# Statistical Methods for Quantitative MS-based Proteomics: Part II. Differential Abundance Analysis

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## Contents



This is part of the online course Proteomics Data Analysis (PDA)

- Playlist PDA Preprocessing

## Outline

- Francisella tularensis Example
- Hypothesis testing
- Multiple testing

- Moderated statistics
- Experimental design

Note, that the R-code is included for learners who are aiming to develop R/markdown scripts to automate their quantitative proteomics data analyses. According to the target audience of the course we either work with a graphical user interface (GUI) in a R/shiny App msqrob2gui (e.g. Proteomics Bioinformatics course of the EBI and the Proteomics Data Analysis course at the Gulbenkian institute) or with R/markdowns scripts (e.g. Bioinformatics Summer School at UCLouvain or the Statistical Genomics Course at Ghent University).

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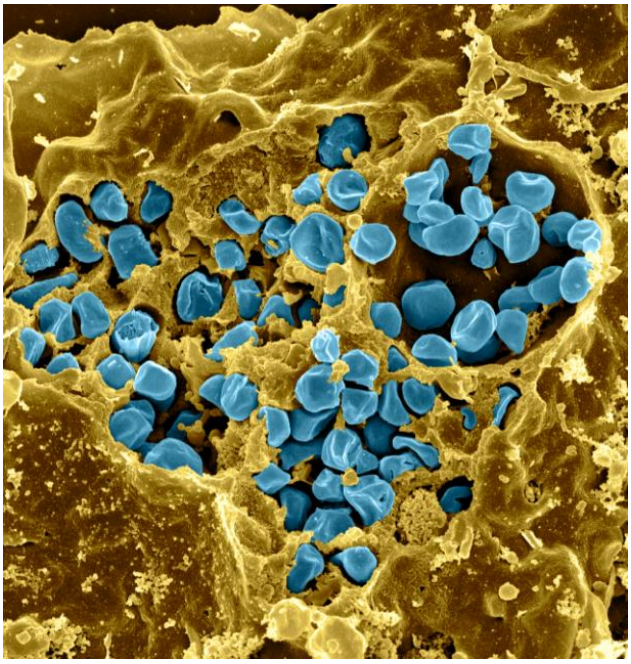
# 1 Francisella tularensis experiment



- Pathogen: causes tularemia
- Metabolic adaptation key for intracellular life cycle of pathogenic microorganisms.
- Upon entry into host cells quick phasomal escape and active multiplication in cytosolic compartment.
- Franciscella is auxotroph for several amino acids, including arginine.
- Inactivation of arginine transporter delayed bacterial phagosomal escape and intracellular multiplication.
- Experiment to assess difference in proteome using 3 WT vs 3 ArgP KO mutants

## 1.1 Import the data in R

Click to see code

1. Load libraries

```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
library(plotly)
library(ggplot2)
```

2. We use a peptides.txt file from MS-data quantified with maxquant that contains MS1 intensities summarized at the peptide level.

```
peptidesFile <- "https://raw.githubusercontent.com/statOmics/PDA/data/quantification/francisella/peptid
```

3. Maxquant stores the intensity data for the different samples in columnns that start with Intensity. We can retreive the column names with the intensity data with the code below:

```
ecols <- grep("Intensity\\.", names(read.delim(peptidesFile)))
```

4. Read the data and store it in QFeatures object

```
pe <- readQFeatures(
  table = peptidesFile,
  fnames = 1,
  ecol = ecols,
  name = "peptideRaw", sep="\t")
```

5. Update data with information on design

```
colData(pe)$genotype <- pe[[1]] %>%
  colnames %>%
  substr(12,13) %>%
  as.factor %>%
  relevel("WT")
pe %>% colData
```

```
## DataFrame with 6 rows and 1 column
##                           genotype
##                           <factor>
## Intensity.1WT_20_2h_n3_3        WT
## Intensity.1WT_20_2h_n4_3        WT
## Intensity.1WT_20_2h_n5_3        WT
## Intensity.3D8_20_2h_n3_3        D8
## Intensity.3D8_20_2h_n4_3        D8
## Intensity.3D8_20_2h_n5_3        D8
```

## 1.2 Preprocessing

Click to see code to log-transfrom the data

1. Log transform

- Calculate number of non zero intensities for each peptide

```
rowData(pe[["peptideRaw"]])$nNonZero <- rowSums(assay(pe[["peptideRaw"]]) > 0)
```

- Peptides with zero intensities are missing peptides and should be represent with a `NA` value rather than `0`.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")
```

2. Filtering

- Handling overlapping protein groups

```
pe <- filterFeatures(pe, ~ Proteins %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$Proteins))
```

- Remove reverse sequences (decoys) and contaminants. Note that this is indicated by the column names Reverse and depending on the version of maxQuant with Potential.contaminants or Contaminants.

```
pe <- filterFeatures(pe,~Reverse != "+")
pe <- filterFeatures(pe,~ Contaminant != "+")
```

- Drop peptides that were only identified in one sample

```
pe <- filterFeatures(pe,~ nNonZero >=2)
nrow(pe[["peptideLog"]])
```

```
## [1] 6525
```

We keep 6525 peptides upon filtering.

3. Normalization by median centering

```
pe <- normalize(pe,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")
```

4. Summarization. We use the standard sumarisation in aggregateFeatures, which is a robust summarisation method.

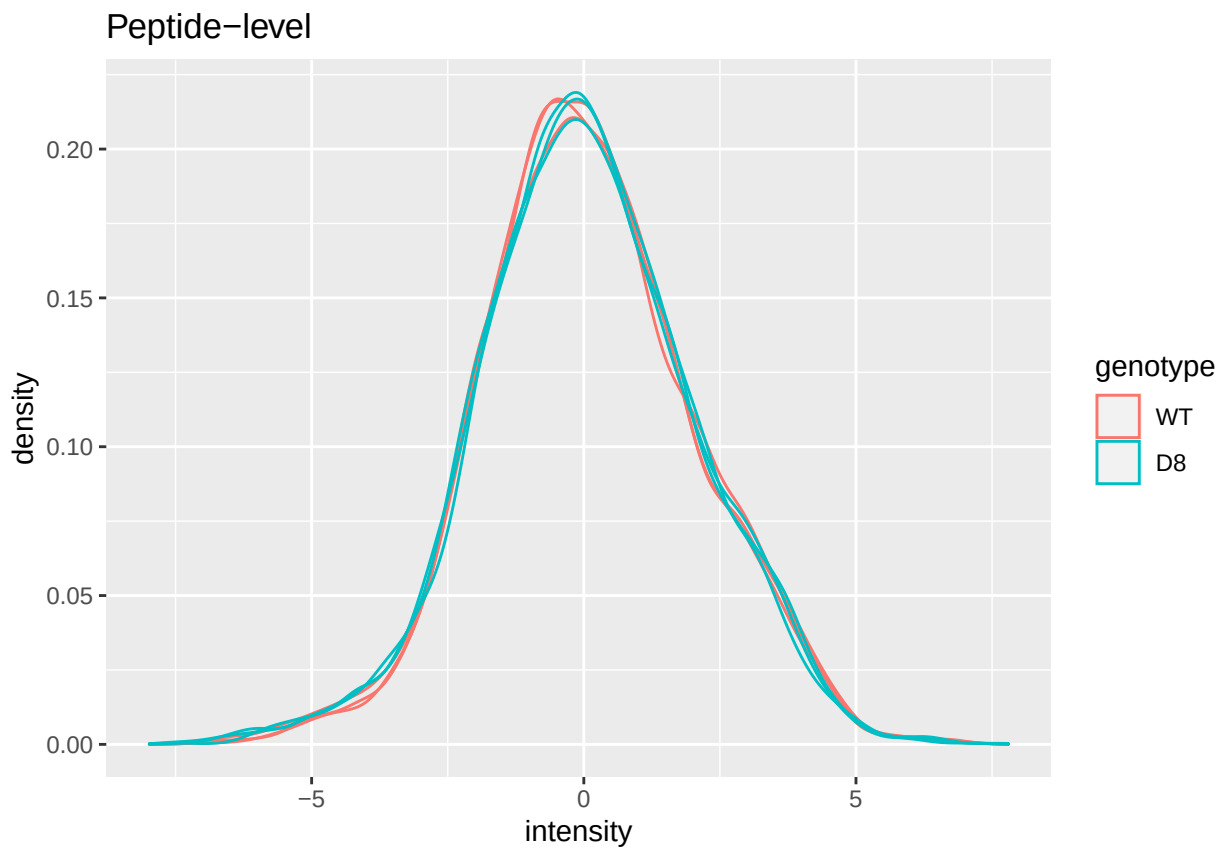
```
pe <- aggregateFeatures(pe,
    i = "peptideNorm",
    fcol = "Proteins",
    na.rm = TRUE,
    name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

Plot of preprocessed data

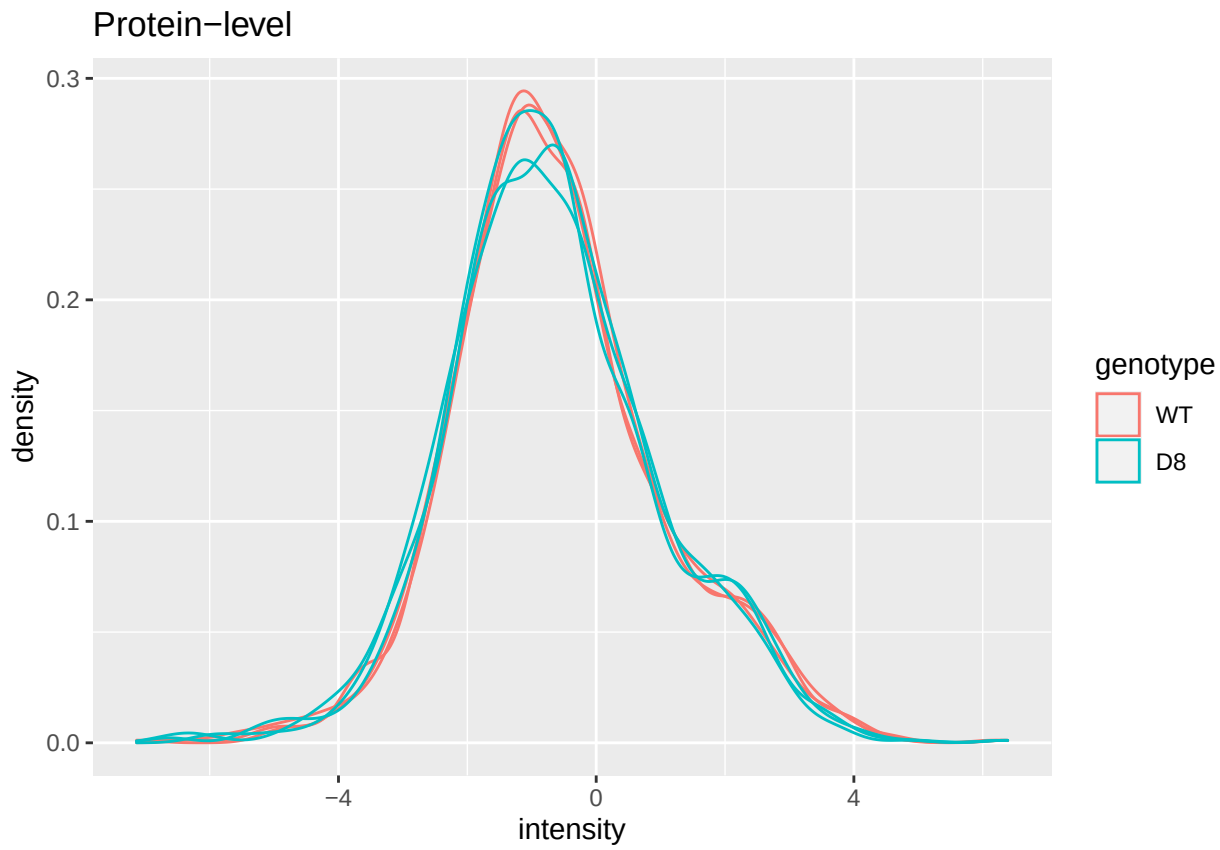
```
pe[["peptideNorm"]] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(genotype = colData(pe)[sample,"genotype"]) %>%
  ggplot(aes(x = intensity,group = sample,color = genotype)) +
    geom_density() +
    ggtitle("Peptide-level")
```

```
## Warning: Removed 7561 rows containing non-finite values (stat_density).
```



```
pe[["protein"]] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(genotype = colData(pe)[sample,"genotype"]) %>%
  ggplot(aes(x = intensity,group = sample,color = genotype)) +
    geom_density() +
    ggtitle("Protein-level")
```

```
## Warning: Removed 428 rows containing non-finite values (stat_density).
```




## 1.3 Summarized data structure

### 1.3.1 Design

```
pe %>%
  colData %>%
  knitr::kable()
```

| | genotype |
|---|---|
| Intensity.1WT_20_2h_n3_3 | WT |
| Intensity.1WT_20_2h_n4_3 | WT |
| Intensity.1WT_20_2h_n5_3 | WT |
| Intensity.3D8_20_2h_n3_3 | D8 |
| Intensity.3D8_20_2h_n4_3 | D8 |
| Intensity.3D8_20_2h_n5_3 | D8 |

