# 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
```

```
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)
```

```
# 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"
```

```
attributes(txi)
```

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

```
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.

```
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?

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

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

```
# 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
```

```
### 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.*

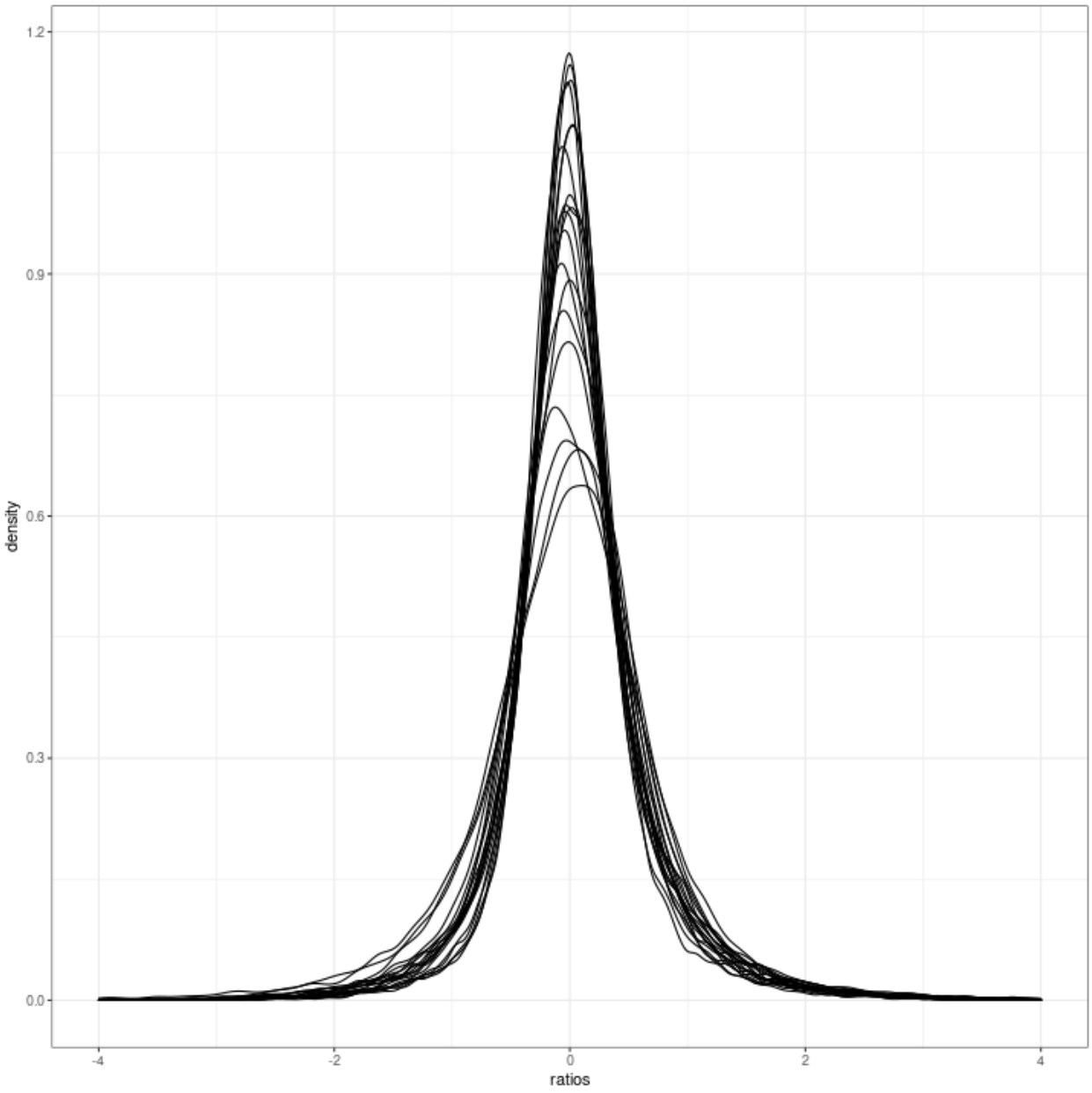
```
## Create DESeq2Dataset object
dds_file <- "data/dds.day1.RDS"
meta$treatment <- as.factor(meta$treatment)
meta$response <- as.factor(meta$response)
meta$er <- as.factor(meta$er)
meta$date_of <- as.factor(meta$date_of)
