                  backports_1.2.1
##   [3] circlize_0.4.12               plyr_1.8.6
##   [5] lazyeval_0.2.2                ConsensusClusterPlus_1.54.0
##   [7] splines_4.0.3                 BiocParallel_1.24.1
##   [9] digest_0.6.27                 htmltools_0.5.1.1
##  [11] fansi_0.4.2                   magrittr_2.0.1
##  [13] memoise_2.0.0              cluster_2.1.0
##  [15] limma_3.46.0               ComplexHeatmap_2.6.2
##  [17] Biostrings_2.58.0          annotate_1.68.0
##  [19] Nozzle.R1_1.1-1            modelr_0.1.8
##  [21] askpass_1.1                prettyunits_1.1.1
##  [23] colorspace_2.0-0           blob_1.2.1
##  [25] rvest_1.0.0                rappdirs_0.3.3
##  [27] haven_2.3.1                xfun_0.19
##  [29] crayon_1.4.1               RCurl_1.98-1.2
##  [31] jsonlite_1.7.1             genefilter_1.72.1
##  [33] survival_3.2-7             glue_1.4.2
##  [35] gtable_0.3.0               zlibbioc_1.36.0
##  [37] XVector_0.30.0             GetoptLong_1.0.5
##  [39] DelayedArray_0.16.2        shape_1.4.5
##  [41] scales_1.1.1               DBI_1.1.1
##  [43] edgeR_3.32.1               Rcpp_1.0.6
##  [45] progress_1.2.2             xtable_1.8-4
##  [47] lasso2_1.2-21.1            tmvnsim_1.0-2
##  [49] clue_0.3-58                bit_4.0.4
##  [51] httr_1.4.2                 ellipsis_0.3.1
##  [53] farver_2.1.0               pkgconfig_2.0.3
##  [55] reshape_0.8.8              XML_3.99-0.5
##  [57] locfit_1.5-9.4             utf8_1.1.4
##  [59] labeling_0.4.2             tidyselect_1.1.0
##  [61] rlang_0.4.10               later_1.1.0.1
##  [63] munsell_0.5.0              BiocVersion_3.12.0
##  [65] cellranger_1.1.0           tools_4.0.3
##  [67] cachem_1.0.4               cli_2.3.1
##  [69] generics_0.1.0             RSQLite_2.2.3
##  [71] broom_0.7.5                evaluate_0.14
##  [73] fastmap_1.1.0              ggdendro_0.1.22
##  [75] yaml_2.2.1                 bit64_4.0.5
##  [77] fs_1.5.0                   nlme_3.1-149
##  [79] mime_0.9                   xml2_1.3.2
##  [81] biomaRt_2.46.3             compiler_4.0.3
##  [83] rstudioapi_0.13            curl_4.3
##  [85] png_0.1-7                  interactiveDisplayBase_1.28.0
##  [87] reprex_1.0.0               geneplotter_1.68.0
##  [89] stringi_1.5.3              lattice_0.20-41
##  [91] ProtGenerics_1.22.0        Matrix_1.2-18
##  [93] psych_2.0.12               vctrs_0.3.6
##  [95] pillar_1.5.1               lifecycle_1.0.0
##  [97] BiocManager_1.30.10        GlobalOptions_0.1.2
##  [99] cowplot_1.1.1              bitops_1.0-6
## [101] rtracklayer_1.50.0         httpuv_1.5.5
## [103] R6_2.5.0                   promises_1.2.0.1
## [105] MASS_7.3-53                assertthat_0.2.1
## [107] openssl_1.4.3              rjson_0.2.20
## [109] withr_2.4.1                GenomicAlignments_1.26.0
## [111] Rsamtools_2.6.0            mnormt_2.0.2
## [113] GenomeInfoDbData_1.2.4     hms_1.0.0
## [115] grid_4.0.3                 rmarkdown_2.5
## [117] Cairo_1.5-12.2             logging_0.10-108
## [119] shiny_1.6.0                lubridate_1.7.10
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