- WT vs KO
- 3 vs 3 repeats

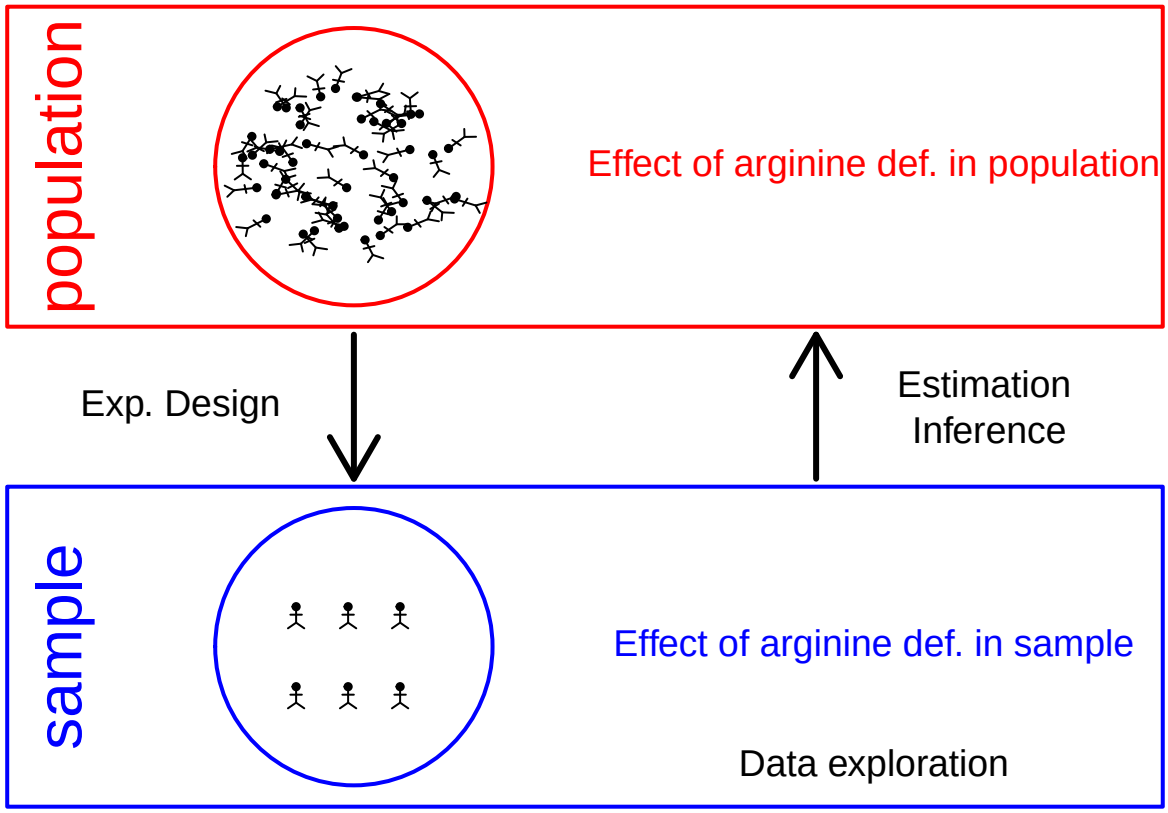
### 1.3.2 Summarized intensity matrix

```
pe[["protein"]] %>% assay() %>% head() %>% knitr::kable()
```

| | Intensity.1WT_20_2h_n3_3 | Intensity.1WT_20_2h_n4_3 | Intensity.1WT_20_2h_n5_3 | Intensity.3D8_20_2h_n3_3 | Intensity.3D8_20_2h_n4_3 | Intensity.3D8_20_2h_n5_3 |
|---|---|---|---|---|---|---|
| WP_003013730 | 31.2748775 | -0.0856247 | 0.1595370 | -0.2809009 | 0.0035526 | 0.0567110 |
| WP_003013860 | NA | NA | -0.2512039 | NA | NA | -0.4865646 |
| WP_003013900 | 26.6851118 | -0.8161658 | -0.7557906 | -0.4591476 | -0.5449424 | -0.4962482 |
| WP_003014068 | 26.6495386 | 0.8522239 | 1.1344852 | 0.5459176 | 0.9187714 | 0.5974741 |
| WP_003014122 | 27.7630863 | -1.0430741 | -0.8091715 | -1.1743951 | -1.1924725 | -1.2565893 |
| WP_003014128 | 32.2051672 | -0.3361704 | -0.2151930 | -0.3855747 | -0.2802011 | -0.5801771 |

- 1115 proteins

### 1.3.3 Hypothesis testing: a single protein




#### 1.3.3.1 T-test

$$\log_2 \text{FC} = \bar{y}_{p1} - \bar{y}_{p2}$$

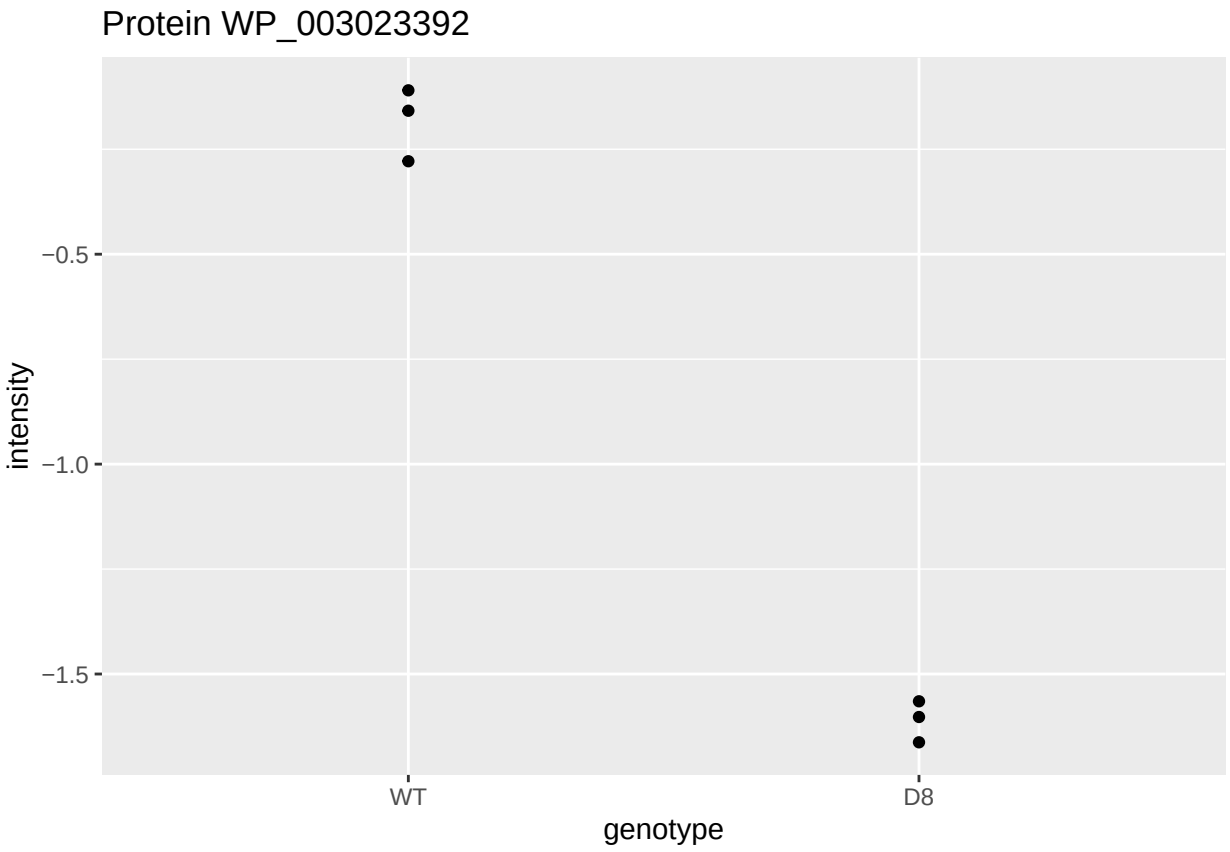
$$T_g = \frac{\log_2 \text{FC}}{\text{se}_{\log_2 \text{FC}}}$$

$$T_g = \frac{\widehat{\text{signal}}}{\widehat{\text{Noise}}}$$

If we can assume equal variance in both treatment groups:

$$\text{se}_{\log_2 \text{FC}} = \text{SD}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

```
WP_003023392 <- data.frame(
    intensity = assay(pe[["protein"]]["WP_003023392",]) %>% c(),
    genotype = colData(pe)[,1])

WP_003023392 %>%
  ggplot(aes(x=genotype,y=intensity)) +
  geom_point() +
  ggtitle("Protein WP_003023392")
```




$$t = \frac{\log_2 \widehat{\mathrm{FC}}}{\mathrm{se}_{\log_2 \widehat{\mathrm{FC}}}} = \frac{-1.43}{0.0577} = -24.7$$

- Is t = -24.7 indicating that there is an effect?
- How likely is it to observe t = -24.7 when there is no effect of the argP KO on the protein expression?

#### 1.3.3.2 Null hypothesis ($H_0$) and alternative hypothesis ($H_1$)

- With data we can never prove a hypothesis (falsification principle of Popper)
- With data we can only reject a hypothesis
- In general we start from *alternative hypothese* $H_1$: we want to show an effect of the KO on a protein

$H_1$: On average the protein abundance in WT is different from that in KO

- But, we will assess this by falsifying the opposite:
  $H_0$: On average the protein abundance in WT is equal to that in KO<-

```
t.test(intensity ~ genotype, data = WP_003023392, var.equal=TRUE)
```

```
##
##  Two Sample t-test
```

```
##
## data:  intensity by genotype
## t = 24.747, df = 4, p-value = 1.582e-05
## alternative hypothesis: true difference in means between group WT and group D8 is not equal to 0
## 95 percent confidence interval:
##  1.267666 1.588058
## sample estimates:
## mean in group WT mean in group D8
##       -0.1821147       -1.6099769
```

- How likely is it to observe an equal or more extreme effect than the one observed in the sample when the null hypothesis is true?
- When we make assumptions about the distribution of our test statistic we can quantify this probability: *p-value*. The p-value will only be calculated correctly if the underlying assumptions hold!
- When we repeat the experiment, the probability to observe a fold change for this gene that is more extreme than a 2.69 fold ($\log_2 FC = -1.43$) down or up regulation by random change (if $H_0$ is true) is 16 out of 1 000 000.

- If the p-value is below a significance threshold $\alpha$ we reject the null hypothesis. *We control the probability on a false positive result at the $\alpha$-level (type I error)*
- Note, that the p-values are uniform under the null hypothesis, i.e. when $H_0$ is true all p-values are equally likely.

## 1.4 Multiple hypothesis testing

- Consider testing DA for all $m = 1066$ proteins simultaneously
- What if we assess each individual test at level $\alpha$? $\rightarrow$ Probability to have a false positive (FP) among all m simultatenous test $>>> \alpha = 0.05$
- Indeed for each non DA protein we have a probability of 5% to return a FP.
- In a typical experiment the majority of the proteins are non DA.
- So an upperbound of the expected FP is $m \times \alpha$ or $1066 \times 0.05 = 53$.

$\rightarrow$ Hence, we are bound to call many false positive proteins each time we run the experiment.

### 1.4.1 Multiple testing

**1.4.1.1 Family-wise error rate** The family-wise error rate (FWER) addresses the multiple testing issue by no longer controlling the individual type I error for each protein, instead it controls:

$$\text{FWER} = \text{P}\left[FP \geq 1\right].$$

The Bonferroni method is widely used to control the type I error:

- assess each test at

$$\alpha_{\text{adj}} = \frac{\alpha}{m}$$

- or use adjusted p-values and compare them to $\alpha$:

$$p_{\text{adj}} = \min(p \times m, 1)$$

Problem, the method is very conservative!

#### 1.4.1.2 False discovery rate

- FDR: Expected proportion of false positives on the total number of positives you return.
- An FDR of 1% means that on average we expect 1% false positive proteins in the list of proteins that are called significant.
- Defined by Benjamini and Hochberg in their seminal paper Benjamini, Y. and Hochberg, Y. (1995). "Controlling the false discovery rate: a practical and powerful approach to multiple testing". Journal of the Royal Statistical Society Series B, 57 (1): 289–300.

The **False Discovery Proportion (FDP)** is the fraction of false positives that are returned, i.e.

$$FDP = \frac{FP}{R}$$

- However, this quantity cannot be observed because in practice we only know the number of proteins for which we rejected $H_0$, $R$.
- But, we do not know the number of false positives, $FP$.

Therefore, Benjamini and Hochberg, 1995, defined The **False Discovery Rate (FDR)** as

$$\text{FDR} = \text{E}\left[\frac{FP}{R}\right] = \text{E}\left[\text{FDP}\right]$$

the expected FDP.

- Controlling the FDR allows for more discoveries (i.e. longer lists with significant results), while the fraction of false discoveries among the significant results in well controlled on average. As a consequence, more of the true positive hypotheses will be detected.

#### 1.4.1.3 Intuition of BH-FDR procedure

Consider $m = 1000$ tests

- Suppose that a researcher rejects all null hypotheses for which $p < 0.01$.
- If we use $p < 0.01$, we expect $0.01 \times m_0$ tests to return false positives.
- A conservative estimate of the number of false positives that we can expect can be obtained by considering that the null hypotheses are true for all features, $m_0 = m = 1000$.
- We then would expect $0.01 \times 1000 = 10$ false positives ($FP = 10$).
- Suppose that the researcher found 200 genes with $p < 0.01$ ($R = 200$).
- The proportion of false positive results (FDP = false positive proportion) among the list of $R = 200$ genes can then be estimated as

$$\widehat{\text{FDP}} = \frac{FP}{R} = \frac{10}{200} = \frac{0.01 \times 1000}{200} = 0.05.$$

#### 1.4.1.4 Benjamini and Hochberg (1995) procedure for controlling the FDR at $\alpha$

1. Let $p_{(1)} \leq \ldots \leq p_{(m)}$ denote the ordered $p$-values.
2. Find the largest integer $k$ so that
$$\frac{p_{(k)} \times m}{k} \leq \alpha$$
or
$$p_{(k)} \leq k \times \alpha/m$$
3. If such a $k$ exists, reject the $k$ null hypotheses associated with $p_{(1)}, \ldots, p_{(k)}$. Otherwise none of the null hypotheses is rejected.

The adjusted $p$-value (also known as the $q$-value in FDR literature):

$$q_{(i)} = \tilde{p}_{(i)} = \min \left[ \min_{j=i,\ldots,m} \left( m p_{(j)}/j \right), 1 \right].$$

In the hypothetical example above: $k = 200$, $p_{(k)} = 0.01$, $m = 1000$ and $\alpha = 0.05$.

#### 1.4.1.5 Francisella Example Click to see code

```
ttestMx <- function(y,group) {
    test <- try(t.test(y[group],y[!group],var.equal=TRUE),silent=TRUE)
    if(is(test,"try-error")) {
      return(c(log2FC=NA,se=NA,tstat=NA,p=NA))
      } else {
      return(c(log2FC= (test$estimate%*%c(1,-1)),se=test$stderr,tstat=test$statistic,pval=test$p.value))
      }
 }

 res <- apply(
    assay(pe[["protein"]]),
    1,
    ttestMx,
    group = colData(pe)$genotype=="D8") %>%
  t
 colnames(res) <- c("logFC","se","tstat","pval")
 res <- res %>% as.data.frame %>% na.exclude %>% arrange(pval)
 res$adjPval <- p.adjust(res$pval, "fdr")
 alpha <- 0.05
res$adjAlphaForm <- paste0(1:nrow(res)," x ",alpha,"/",nrow(res))
res$adjAlpha <- alpha * (1:nrow(res))/nrow(res)
res$"pval < adjAlpha" <- res$pval < res$adjAlpha
res$"adjPval < alpha" <- res$adjPval < alpha
```

FWER: Bonferroni method:$\alpha_{\text{adj}} = \alpha/m = 0.05/1066 = 5 \times 10^{-5}$