meta$tumor_percentage <- as.factor(meta$tumor_percentage)
meta$tumor_percentage_high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

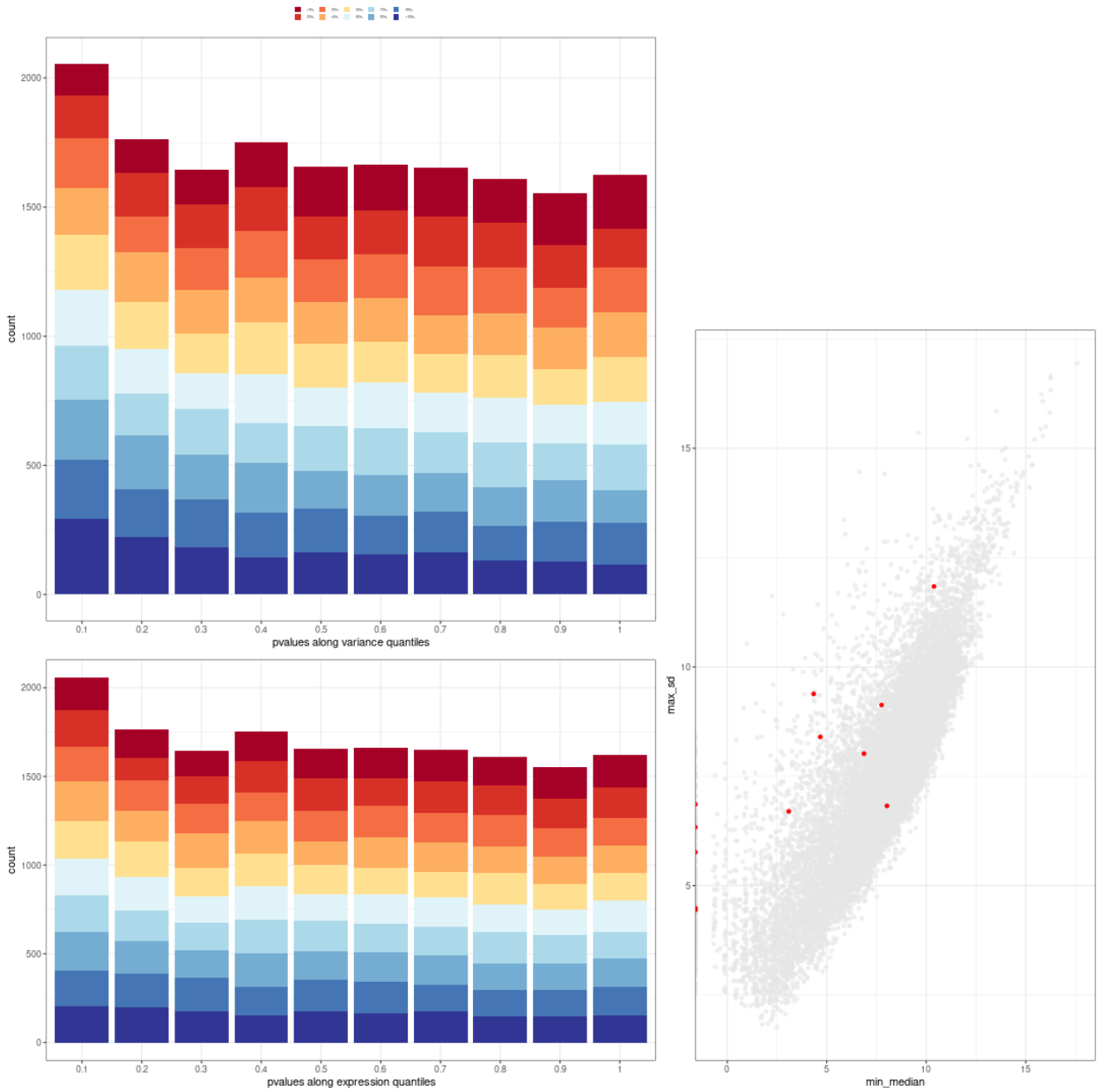
```
counts <- counts(dds, normalized = TRUE)
design <- as.data.frame(colData(dds))
degCheckFactors(counts[, 1:20])
```




# Mean-Variance QC plots

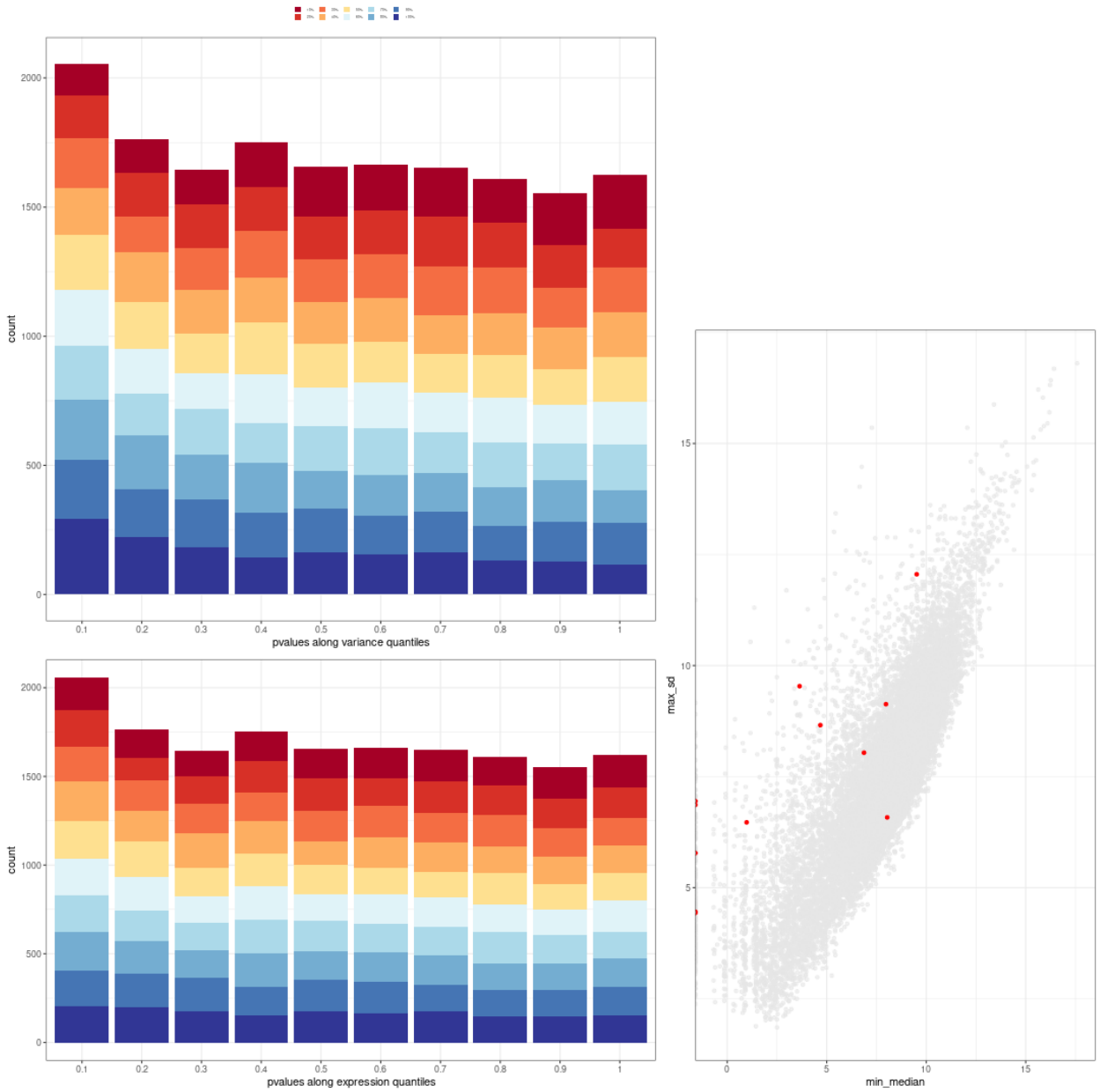
### response

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



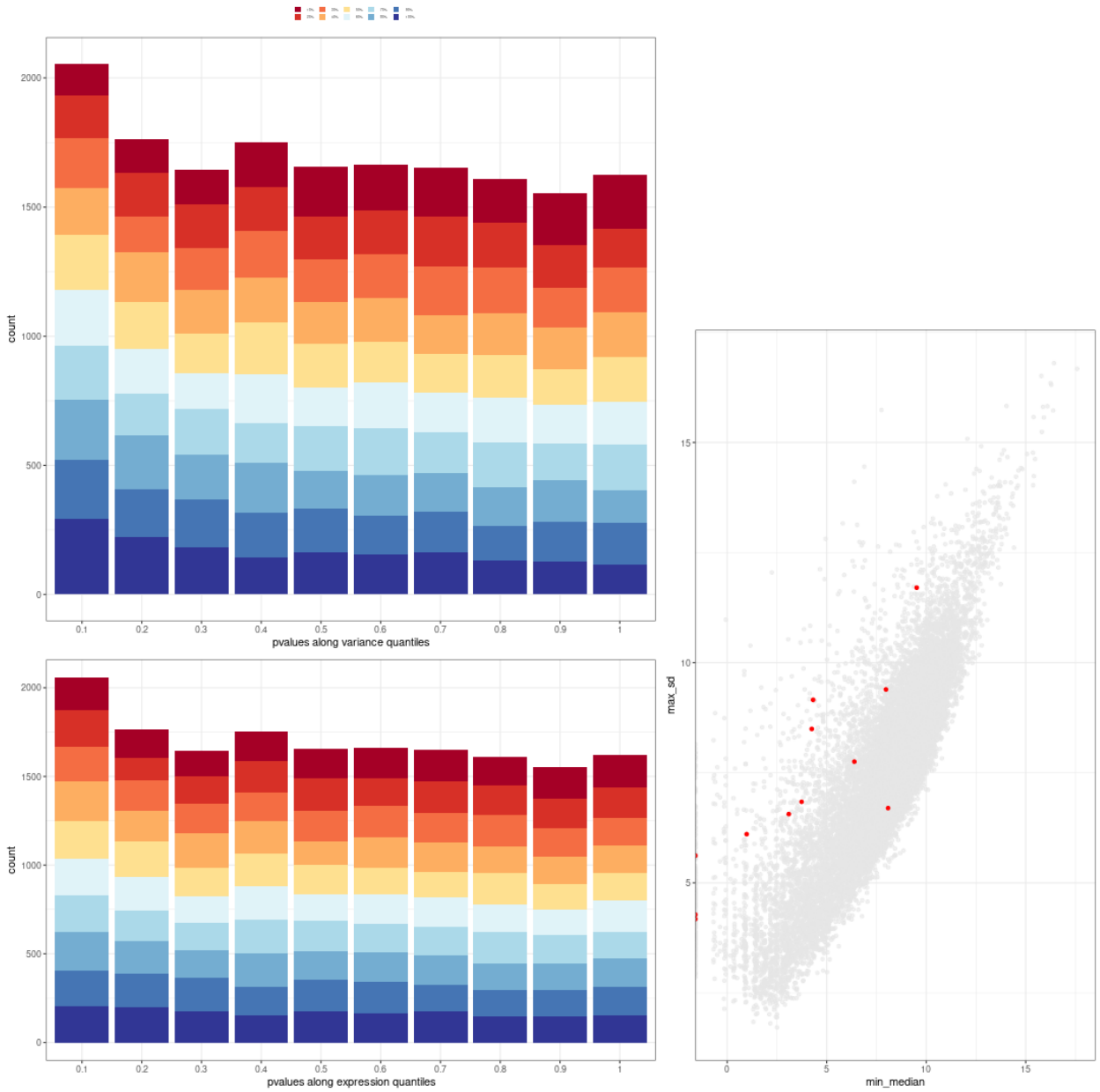

## ER

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




### tumor_percentage_high

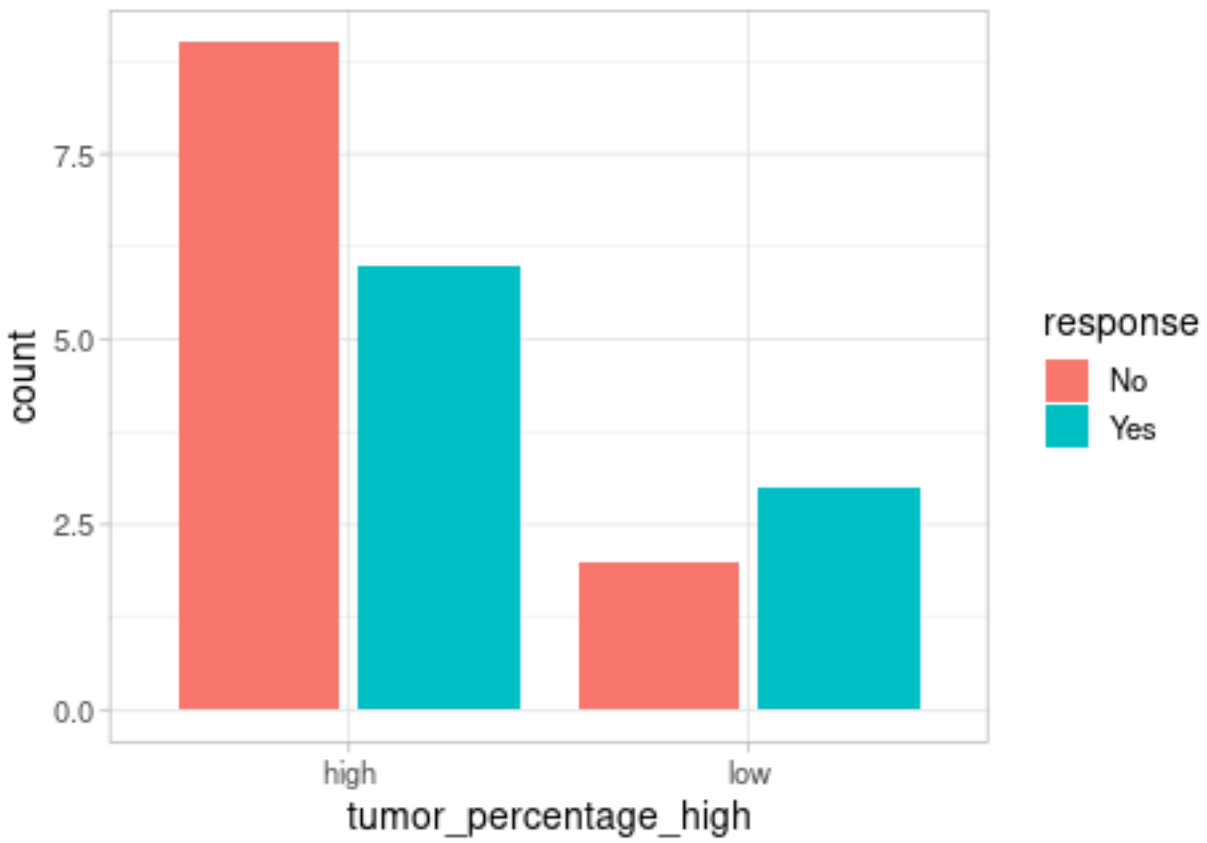
```
degQC(counts, design[["tumor_percentage_high"]], pvalue = res[["pvalue"]])
```




## Covariates effect on count data

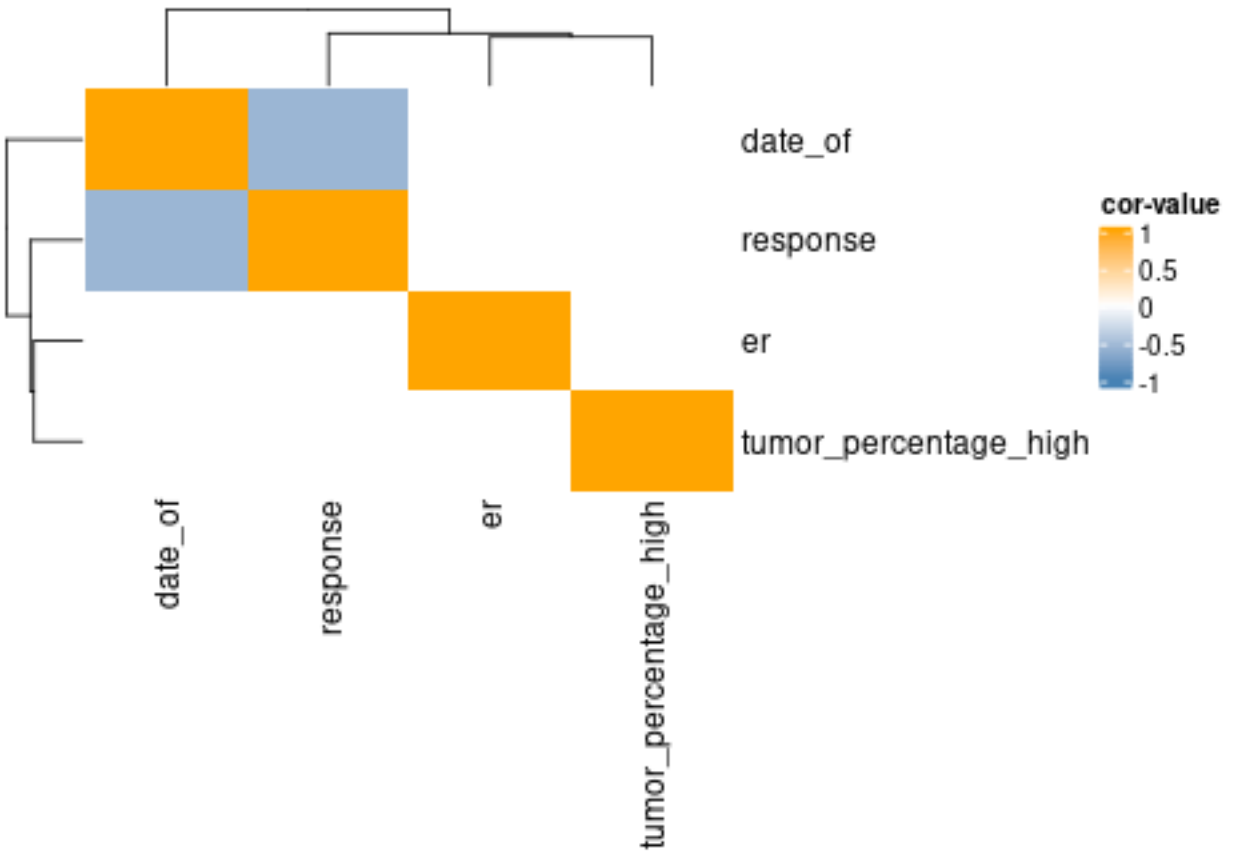
```
mdata <- colData(dds) %>%  as.data.frame()  %>%
  dplyr::select(response, er, date_of, tumor_percentage_high)