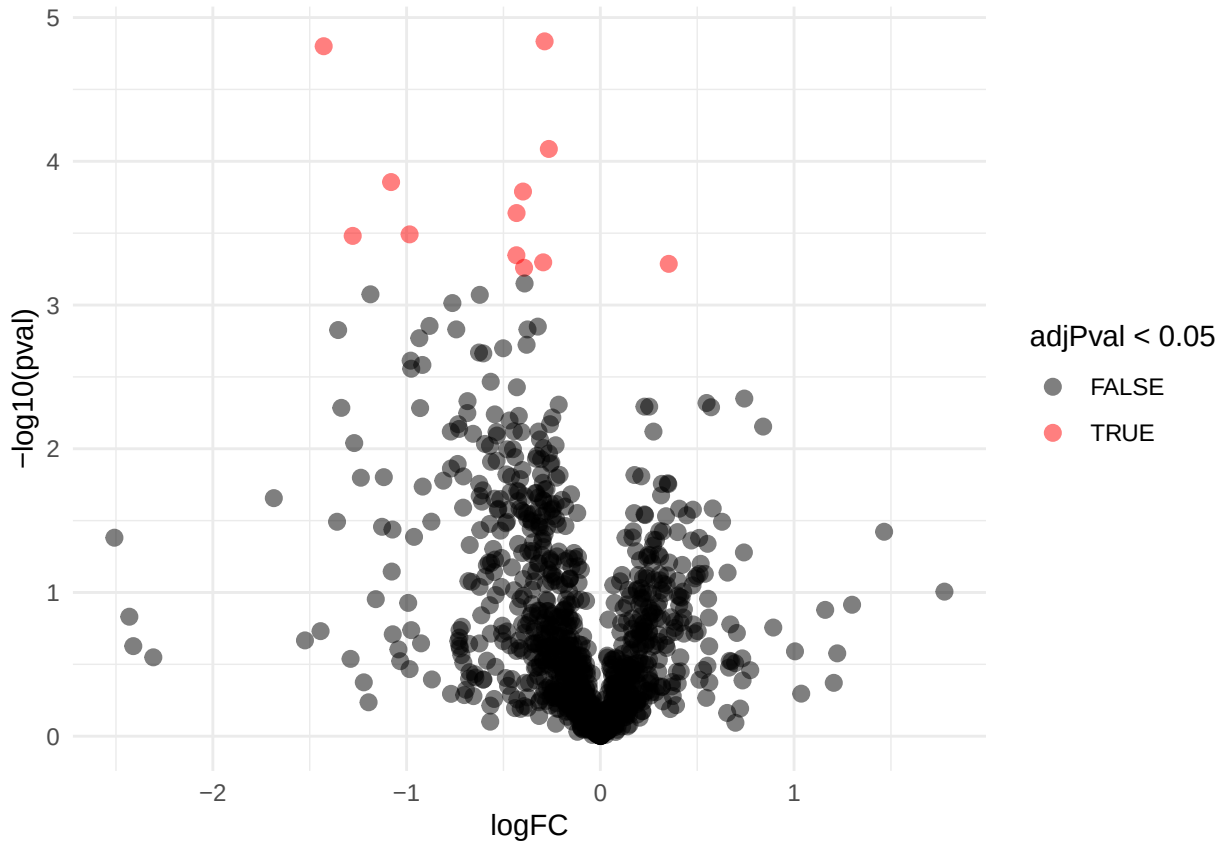
| | logFC | pval | adjPval | adjAlphaForm | adjAlpha | pval < adjAlpha | adjPval < alpha |
|---|---|---|---|---|---|---|---|
| WP_003038940 | -0.2876290 | 0.0000146 | 0.0084347 | 1 x 0.05/1066 | 0.0000469 | TRUE | TRUE |

| | logFC | pval | adjPval | adjAlphaForm | adjAlpha | pval < adjAlpha | adjPval < alpha |
|---|---|---|---|---|---|---|---|
| WP_003023392 | -1.4278622 | 0.0000158 | 0.0084347 | 2 x 0.05/1066 | 0.0000938 | TRUE | TRUE |
| WP_003039212 | -0.2658247 | 0.0000820 | 0.0291520 | 3 x 0.05/1066 | 0.0001407 | TRUE | TRUE |
| WP_003026016 | -1.0800305 | 0.0001395 | 0.0346124 | 4 x 0.05/1066 | 0.0001876 | TRUE | TRUE |
| WP_003039615 | -0.3992190 | 0.0001623 | 0.0346124 | 5 x 0.05/1066 | 0.0002345 | TRUE | TRUE |
| WP_011733588 | -0.4323262 | 0.0002291 | 0.0407034 | 6 x 0.05/1066 | 0.0002814 | TRUE | TRUE |
| WP_003014552 | -0.9843865 | 0.0003224 | 0.0440266 | 7 x 0.05/1066 | 0.0003283 | TRUE | TRUE |
| WP_003040849 | -1.2780743 | 0.0003304 | 0.0440266 | 8 x 0.05/1066 | 0.0003752 | TRUE | TRUE |
| WP_003038430 | -0.4331987 | 0.0004505 | 0.0489078 | 9 x 0.05/1066 | 0.0004221 | FALSE | TRUE |
| WP_003033975 | -0.2949061 | 0.0005047 | 0.0489078 | 10 x 0.05/1066 | 0.0004690 | FALSE | TRUE |
| WP_011733645 | 0.3531405 | 0.0005171 | 0.0489078 | 11 x 0.05/1066 | 0.0005159 | FALSE | TRUE |
| WP_011733723 | -0.3935768 | 0.0005506 | 0.0489078 | 12 x 0.05/1066 | 0.0005629 | TRUE | TRUE |
| WP_003038679 | -0.3909725 | 0.0007083 | 0.0580821 | 13 x 0.05/1066 | 0.0006098 | FALSE | FALSE |
| WP_003033719 | -1.1865453 | 0.0008426 | 0.0603810 | 14 x 0.05/1066 | 0.0006567 | FALSE | FALSE |
| ... | ... | ... | ... | ... | ... | ... | ... |
| WP_003040562 | 0.0039480 | 0.9976429 | 0.9985797 | 1065 x 0.05/1066 | 0.0499531 | FALSE | FALSE |
| WP_003041130 | 0.0002941 | 0.9992812 | 0.9992812 | 1066 x 0.05/1066 | 0.05 | FALSE | FALSE |

#### 1.4.1.6 Results Click to see code

```
volcanoT <- res %>%
  ggplot(aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal()
```

```
volcanoT
```




## 1.5 Moderated Statistics

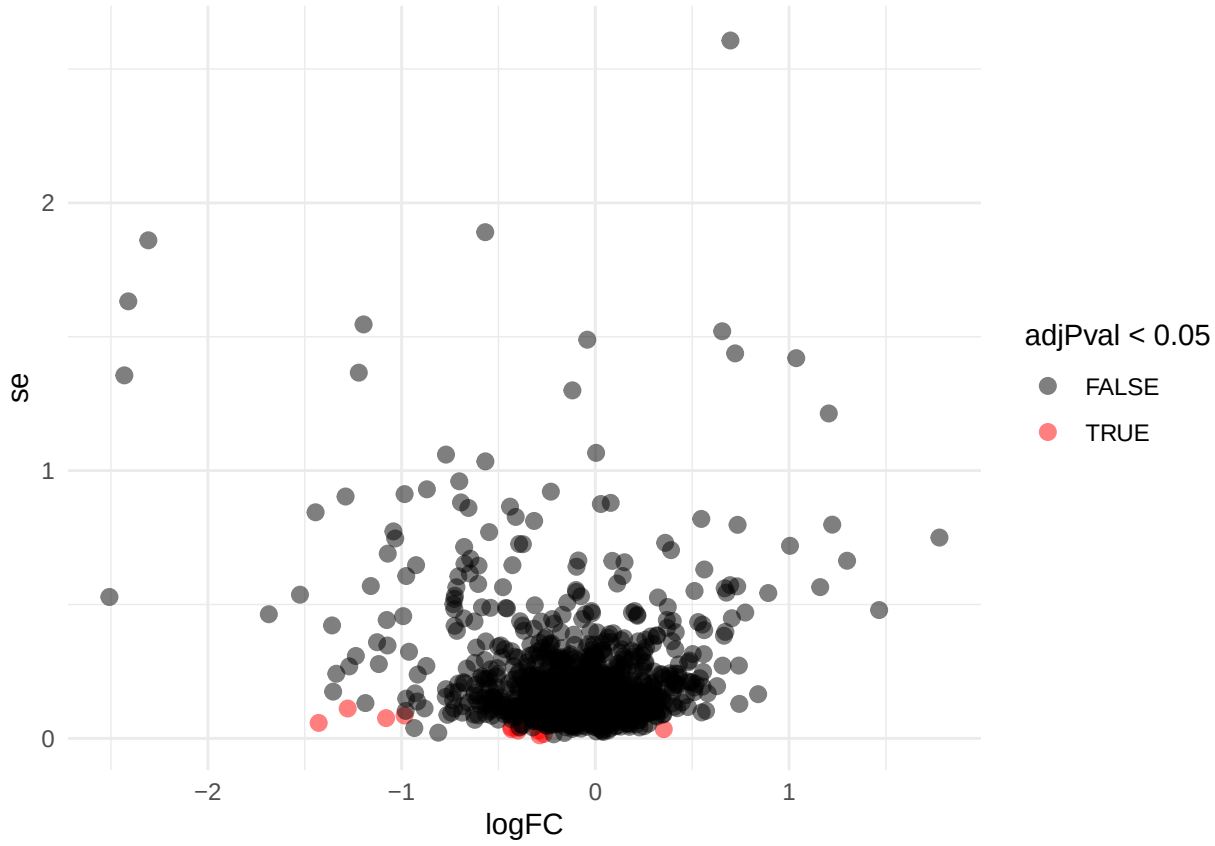
Problems with ordinary t-test

Click to see code

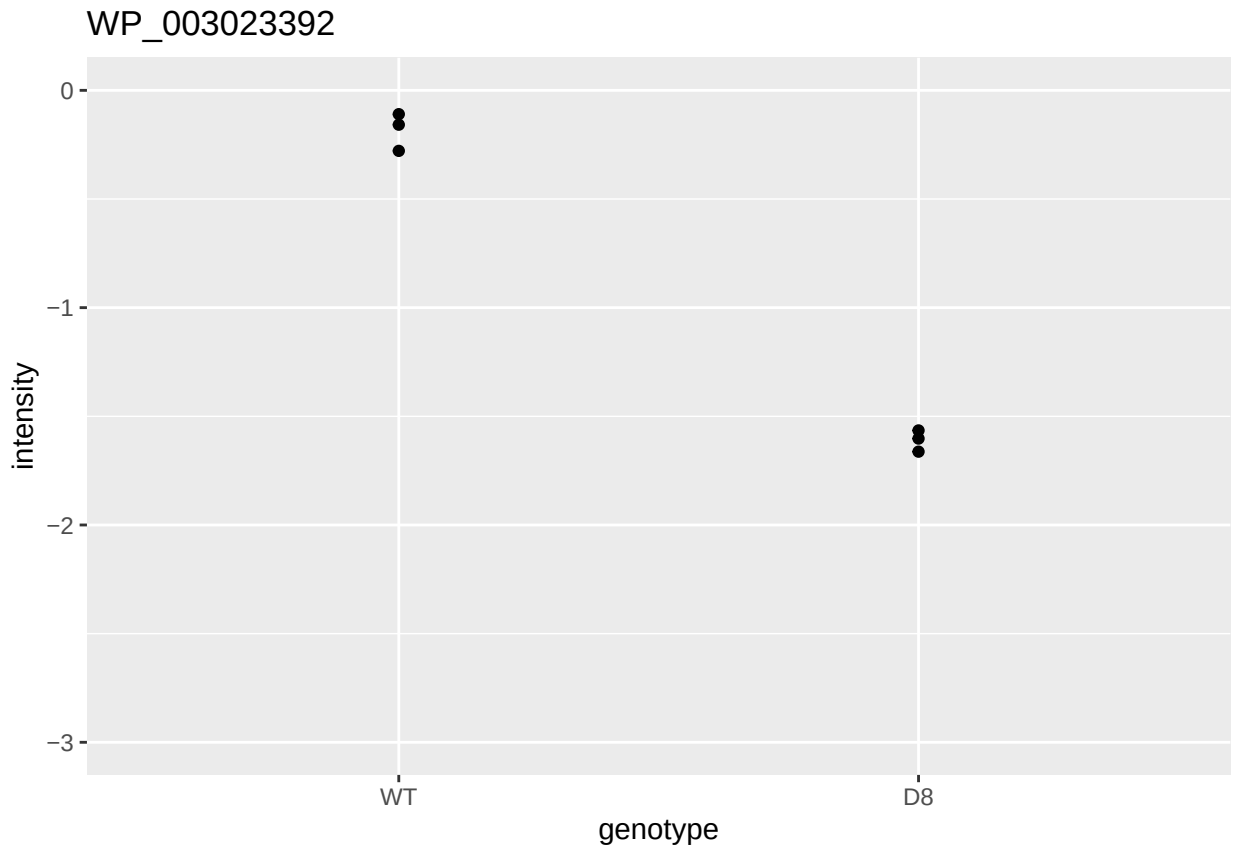
```
problemPlots <- list()
problemPlots[[1]] <- res %>%
  ggplot(aes(x = logFC, y = se, color = adjPval < 0.05)) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal()

for (i in 2:3)
{
problemPlots[[i]] <- colData(pe) %>%
    as.data.frame %>%
    mutate(intensity = pe[["protein"]][rownames(res)[i],] %>%
             assay %>%
             c) %>%
    ggplot(aes(x=genotype,y=intensity)) +
    geom_point() +
    ylim(-3,0) +
    ggtitle(rownames(res)[i])
}
```

```
problemPlots
```

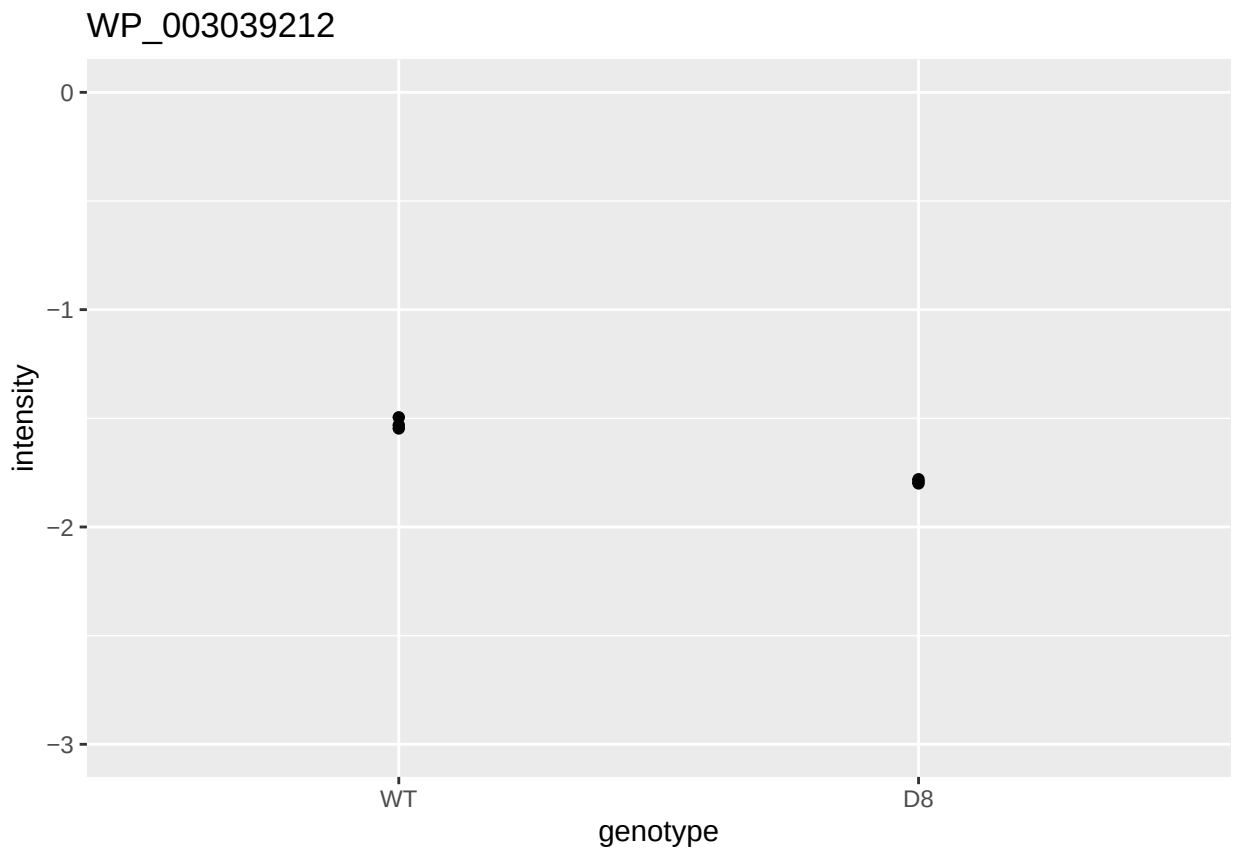
```
## [[1]]
```




```
##
## [[2]]
```




```
##
## [[3]]
```




A general class of moderated test statistics is given by

$$T_g^{mod} = \frac{\bar{Y}_{g1} - \bar{Y}_{g2}}{C \quad \tilde{S}_g},$$

where $\tilde{S}_g$ is a moderated standard deviation estimate.

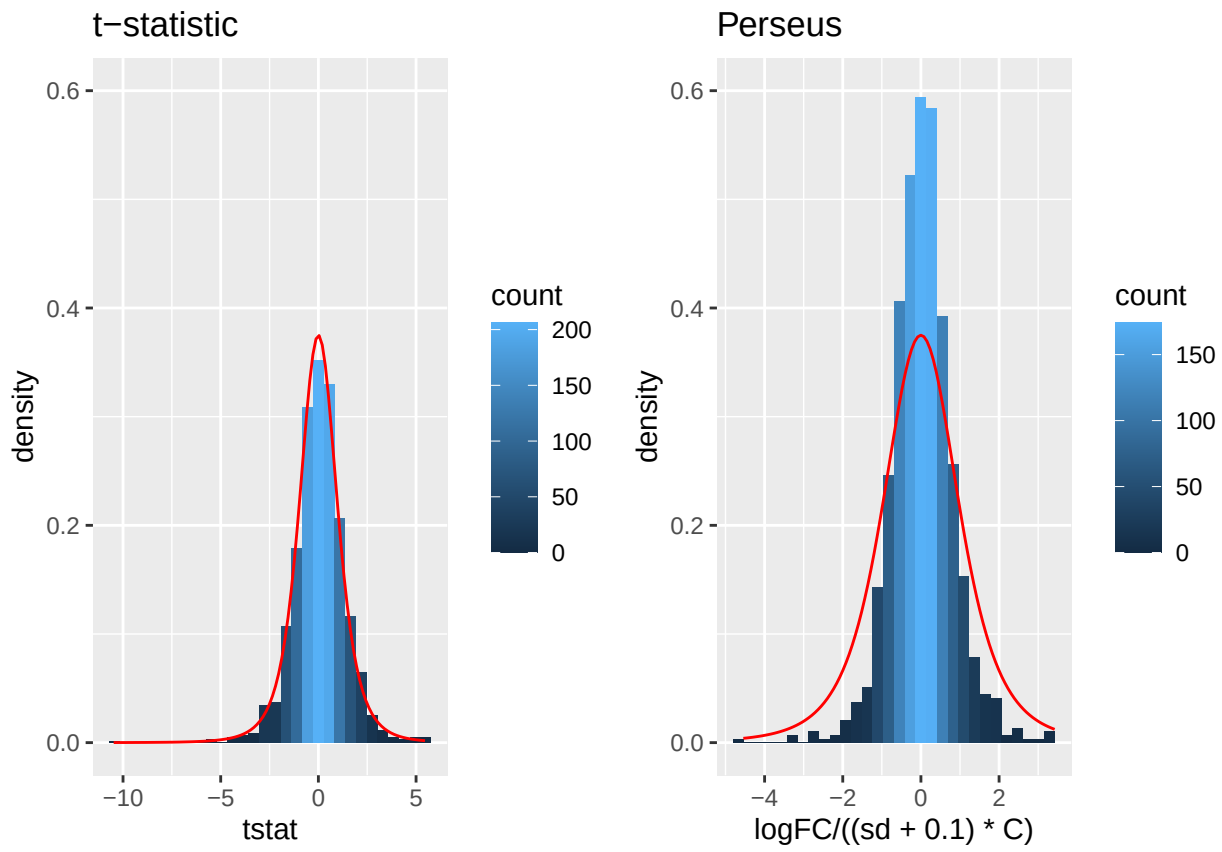
- $C$ is a constant depending on the design e.g. $\sqrt{1/n_1 + 1/n_2}$ for a t-test and of another form for linear models.
- $\tilde{S}_g = S_g + S_0$: add small positive constant to denominator of t-statistic.
- This can be adopted in Perseus.

Click to see code

```
simI<-sapply(res$se/sqrt(1/3+1/3),function(n,mean,sd) rnorm(n,mean,sd),n=6,mean=0) %>% t
resSim <- apply(
    simI,
    1,
    ttestMx,
    group = colData(pe)$genotype=="D8") %>%
  t
 colnames(resSim) <- c("logFC","se","tstat","pval")
 resSim <- as.data.frame(resSim)
 tstatSimPlot <- resSim %>%
   ggplot(aes(x=tstat)) +
     geom_histogram(aes(y=..density.., fill=..count..),bins=30) +
```

```
    stat_function(fun=dt,
   color="red",
   args=list(df=4)) +
  ylim(0,.6) +
  ggtitle("t-statistic")


resSim$C <- sqrt(1/3+1/3)
resSim$sd <- resSim$se/resSim$C
tstatSimPerseus <- resSim %>%
  ggplot(aes(x=logFC/((sd+.1)*C))) +
    geom_histogram(aes(y=..density.., fill=..count..),bins=30) +
    stat_function(fun=dt,
                  color="red",
                 args=list(df=4)) +
    ylim(0,.6) +
   ggtitle("Perseus")
```

```
gridExtra::grid.arrange(tstatSimPlot,tstatSimPerseus,nrow=1)
```




- The choice of $S_0$ in Perseus is ad hoc and the t-statistic is no-longer t-distributed.
- Permutation test, but is difficult for more complex designs.
- Allows for Data Dredging because user can choose $S_0$

### 1.5.1 Empirical Bayes

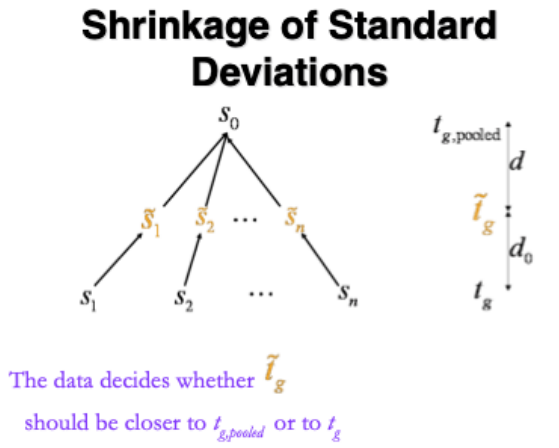



Figure courtesy to Rafael Irizarry

$$T_g^{mod} = \frac{\bar{Y}_{g1} - \bar{Y}_{g2}}{C \quad \tilde{S}_g},$$

- **empirical Bayes** theory provides formal framework for borrowing strength across proteins,
- Implemented in popular bioconductor package **limma** and **msqrob2**

$$\tilde{S}_g = \sqrt{\frac{d_g S_g^2 + d_0 S_0^2}{d_g + d_0}},$$

- $S_0^2$: common variance (over all proteins)
- Moderated t-statistic is t-distributed with $d_0 + d_g$ degrees of freedom.
- Note that the degrees of freedom increase by borrowing strength across proteins!

Click to see the code

1. We model the protein level expression values using the `msqrob` function. By default `msqrob2` estimates the model parameters using robust regression.

We will model the data with a different group mean for every genotype. The group is incoded in the variable `genotype` of the colData. We can specify this model by using a formula with the factor `genotype` as its predictor: `formula = ~genotype`.

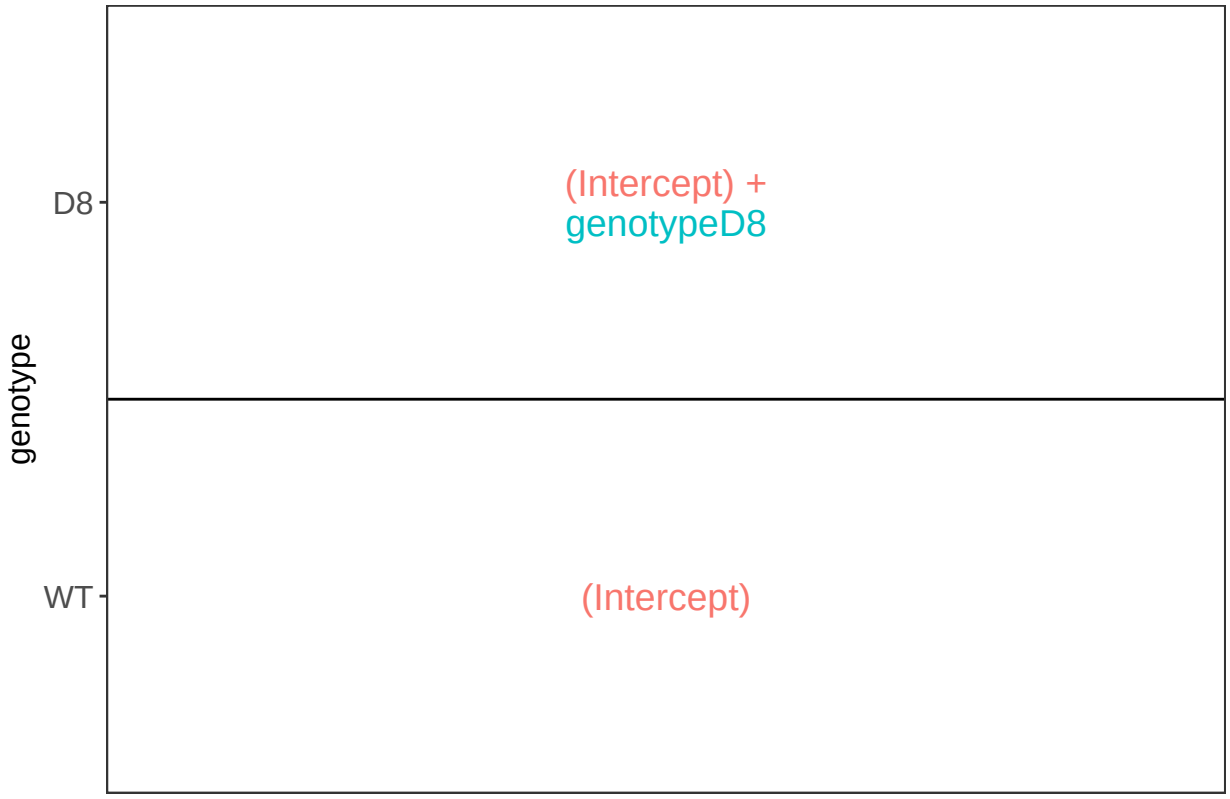
Note, that a formula always starts with a symbol '~'.

```
pe <- msqrob(object = pe, i = "protein", formula = ~genotype)
```

2. Inference

We first explore the design of the model that we specified using the the package `ExploreModelMatrix`

```
library(ExploreModelMatrix)
VisualizeDesign(colData(pe),~genotype)$plotlist[[1]]
```




We have two model parameters, the (Intercept) and genotypeD8. This results in a model with two group means:

1. For the wild type (WT) the expected value (mean) of the log2 transformed intensity y for a protein will be modelled using

$$\mathrm{E}[Y|\text{genotype} = \text{WT}] = (\text{Intercept})$$

2. For the knockout genotype D8 the expected value (mean) of the log2 transformed intensity y for a protein will be modelled using

$$\mathrm{E}[Y|\text{genotype} = \text{D8}] = (\text{Intercept}) + \text{genotypeD8}$$

The average log2FC between D8 and WT is thus

$$\log_2 \mathrm{FC}_{D8-WT} = \mathrm{E}[Y|\text{genotype} = \text{D8}] - \mathrm{E}[Y|\text{genotype} = \text{WT}] = \text{genotypeD8}$$

Hence, assessing the null hypothesis that there is no differential abundance between D8 and WT can be reformulated as

$$H_0 : \text{genotypeD8} = 0$$

We can implement a hypothesis test for each protein in msqrob2 using the code below:

```
L <- makeContrast("genotypeD8 = 0", parameterNames = c("genotypeD8"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L)
```

We can show the list with all significant DE proteins at the 5% FDR using

```
rowData(pe[["protein"]])$genotypeD8 %>%
  arrange(pval) %>%
  filter(adjPval<0.05)
```

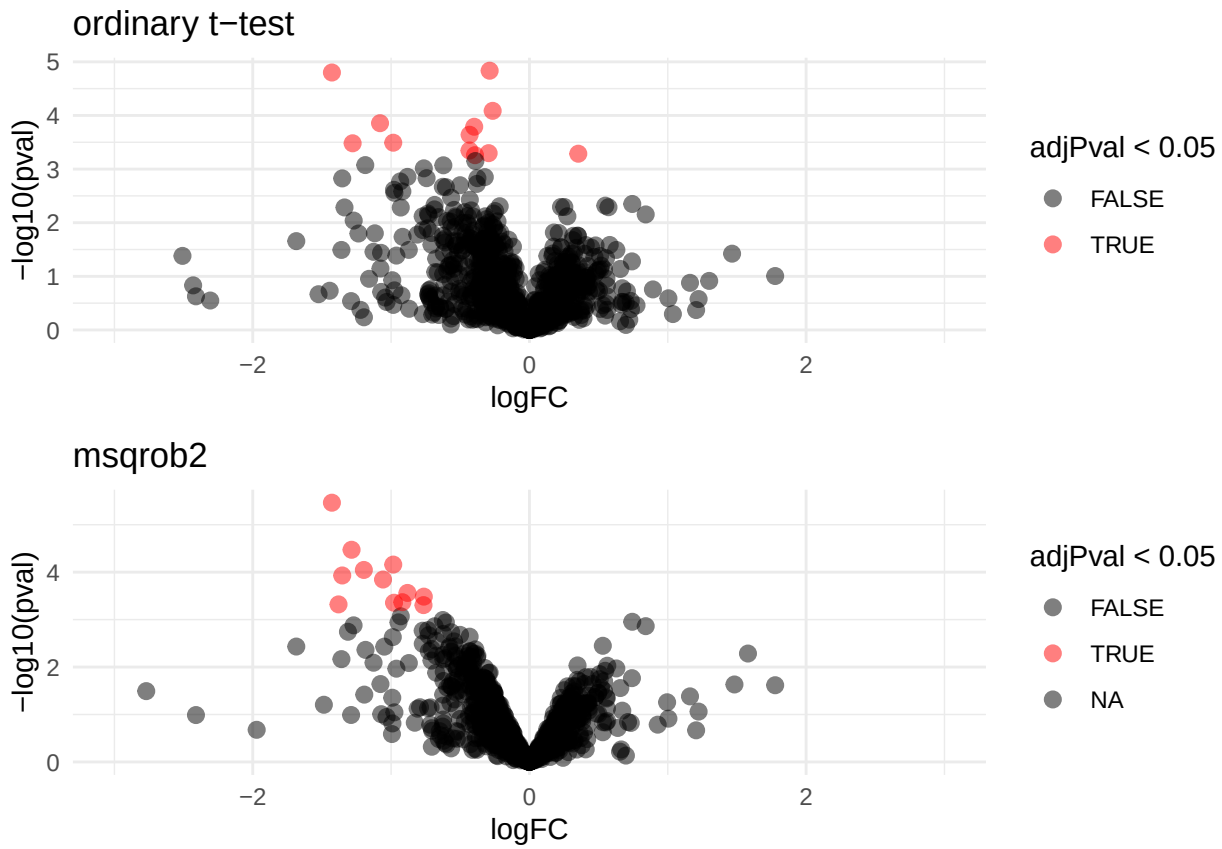
```
##                   logFC         se       df          t         pval     adjPval
## WP_003023392 -1.4278622 0.09546104 6.353267 -14.957538 3.435824e-06 0.003456439
## WP_003040849 -1.2857733 0.12226820 6.236222 -10.516007 3.372825e-05 0.016965307
## WP_003014552 -0.9843865 0.10770682 6.353267  -9.139501 6.920701e-05 0.022593404
## WP_003033719 -1.1970073 0.13408775 6.220667  -8.927044 8.983461e-05 0.022593404
## WP_003040790 -1.3531336 0.16187947 6.353267  -8.358896 1.174333e-04 0.023627585
## WP_003026016 -1.0584448 0.11240892 5.442252  -9.416022 1.429981e-04 0.023976009
## WP_003033905 -0.8815374 0.12199689 6.353267  -7.225901 2.740575e-04 0.039385984
## WP_003039713 -0.7635044 0.10902744 6.353267  -7.002865 3.279672e-04 0.041241874
## WP_003039530 -0.9184399 0.13755363 6.353267  -6.676959 4.299645e-04 0.041477282
## WP_003014581 -0.9789058 0.12767782 5.353267  -7.667000 4.409838e-04 0.041477282
## WP_003038816 -1.3800203 0.20369907 6.080188  -6.774799 4.777868e-04 0.041477282
## WP_003033046 -0.7657848 0.11307420 6.033034  -6.772410 4.947588e-04 0.041477282
```