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

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




## Covariates correlation with metrics

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




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


## Sample-level QC analysis

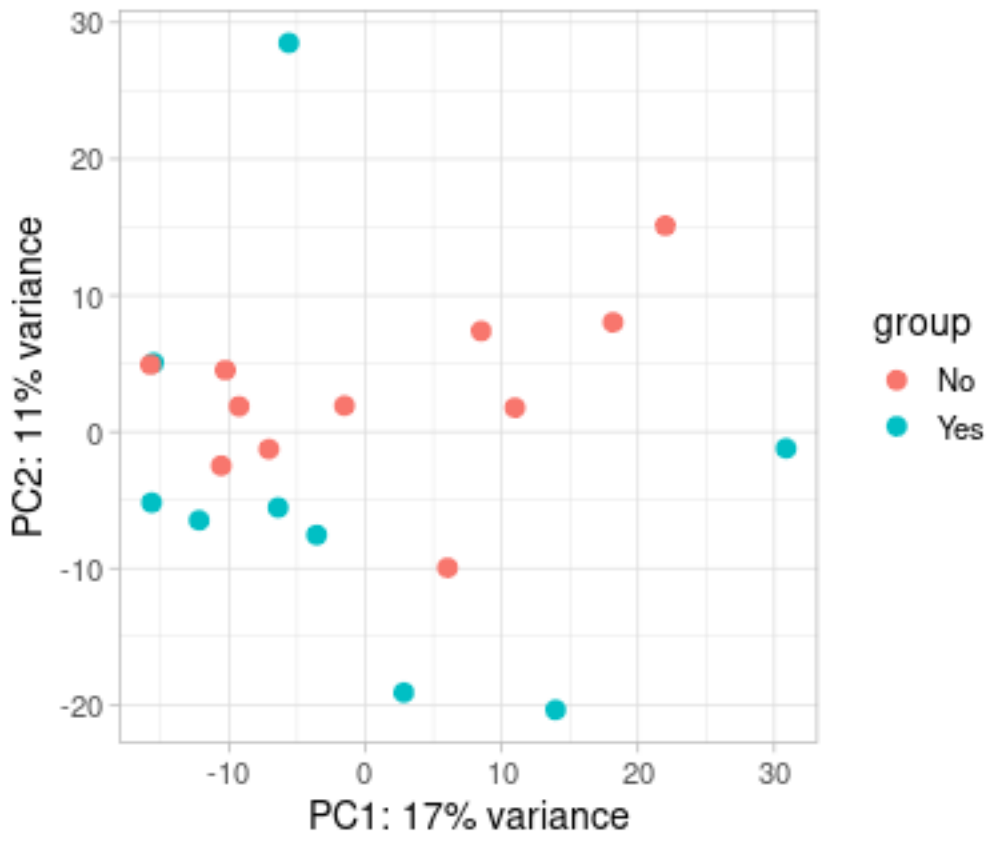
```
### Transform counts for data visualization (unsupervised analysis)
rld_file <- "data/rld.day1.RDS"
if (file.exists(rld_file)){
    rld <- readRDS(rld_file)
}else{
    rld <- rlog(dds, blind = TRUE)
    saveRDS(rld, rld_file)
}
class(rld) # what type of object is this
```