We can also visualise the results using a volcanoplot

```
volcano <- ggplot(
    rowData(pe[["protein"]])$genotypeD8,
    aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)
) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal() +
    ggtitle("msqrob2")
```

```
gridExtra::grid.arrange(
  volcanoT +
    xlim(-3,3) +
  ggtitle("ordinary t-test"),
  volcano +
    xlim(-3,3)
,nrow=2)
```

```
## Warning: Removed 109 rows containing missing values (geom_point).
```

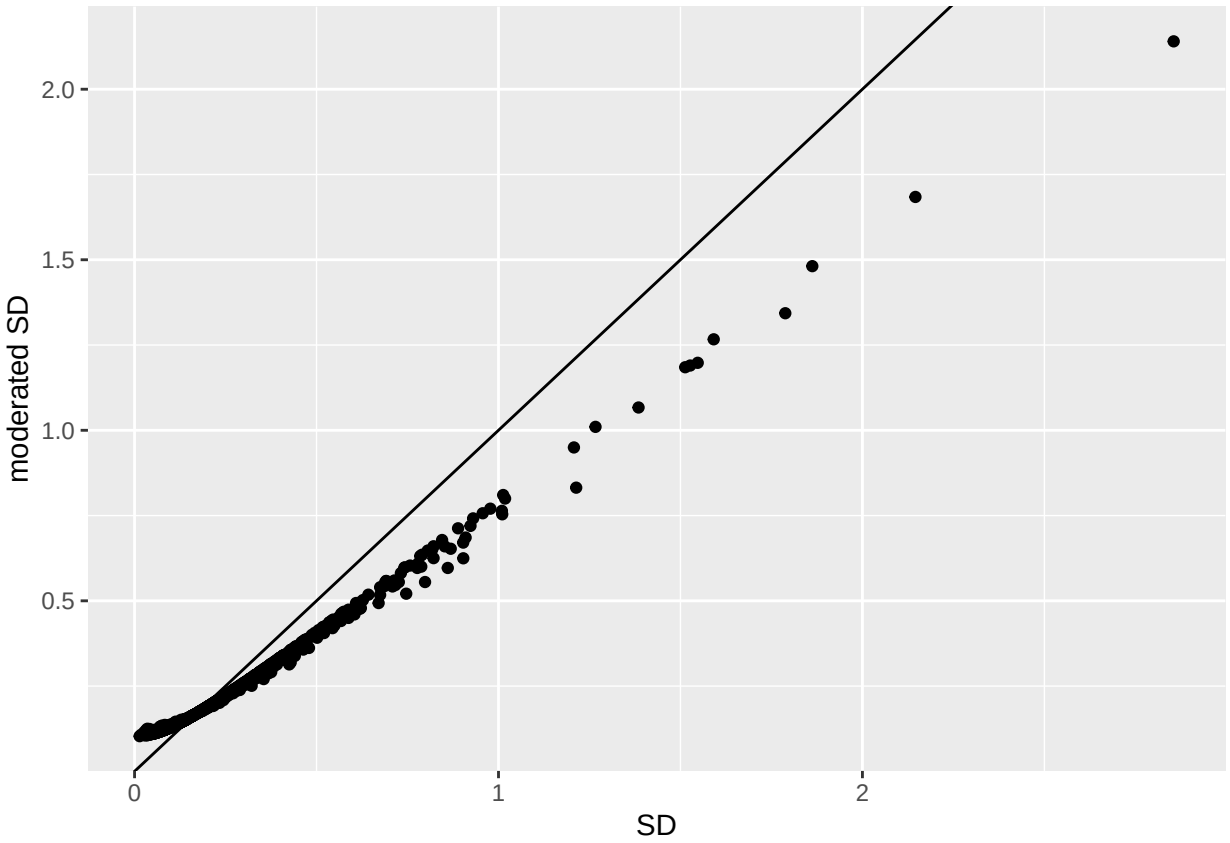



- The volcano plot opens up when using the EB variance estimator
- Borrowing strength to estimate the variance using empirical Bayes solves the issue of returning proteins with a low fold change as significant due to a low variance.

### 1.5.2 Shrinkage of the variance and moderated t-statistics

```
qplot(
  sapply(rowData(pe[["protein"]])$msqrobModels,getSigma),
  sapply(rowData(pe[["protein"]])$msqrobModels,getSigmaPosterior)) +
  xlab("SD") +
  ylab("moderated SD") +
  geom_abline(intercept = 0,slope = 1) +
  geom_hline(yintercept = )
```

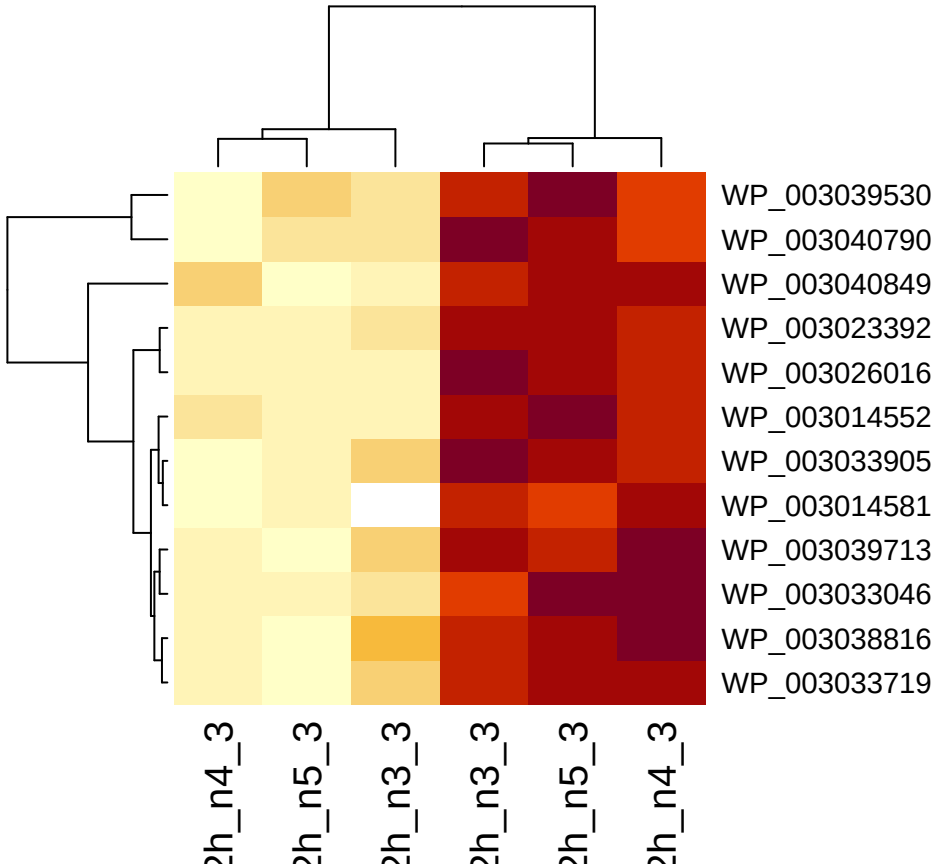
```
## Warning: Removed 109 rows containing missing values (geom_point).
```




- Small variances are shrunken towards the common variance resulting in large EB variance estimates
- Large variances are shrunken towards the common variance resulting in smaller EB variance estimates
- Pooled degrees of freedom of the EB variance estimator are larger because information is borrowed across proteins to estimate the variance

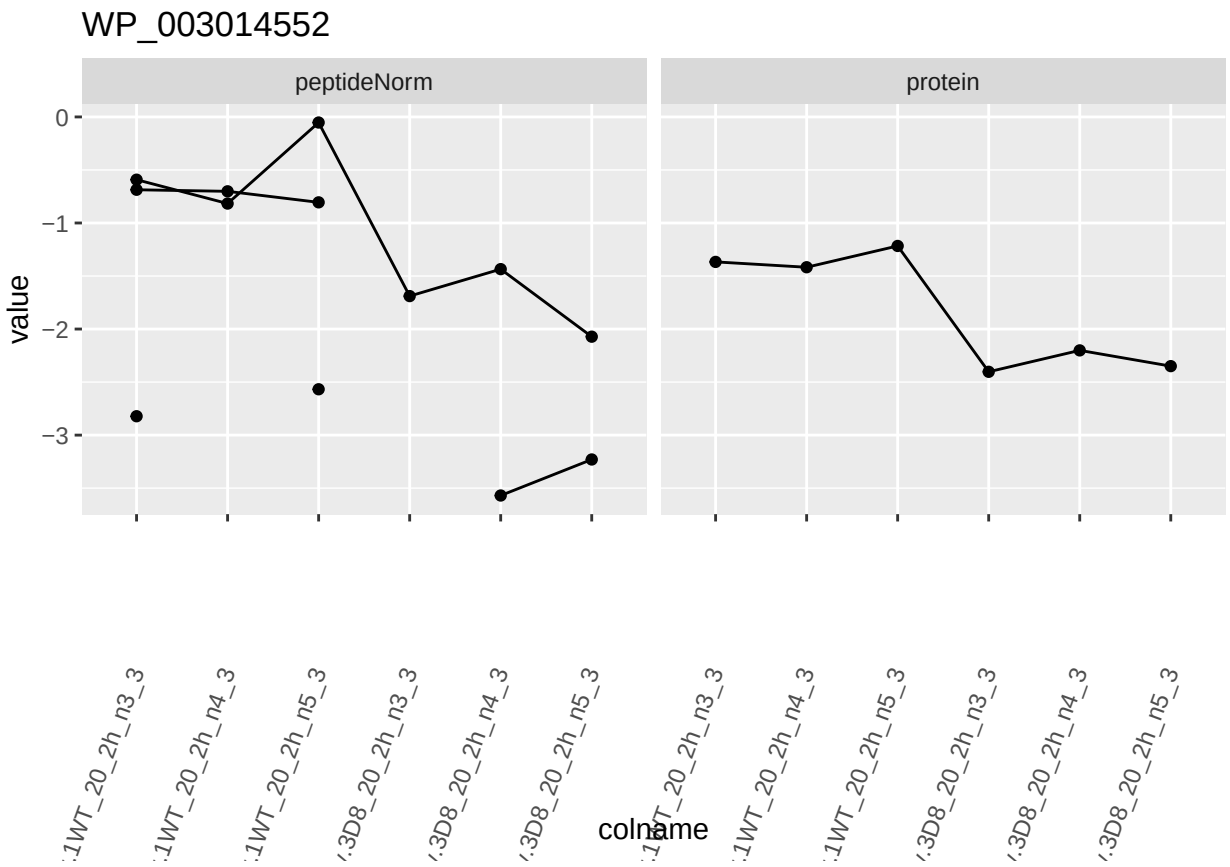
## 1.6 Plots

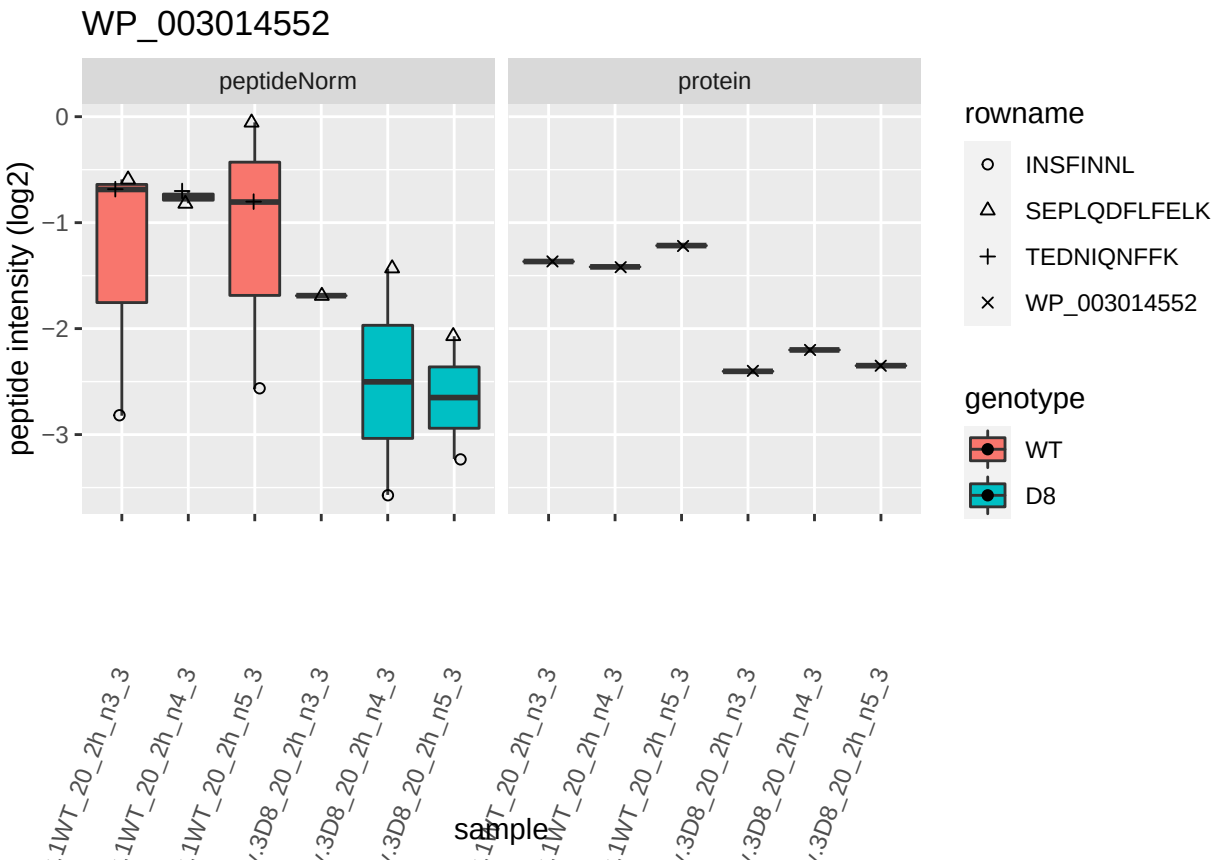
```
sigNames <- rowData(pe[["protein"]])$genotypeD8 %>%
    rownames_to_column("protein") %>%
    filter(adjPval < 0.05) %>%
    pull(protein)
heatmap(assay(pe[["protein"]])[sigNames, ])
```




```
for (protName in sigNames)
    {
        pePlot <- pe[protName, , c("peptideNorm", "protein")]
        pePlotDf <- data.frame(longFormat(pePlot))
        pePlotDf$assay <- factor(pePlotDf$assay,
            levels = c("peptideNorm", "protein")
        )
        pePlotDf$genotype <- as.factor(colData(pePlot)[pePlotDf$colname, "genotype"])

        # plotting
        p1 <- ggplot(
            data = pePlotDf,
            aes(x = colname, y = value, group = rowname)
        ) +
            geom_line() +
            geom_point() +
            facet_grid(~assay) +
            theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
            ggtitle(protName)
        print(p1)

        # plotting 2
        p2 <- ggplot(pePlotDf, aes(x = colname, y = value, fill = genotype)) +
            geom_boxplot(outlier.shape = NA) +
            geom_point(
                position = position_jitter(width = .1),
```

```
                aes(shape = rowname)
            ) +
            scale_shape_manual(values = 1:nrow(pePlotDf)) +
            labs(title = protName, x = "sample", y = "peptide intensity (log2)") +
            theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
            facet_grid(~assay)
        print(p2)
}
```