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

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

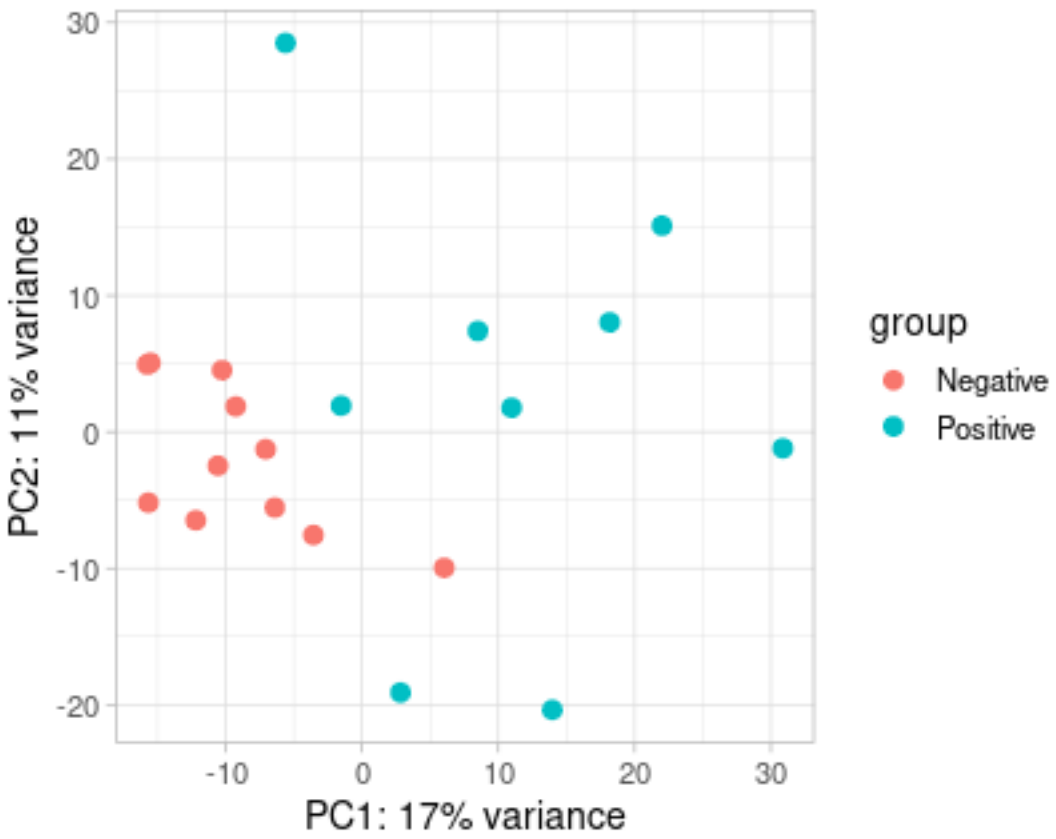
### PCA - response

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



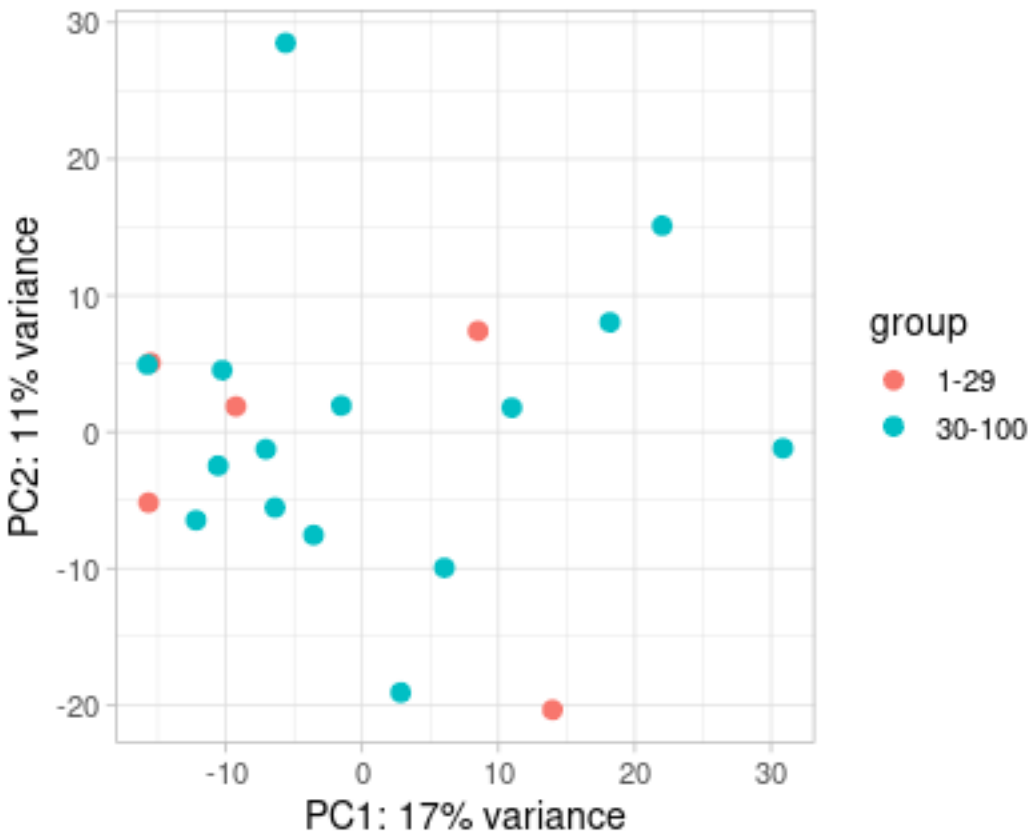

### PCA - ER

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



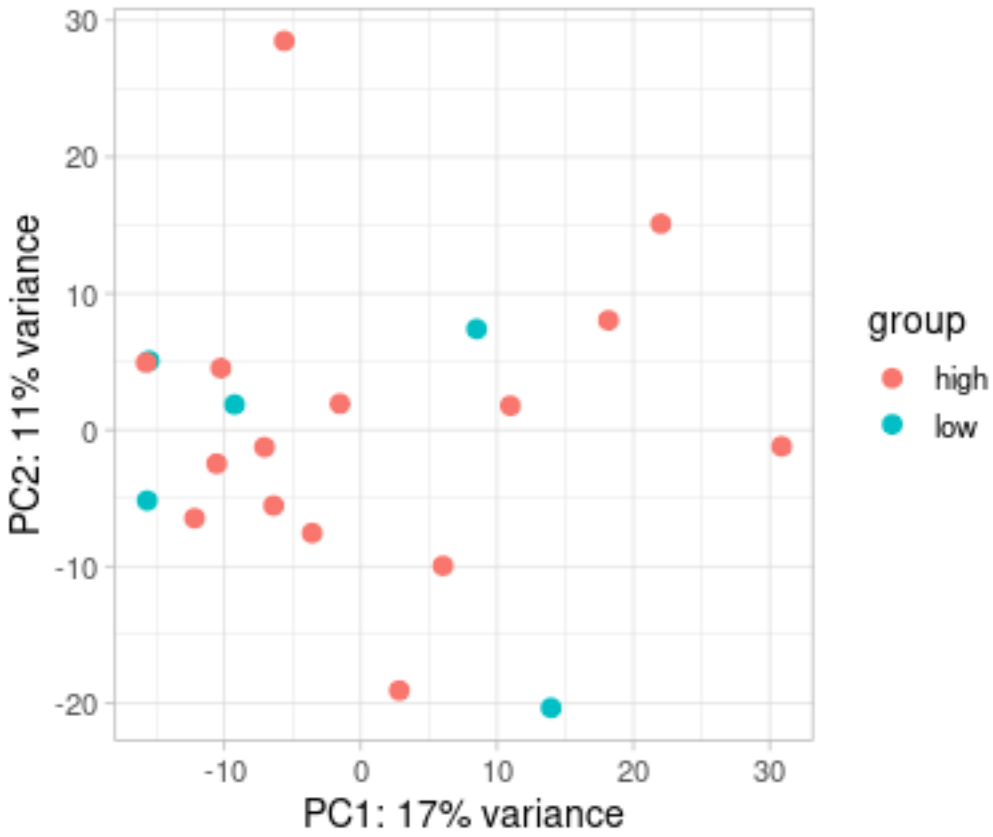

### PCA - tumor_percentage

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



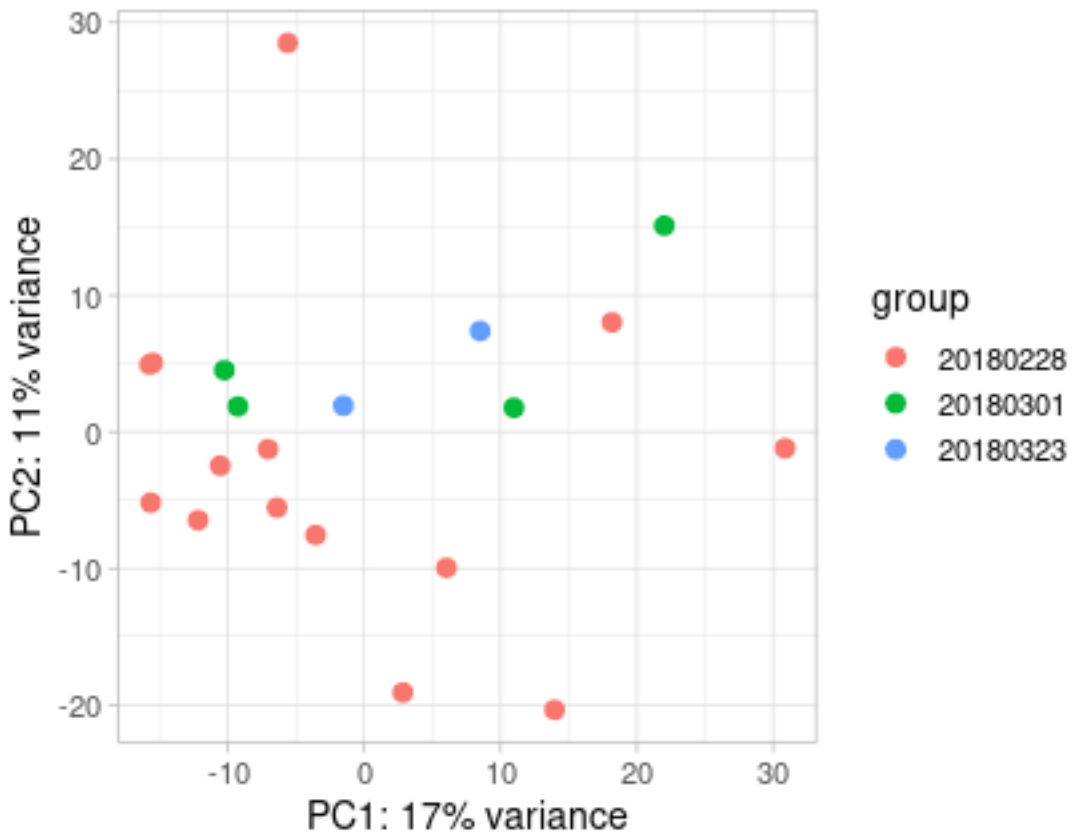

### PCA - tumor_percentage_high

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




### PCA - date_of

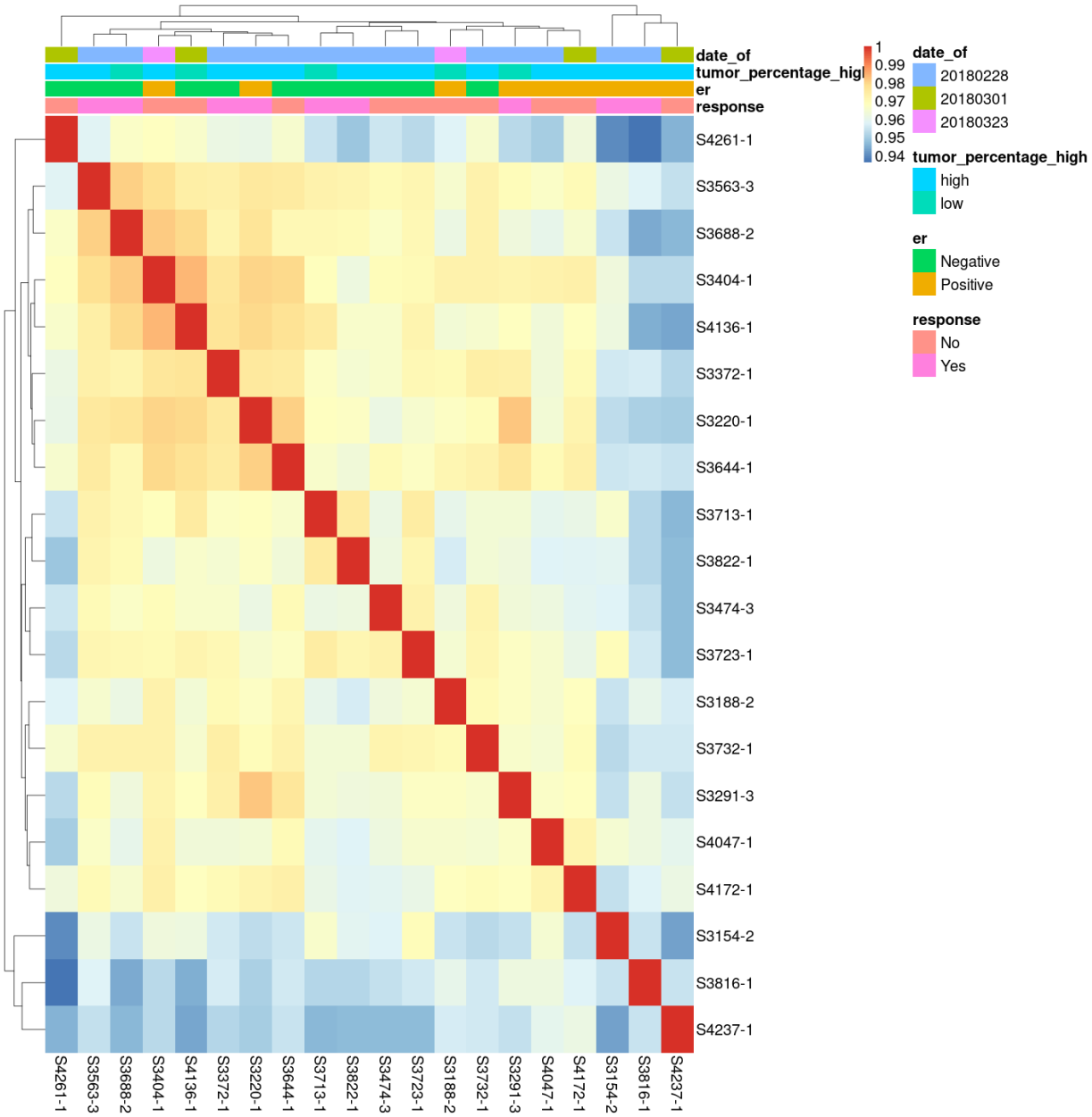
```
# Use the DESeq2 function
plotPCA(rld, intgroup = c("date_of"))
```