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protein
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−3
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rowname
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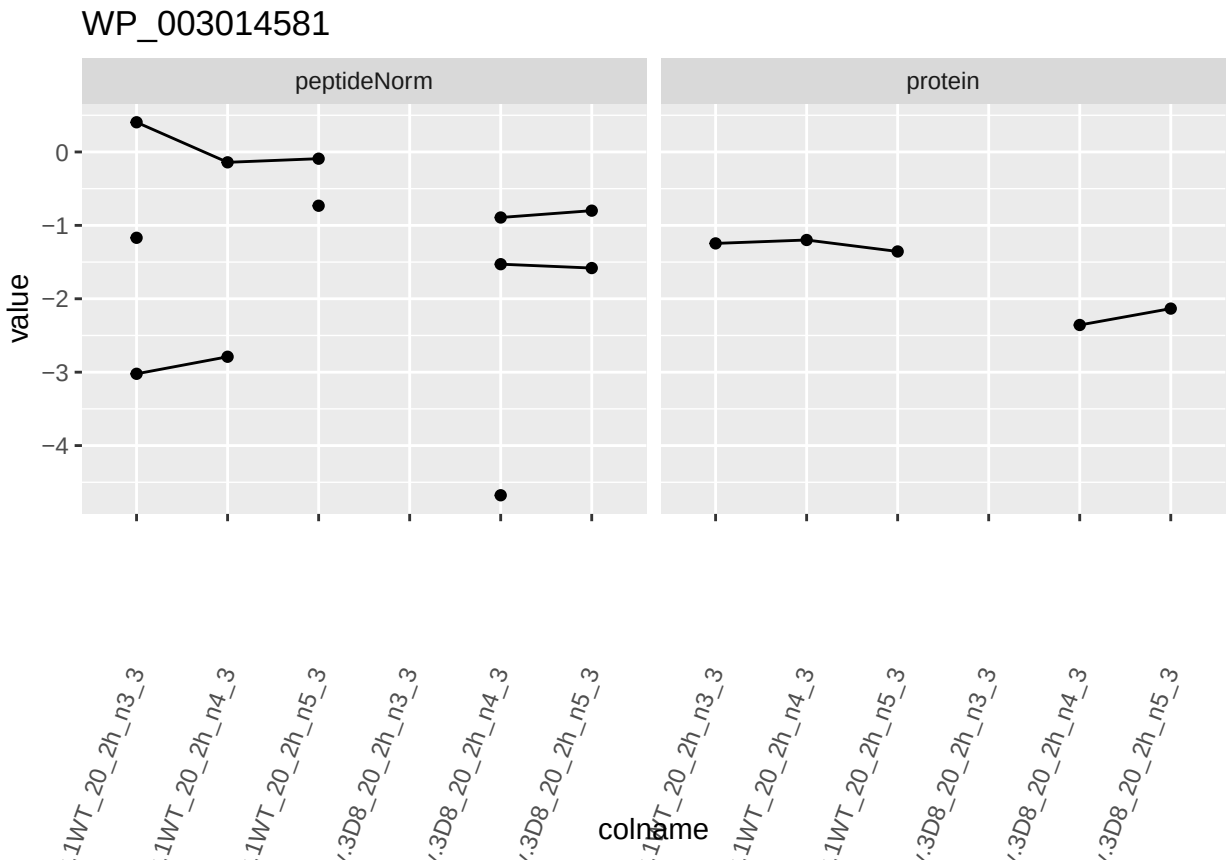
TEDNIQNFFK
WP_003014552
genotype
WT
D8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protein
value
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−4
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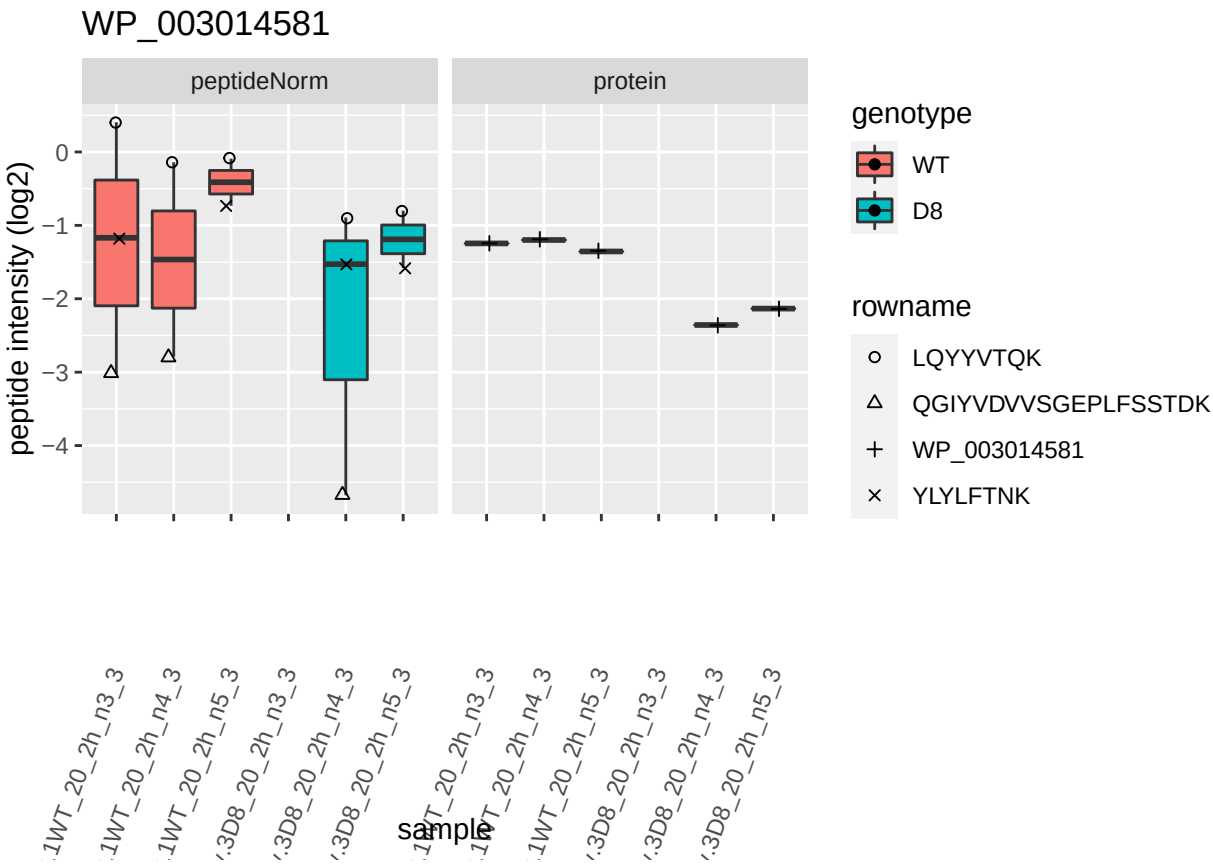
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
colname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protein
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−4
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WT
D8
rowname
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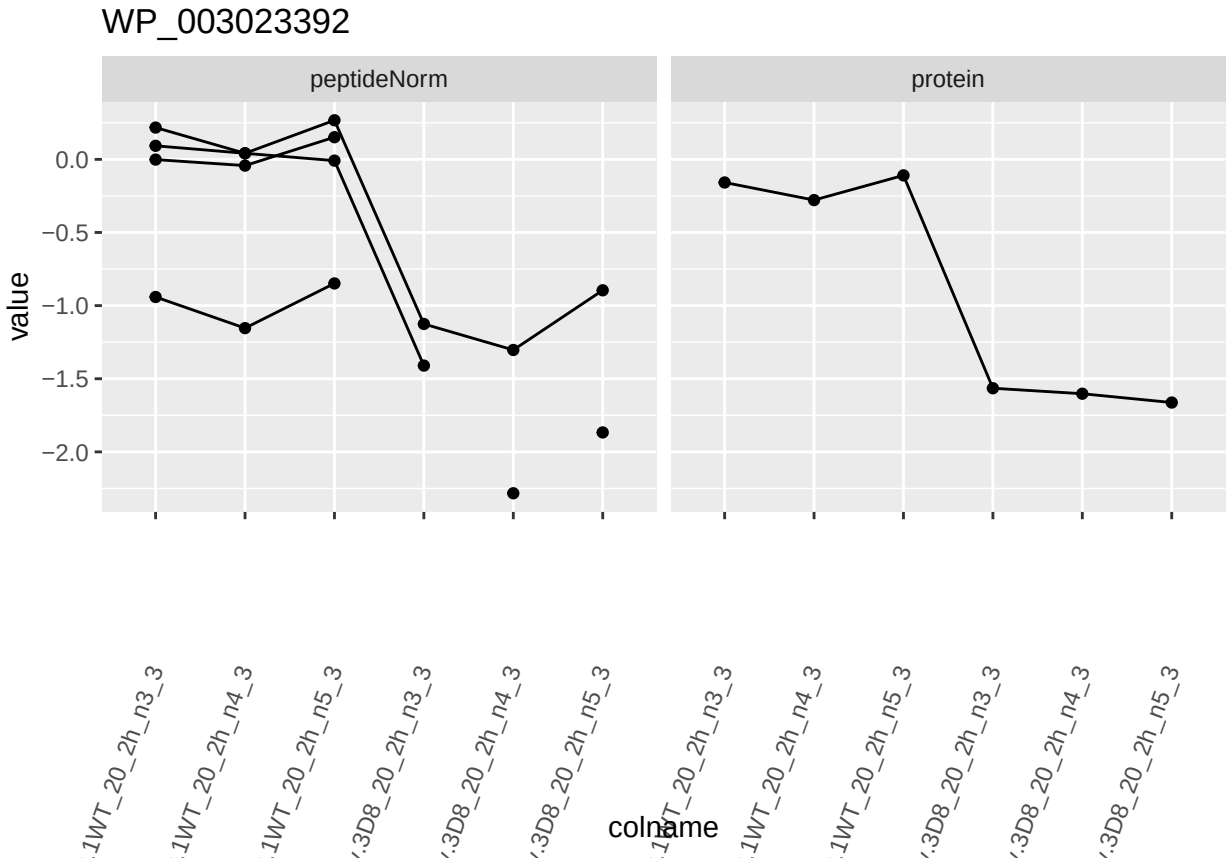
WP_003014581
YLYLFTNK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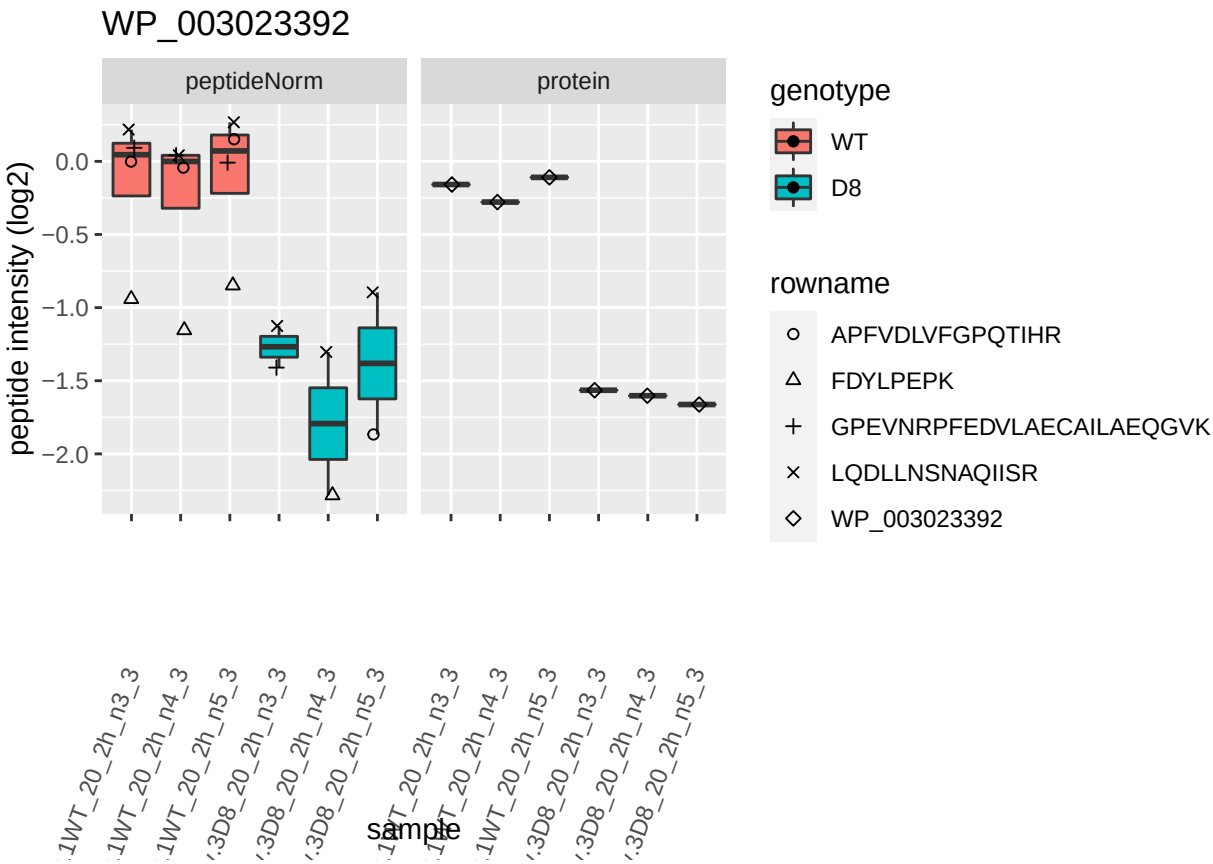
colname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sample
genotype
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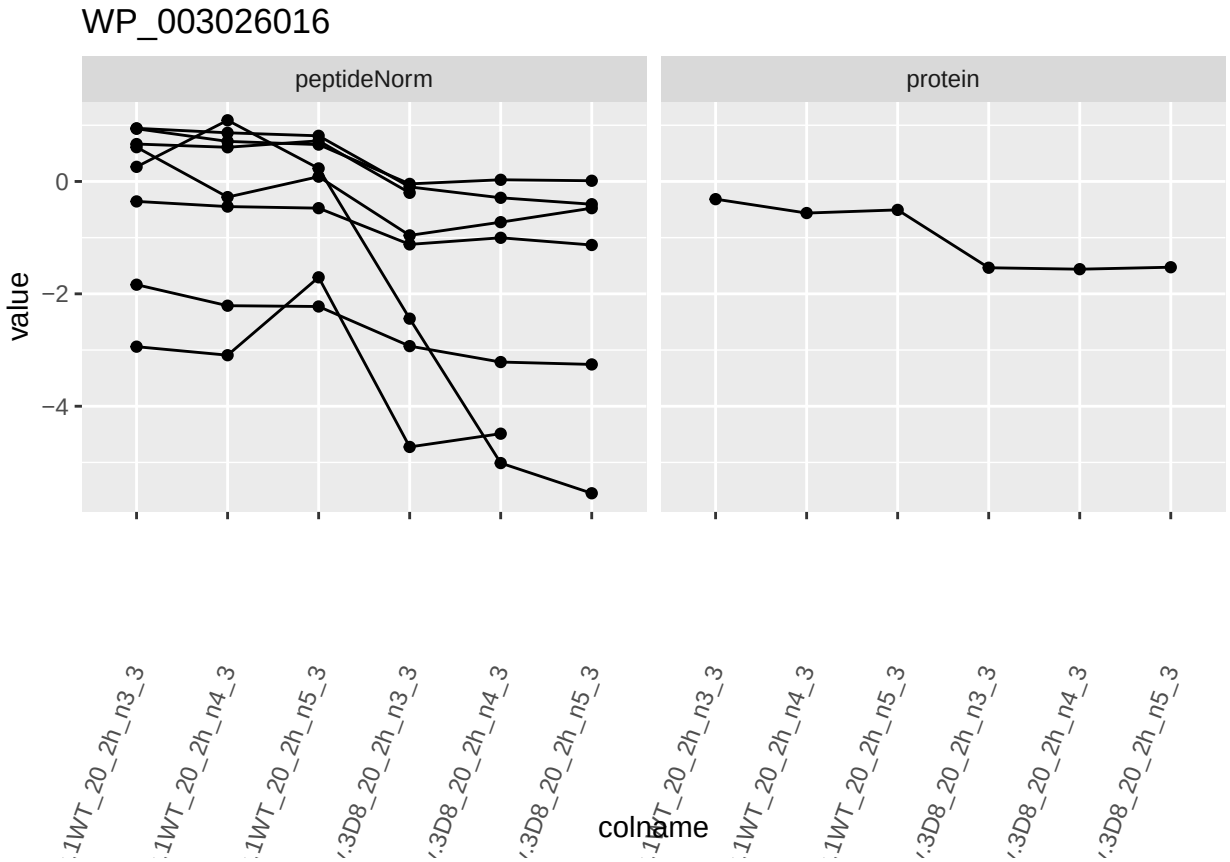
WP_003023392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protein
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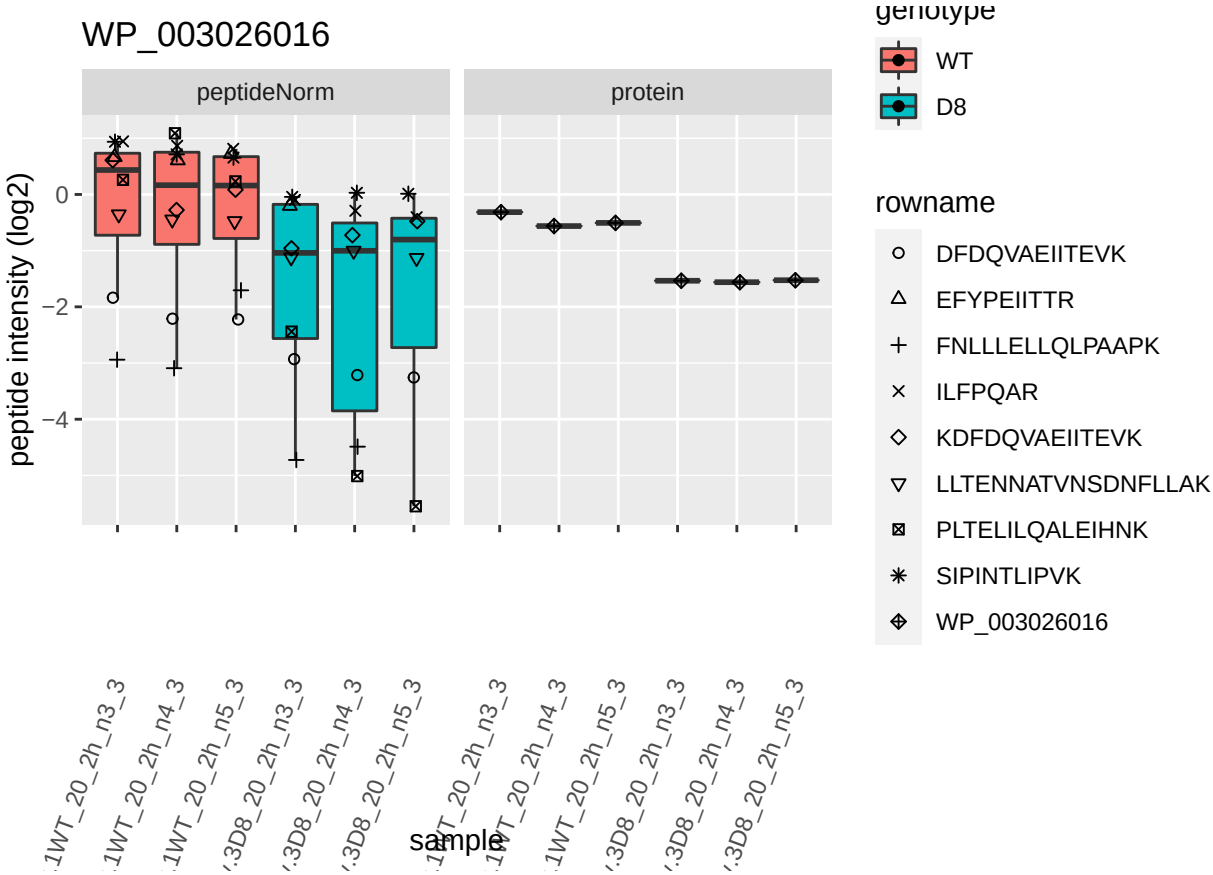
−4
colname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D8
rowname
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EFYPEIITTR
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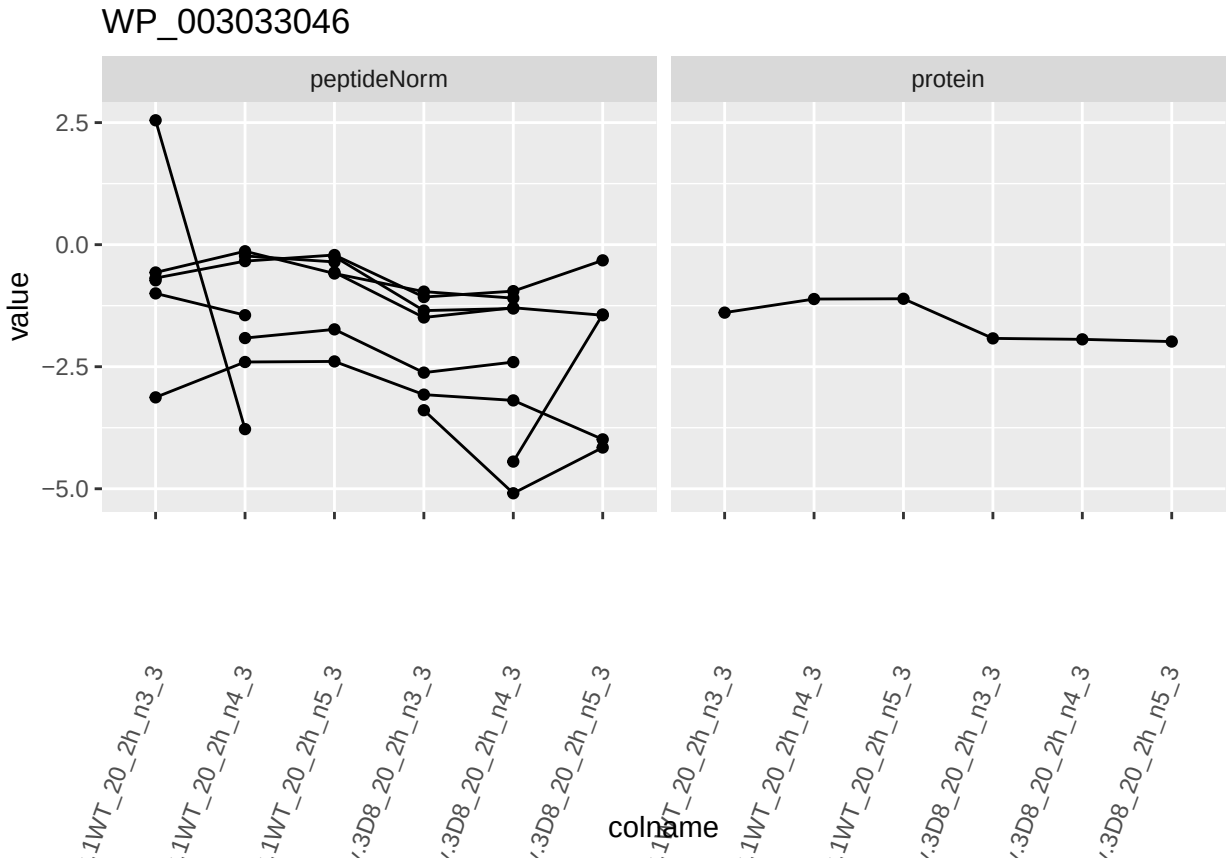
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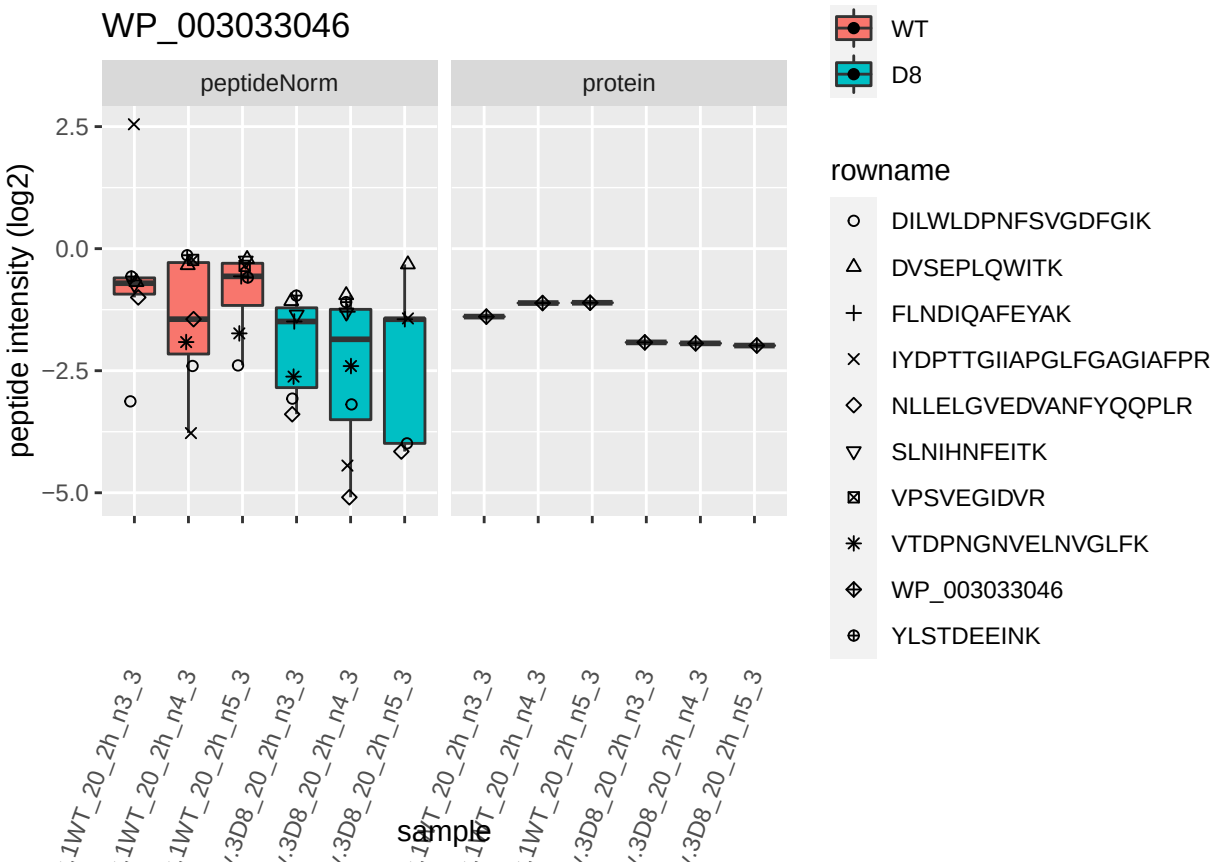
value
colname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protein
peptide intensity (log2)
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0.0
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−5.0
sample
WT
D8
rowname
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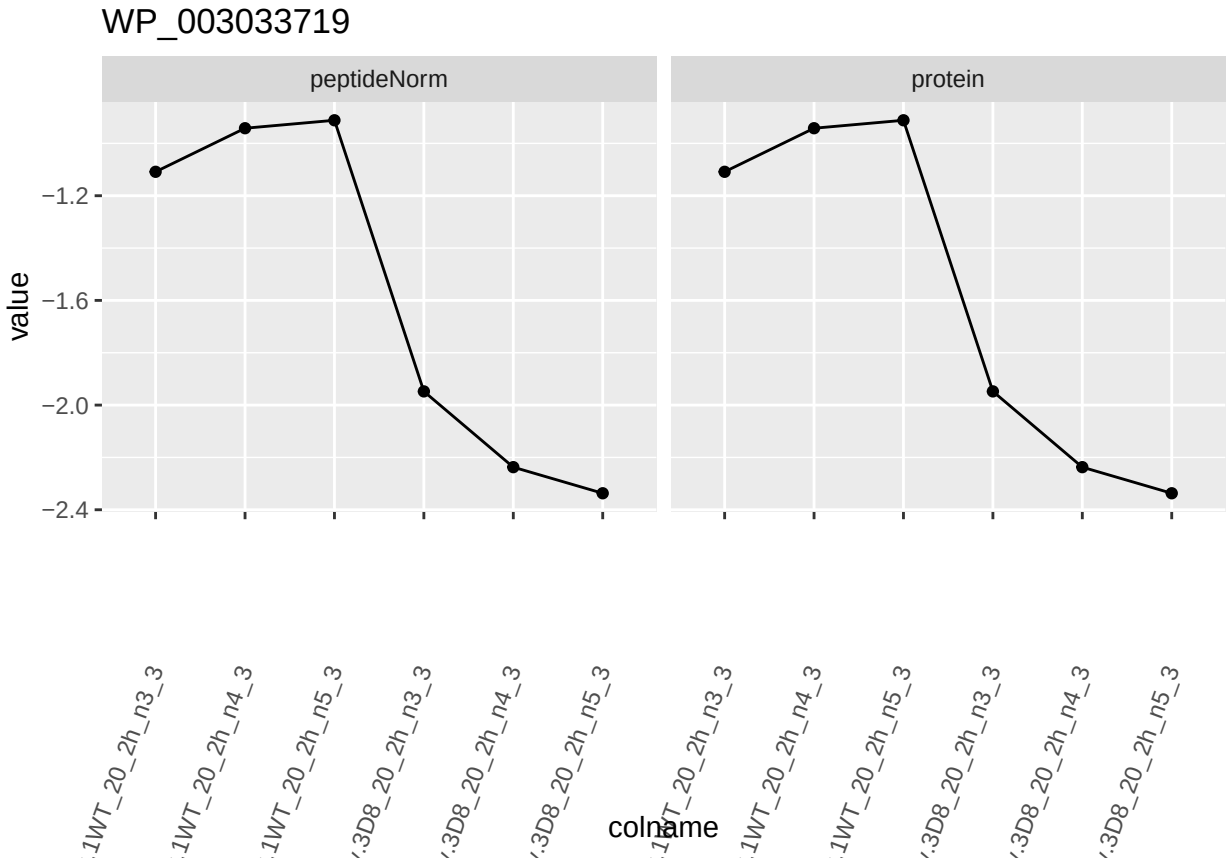
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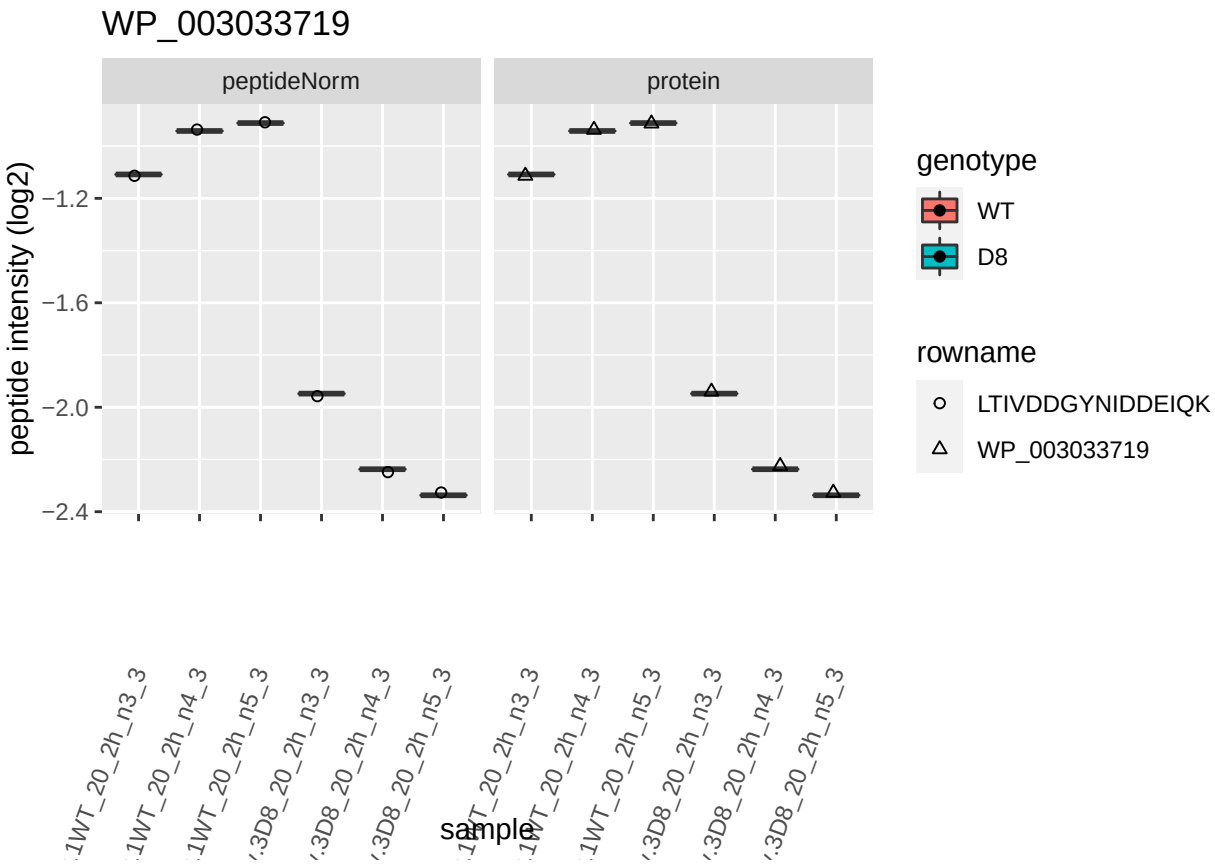
colname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−2.4
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WT
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rowname
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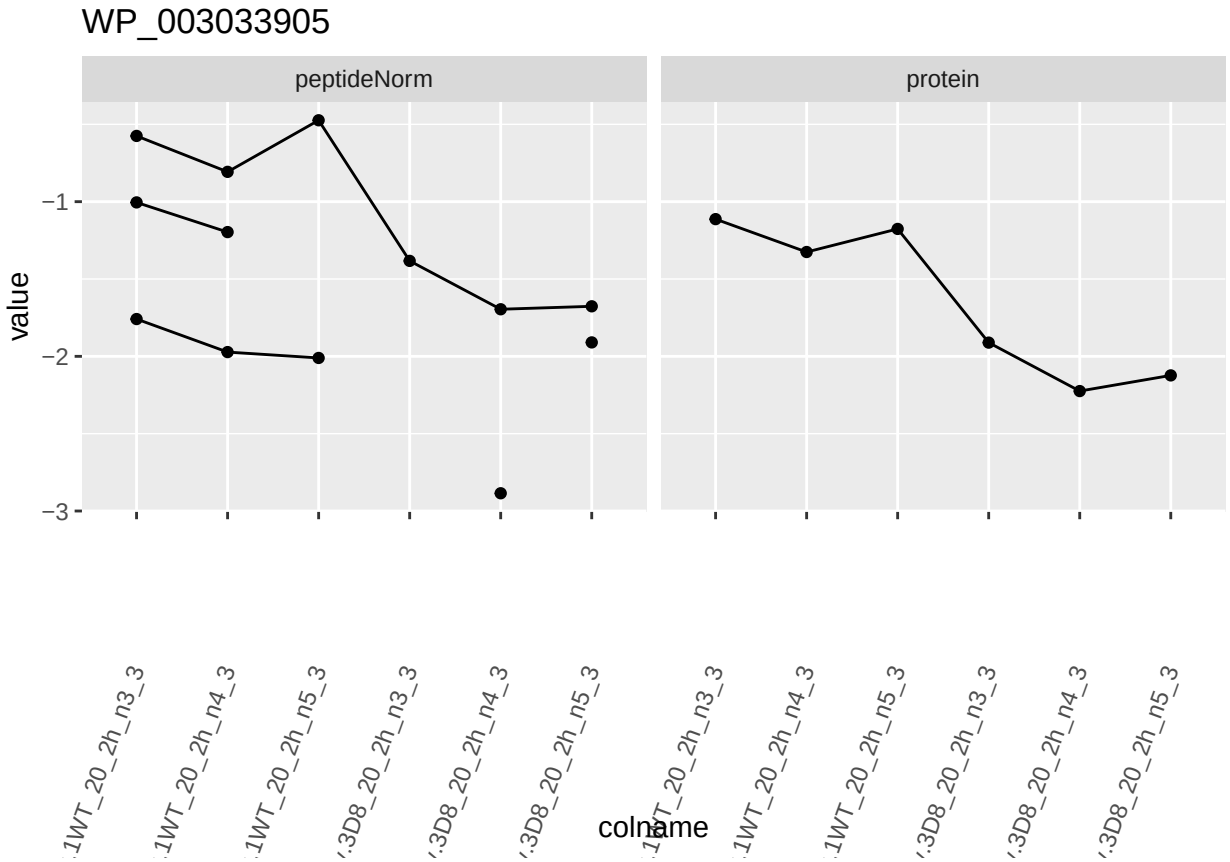
sample