## Inter-correlation analysis

### Without study_id

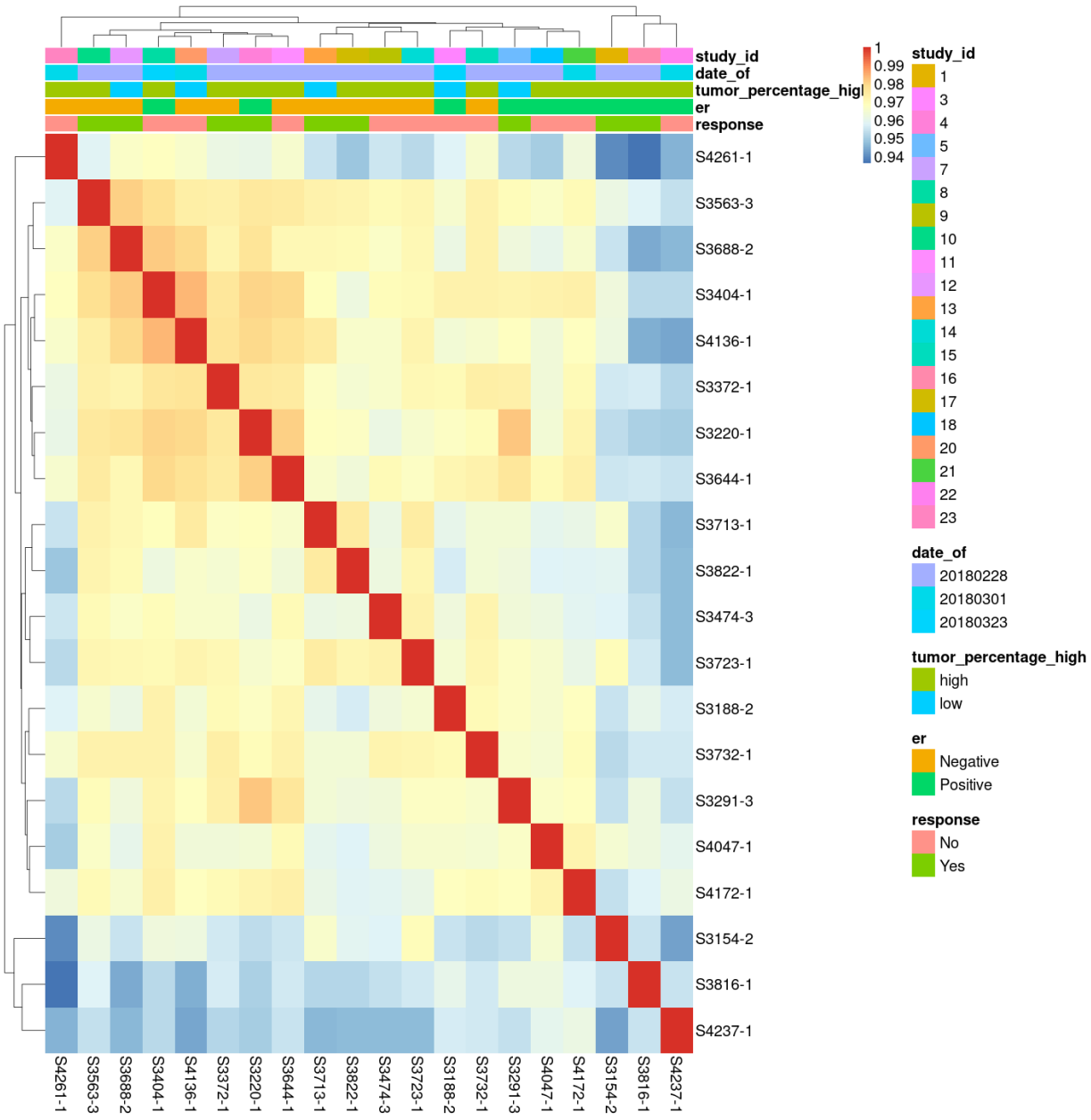
```
# Correlation matrix
rld_cor <- cor(rld_mat)

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
```

```r
# 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 doum-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 < 0
```

## ER : Positive vs Negative for Day1 - Table 10

```r
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
```

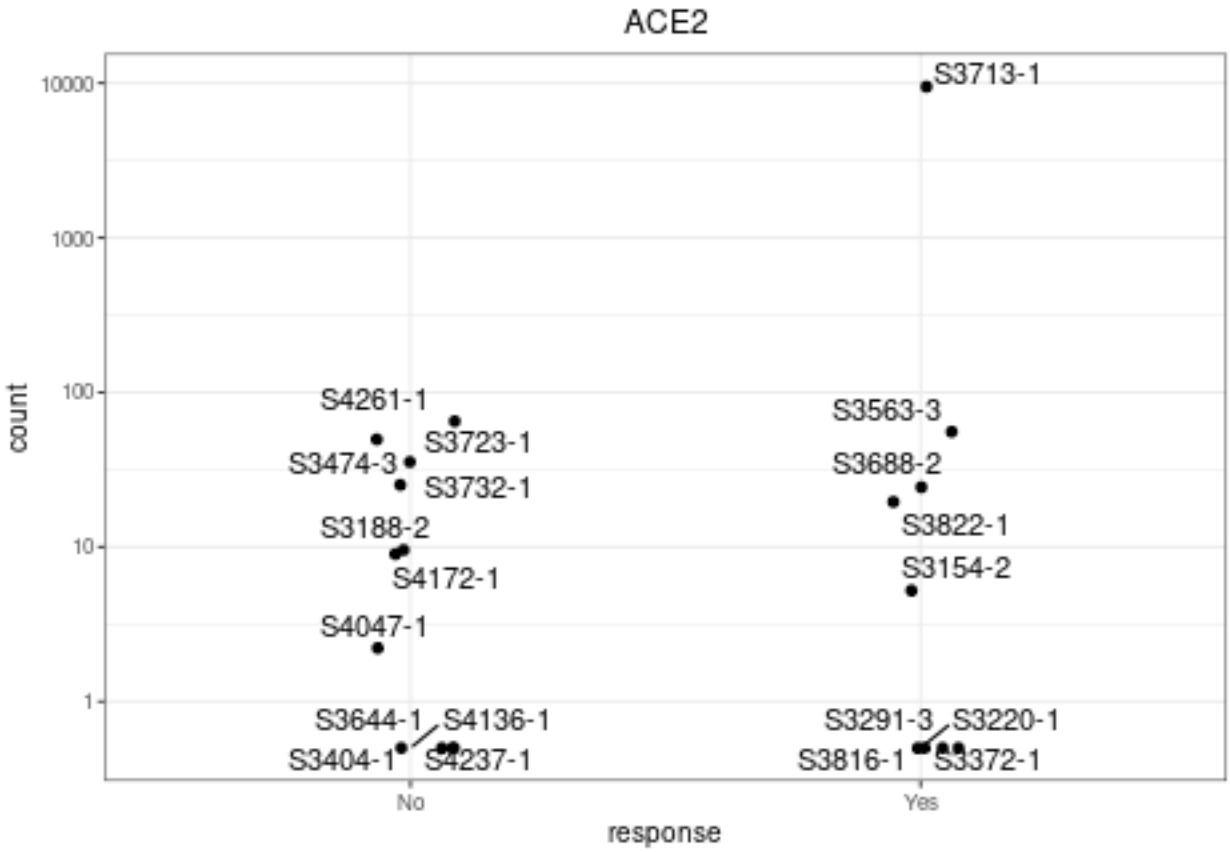
```r
# Add annotations
resDO_tb <- resDO %>%
  data.frame() %>%
  rownames_to_column(var = "gene") %>%
  as_tibble() %>%
  left_join(gene_symbol, by = c("gene" = "gene_id"))

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*

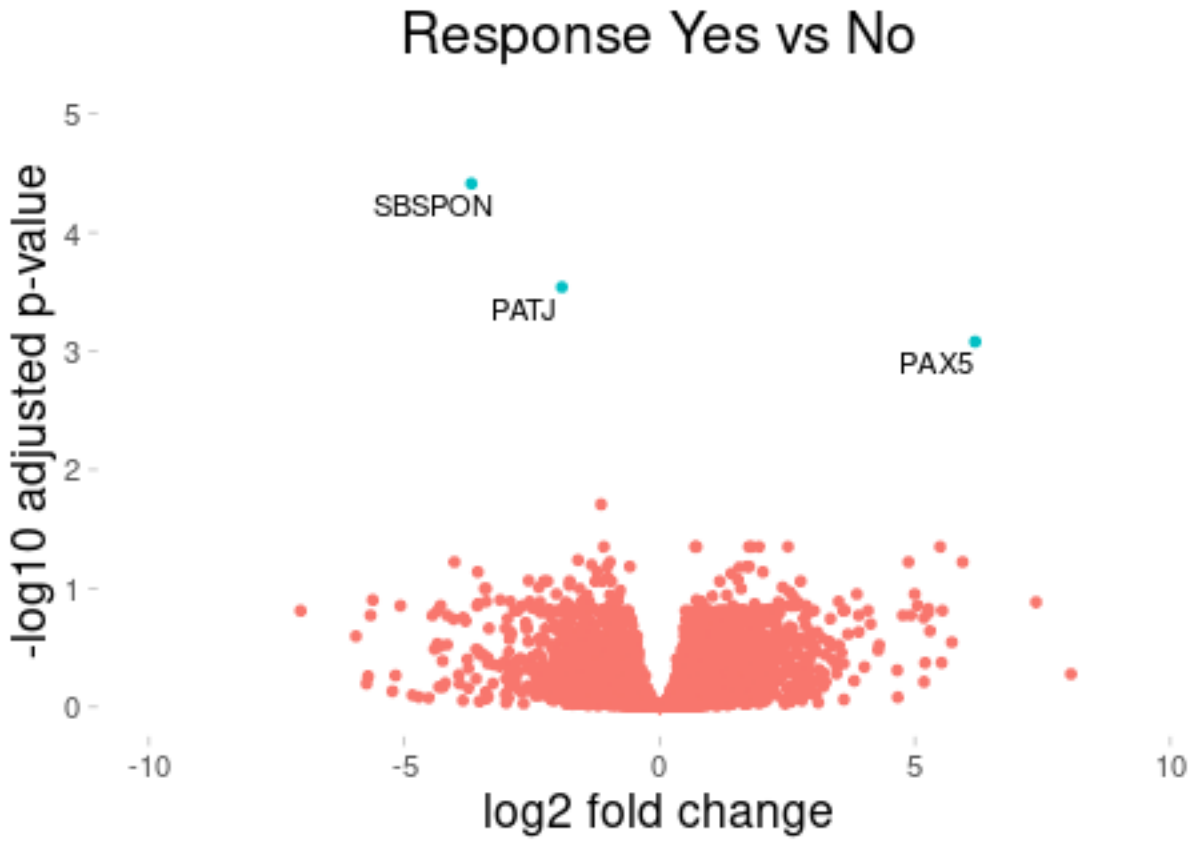
```r
d <- plotCounts(dds,
                gene = "ENSG00000130234",
                intgroup = "response",
                returnData = TRUE)
```

```
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()
```

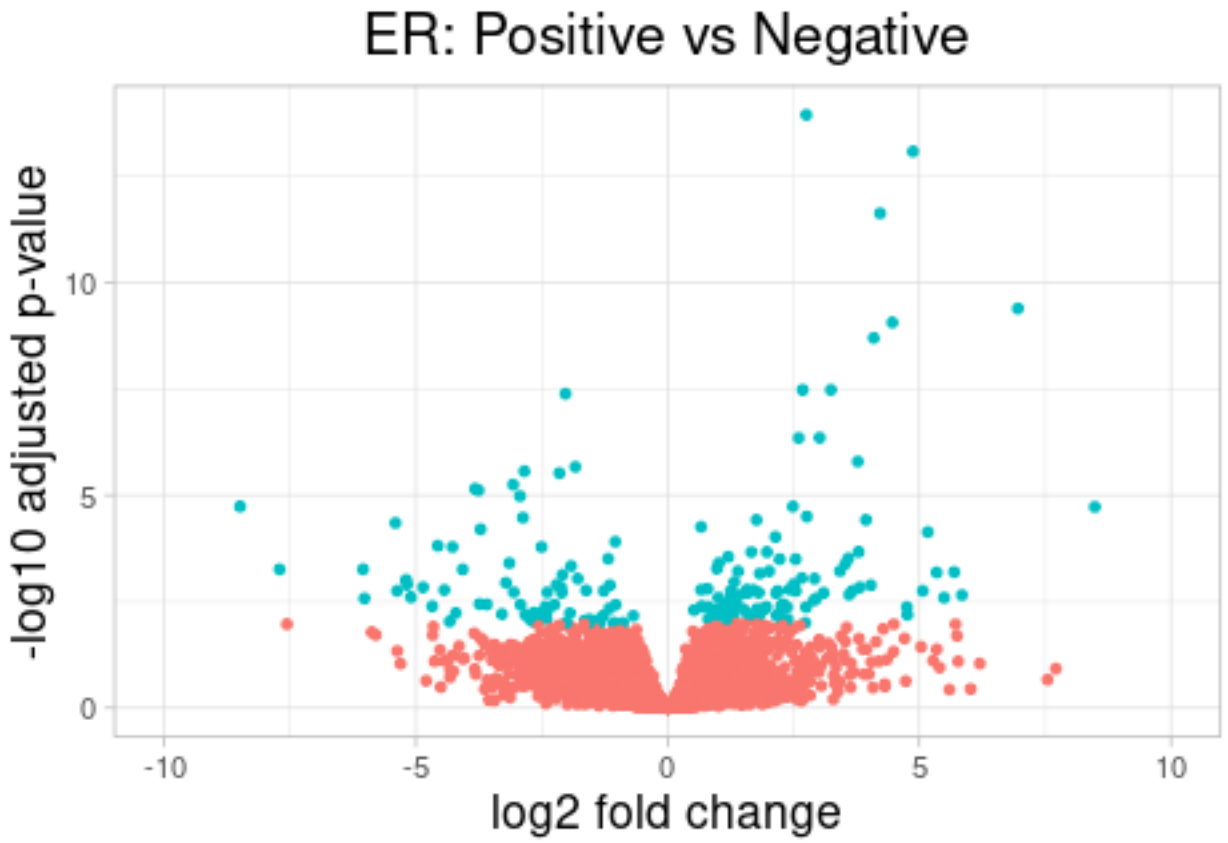



```
# Add a column for significant genes
resResponse_tb_vis <- resResponse_tb %>% mutate(threshold = padj < 0.01)

resResponse_tb_vis$symbol <- ifelse((abs(resResponse_tb_vis$log2FoldChange) > 1.5),
                                resResponse_tb_vis$symbol, NA)

resResponse_tb_vis$symbol <- ifelse(resResponse_tb_vis$threshold,
                                    resResponse_tb_vis$symbol, NA)

ggplot(resResponse_tb_vis,
       aes(log2FoldChange, -log10(padj), label = symbol)) +
  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, 5))+
  theme(legend.position = "none",
```

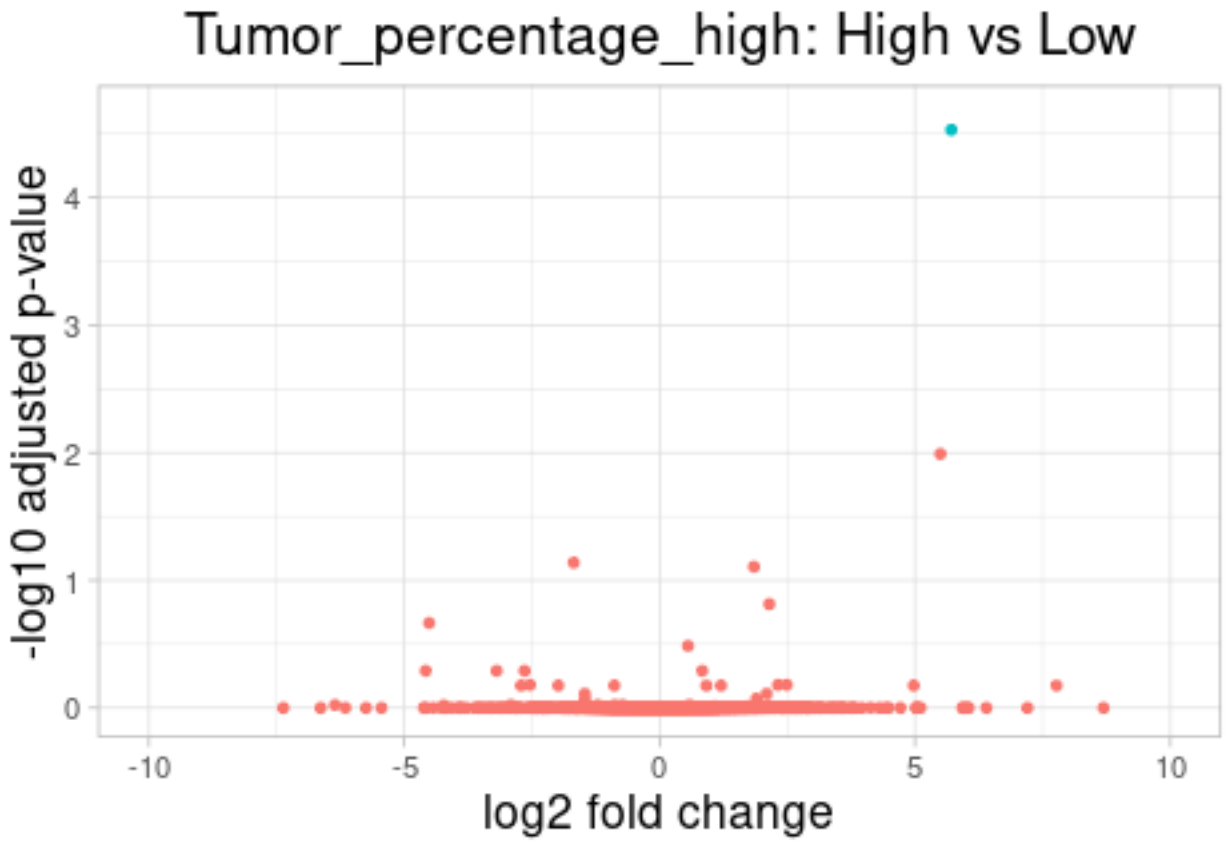
```
        plot.title = element_text(size = rel(1.5), hjust = 0.5),
        axis.title = element_text(size = rel(1.25)),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        panel.border = element_blank(),
        panel.background = element_blank()) +
  geom_text_repel(aes(label = symbol))
```



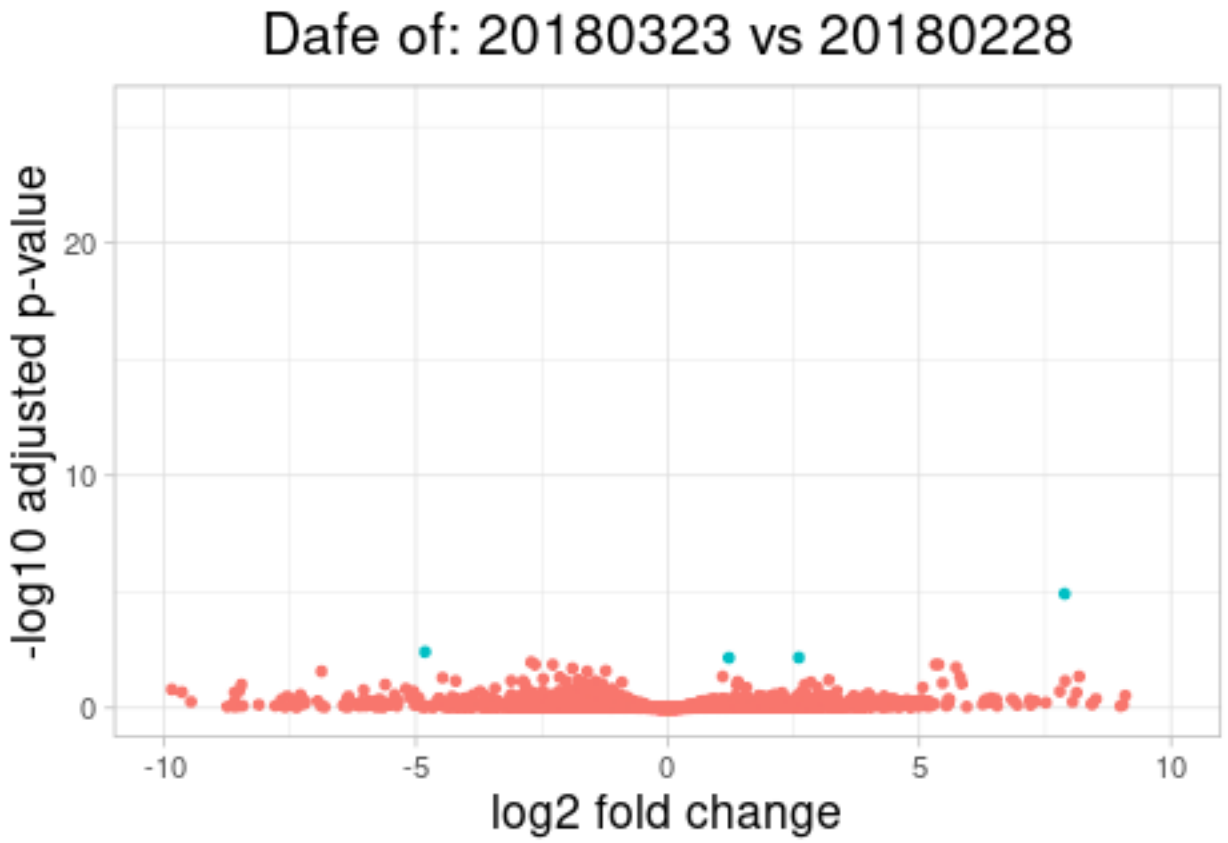

```
# Add a column for significant genes
resER_tb <- resER_tb %>% mutate(threshold = padj < 0.01)

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)))
```



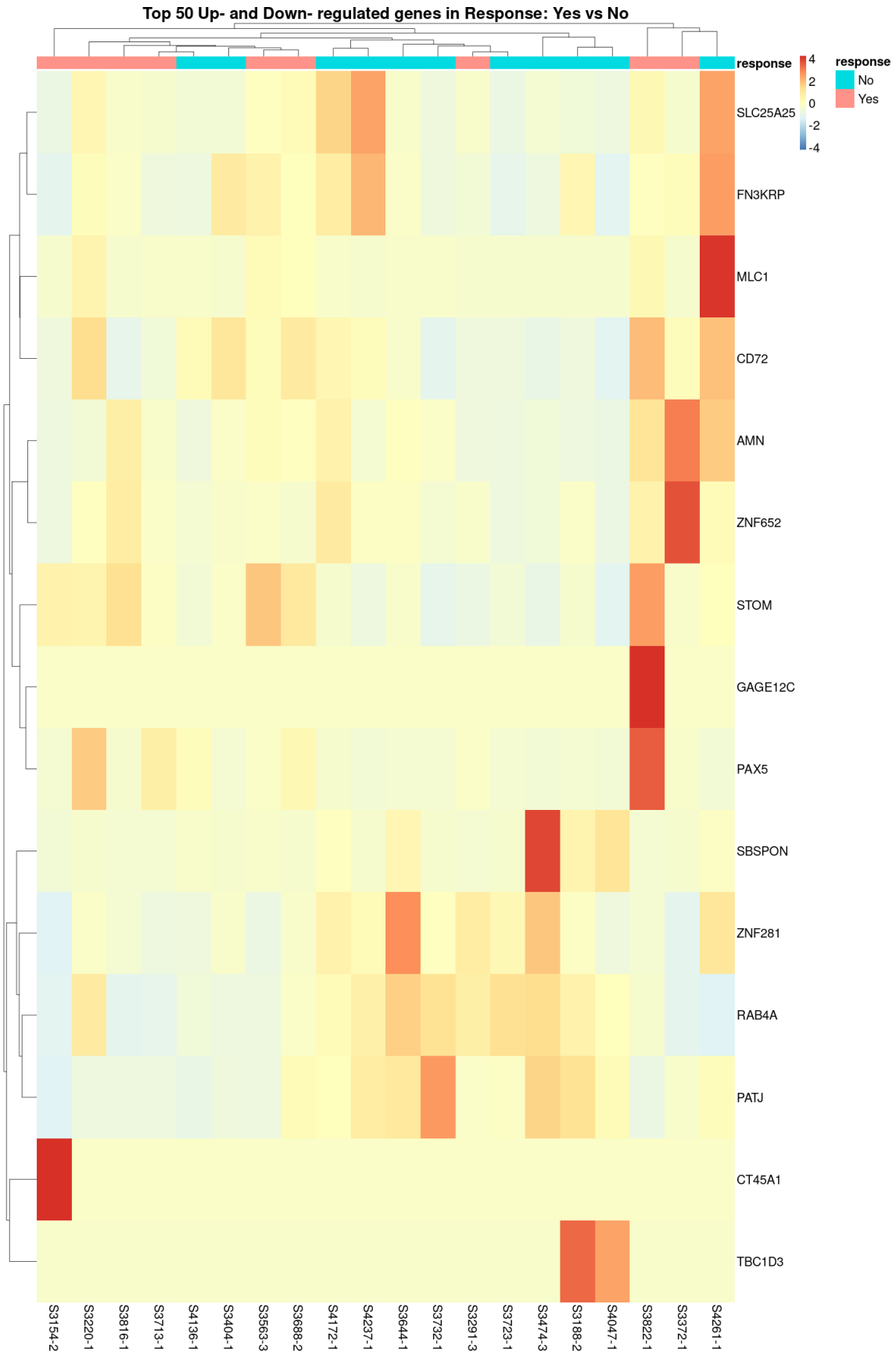

```
# Add a column for significant genes
resDO_tb <- resDO_tb %>% mutate(threshold = padj < 0.01)

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)))
```



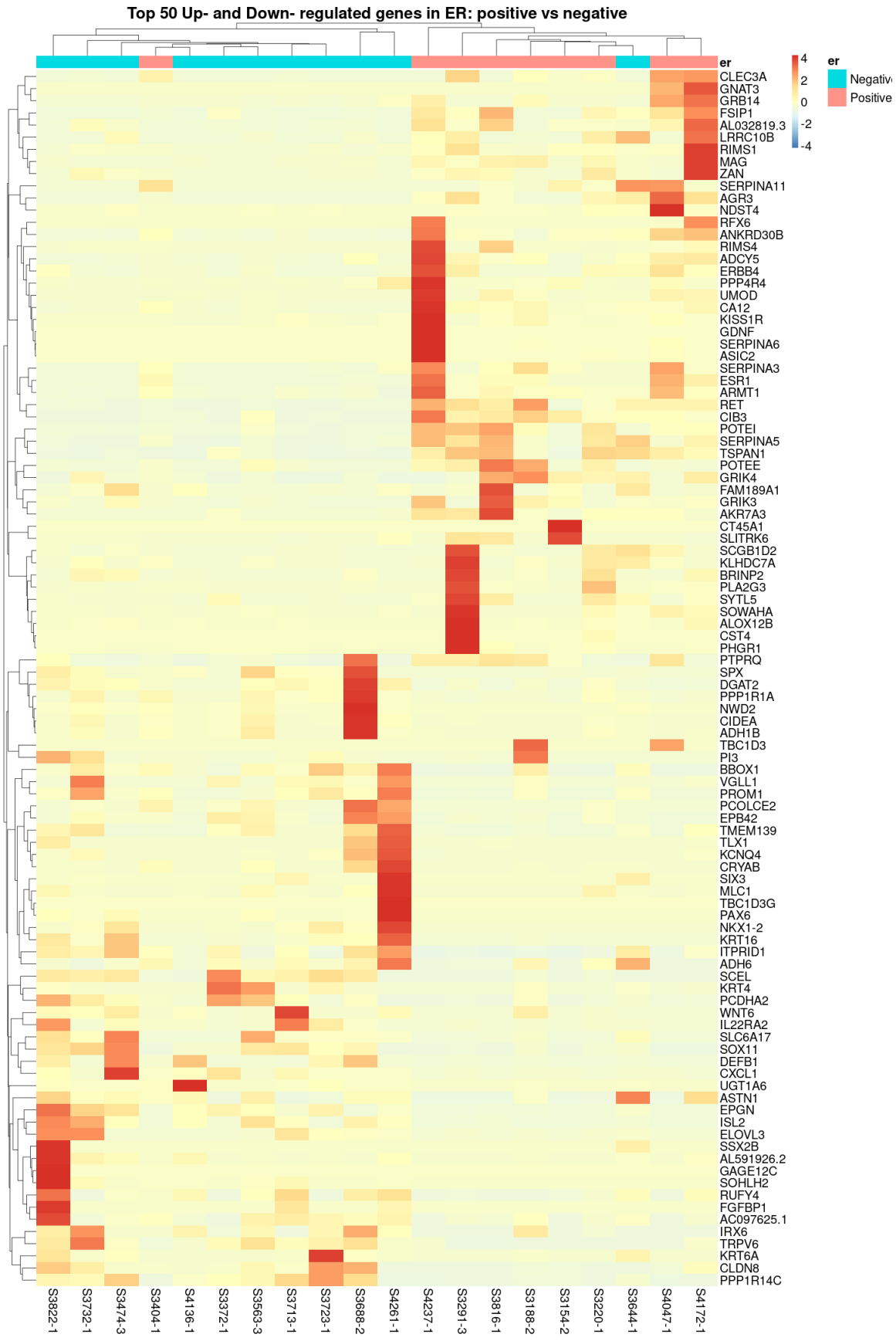

# Heatmaps

```
# 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,
```

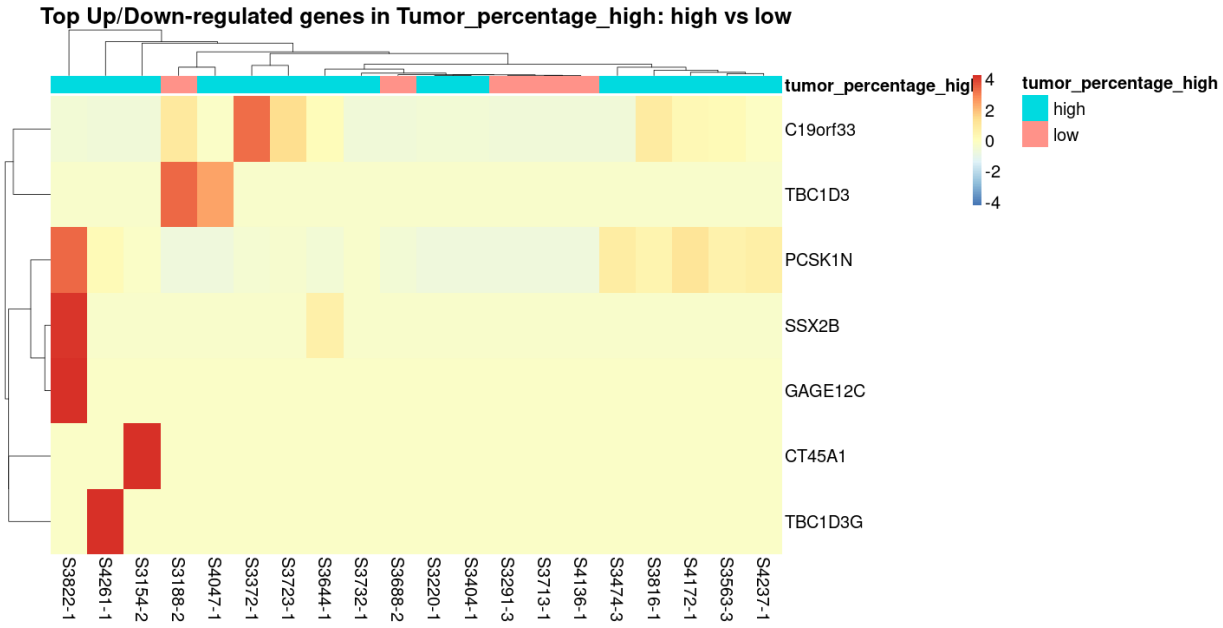
```
         border = FALSE,
         annotation = meta[, c("response"), drop = FALSE],
         main = "Top 50 Up- and Down- regulated genes in Response: Yes vs No",
         fontsize = 20)
```


Top 50 Up- and Down- regulated genes in Response: Yes vs No
response
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
4
2
0
-2
-4
response
No
Yes

```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)
```

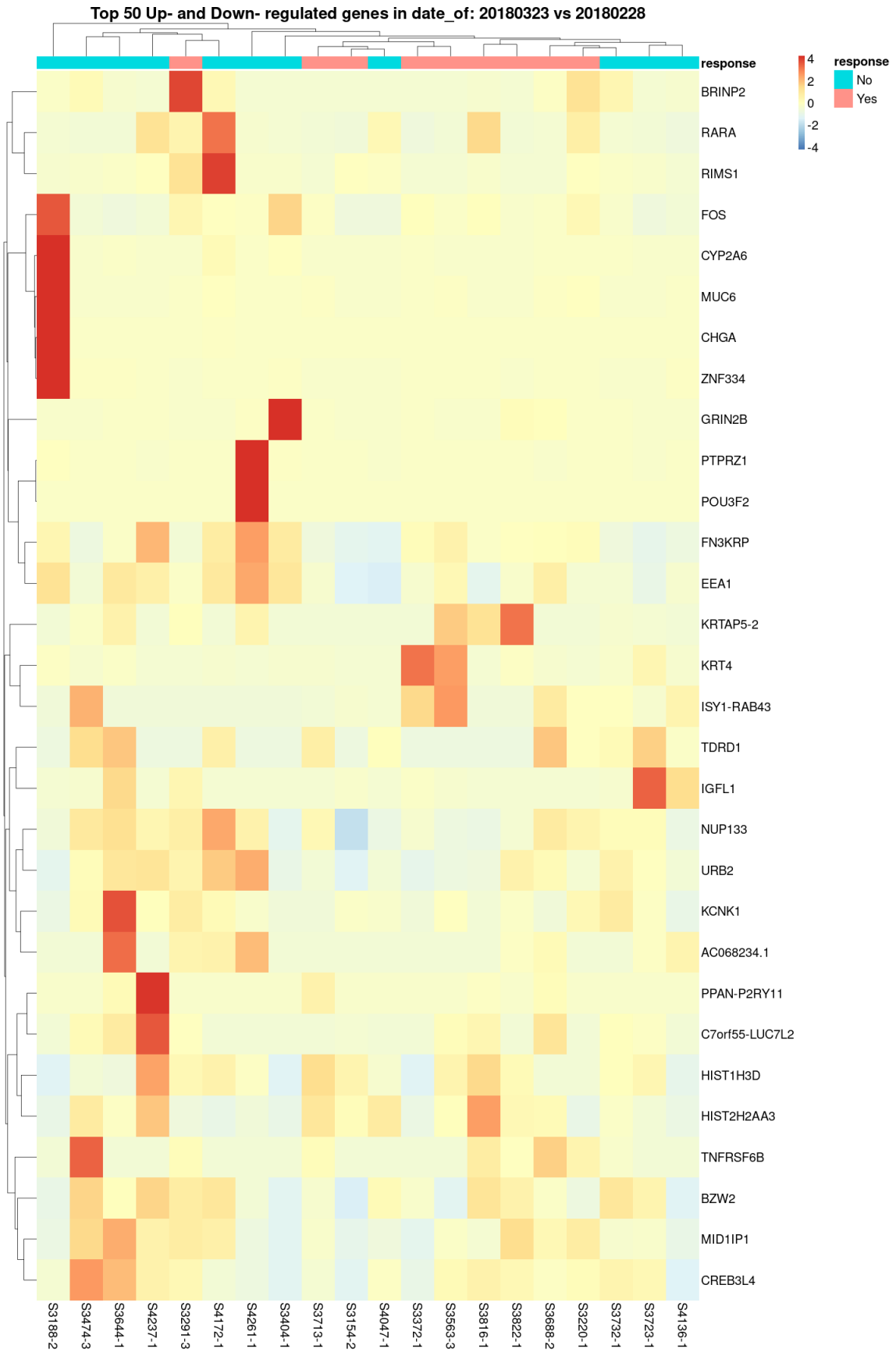

Top 50 Up- and Down- regulated genes in ER: positive vs negative
er
Negativ
Positive
4
2
0
-2
-4
CLEC3A
GNAT3
GRB14
FSIP1
AL032819.3
LRRC10B
RIMS1
MAG
ZAN
SERPINA11
AGR3
NDST4
RFX6
ANKRD30B
RIMS4
ADCY5
ERBB4
PPP4R4
UMOD
CA12
KISS1R
GDNF
SERPINA6
ASIC2
SERPINA3
ESR1
ARMT1
RET
CIB3
POTEI
SERPINA5
TSPAN1
POTEE
GRIK4
FAM189A1
GRIK3
AKR7A3
CT45A1
SLITRK6
SCGB1D2
KLHDC7A
BRINP2
PLA2G3
SYTL5
SOWAHA
ALOX12B
CST4
PHGR1
PTPRQ
SPX
DGAT2
PPP1R1A
NWD2
CIDEA
ADH1B
TBC1D3
PI3
BBOX1
VGLL1
PROM1
PCOLCE2
EPB42
TMEM139
TLX1
KCNQ4
CRYAB
SIX3
MLC1
TBC1D3G
PAX6
NKX1-2
KRT16
ITPRID1
ADH6
SCEL
KRT4
PCDHA2
WNT6
IL22RA2
SLC6A17
SOX11
DEFB1
CXCL1
UGT1A6
ASTN1
EPGN
ISL2
ELOVL3
SSX2B
AL591926.2
GAGE12C
SOHLH2
RUFY4
FGFBP1
AC097625.1
IRX6
TRPV6
KRT6A
CLDN8
PPP1R14C
S3822-1
S3732-1
S3474-3
S3404-1
S4136-1
S3372-1
S3563-3
S3713-1
S3723-1
S3688-2
S4261-1
S4237-1
S3291-3
S3816-1
S3188-2
S3154-2
S3220-1
S3644-1
S4047-1
S4172-1

```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)
```

```
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

## Generate input files for GSEA

```
#  prepares an expression profile for GSEA
#  http://software.broadinstitute.org/cancer/software/gsea/wiki/index.php/Data_formats#Expression_Data_
#  for GSEA it is important to report all genes - genome wide
#  hopefully cpms are better than logcpms
counts <- counts[rowSums(counts)>0,]
result_file <-  paste0("tables/1day.4gsea.txt")

counts_gsea <- counts %>% as.data.frame() %>%
    rownames_to_column(var = "ensembl_gene_id") %>%
    left_join(gene_symbol, by = c("ensembl_gene_id" = "gene_id")) %>%
    dplyr::relocate(symbol)
#%>%
#    dplyr::relocate(ensembl_gene_id)

colnames(counts_gsea)[1:2] <- c("NAME", "DESCRIPTION")

d <-duplicated(counts_gsea$NAME)
o <-  order(rowSums(counts_gsea[,rownames(meta)]),decreasing = T)
counts_gsea <- counts_gsea[o, ]
counts_gsea <- counts_gsea[!d, ]


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

counts_gsea <- counts_gsea[,c("NAME", "DESCRIPTION", samples_yes, samples_no)]
# gsea now supports ENSEMBL_IDs
write_tsv(counts_gsea, result_file)
```

## 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 fu
```

```
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 find
```

```
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"
```

```
nrow(results_down)
```

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

# R session

```
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
```