WP_003033905
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protein
value
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−2
−3
colname
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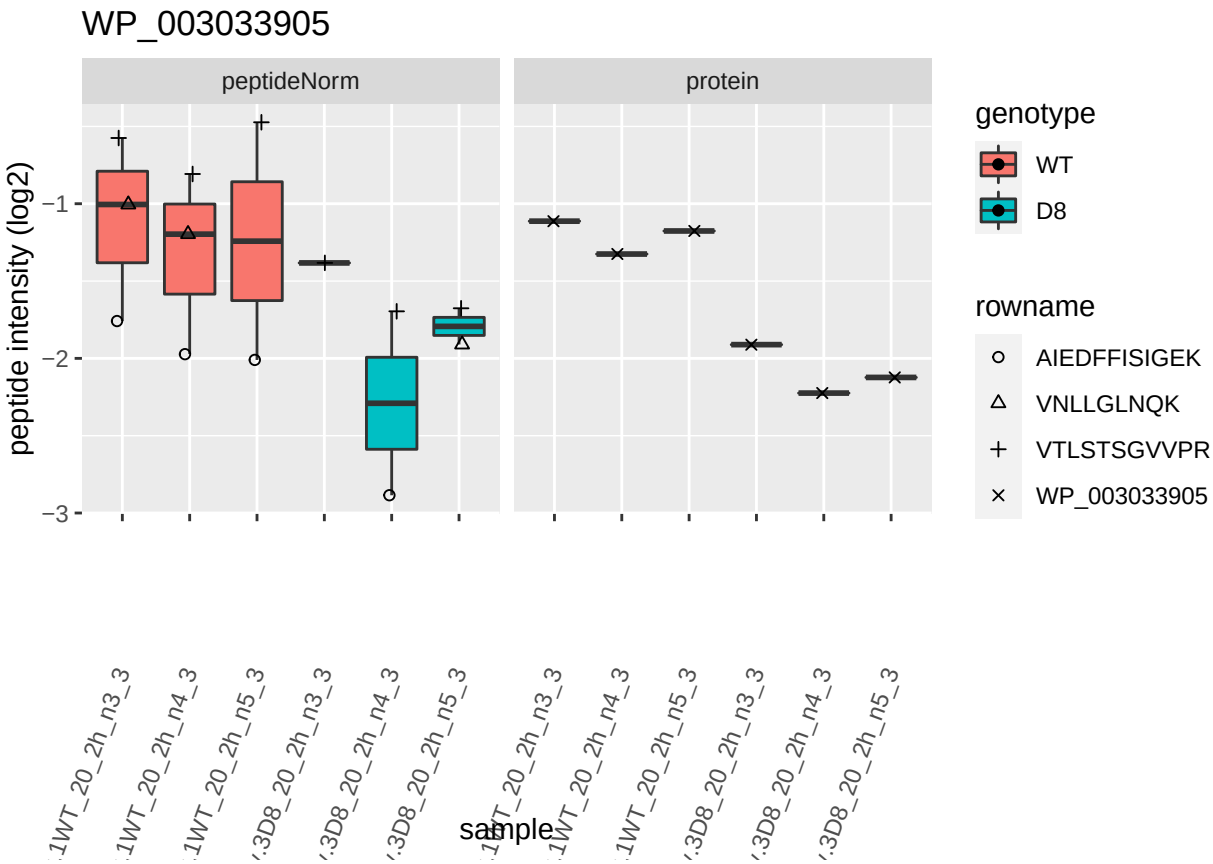
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3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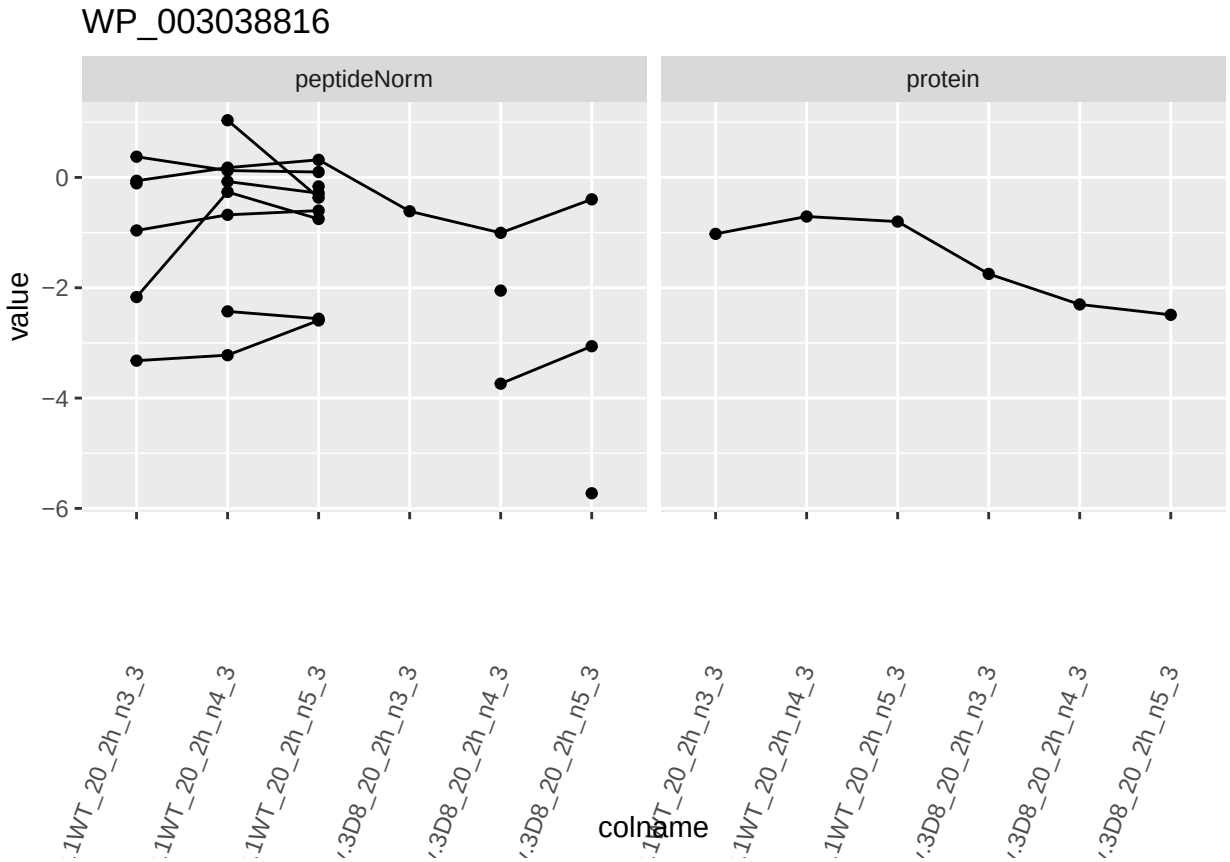
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value
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colname
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1WT_20_2h_n5_3
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3D8_20_2h_n4_3
3D8_20_2h_n5_3
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1WT_20_2h_n4_3
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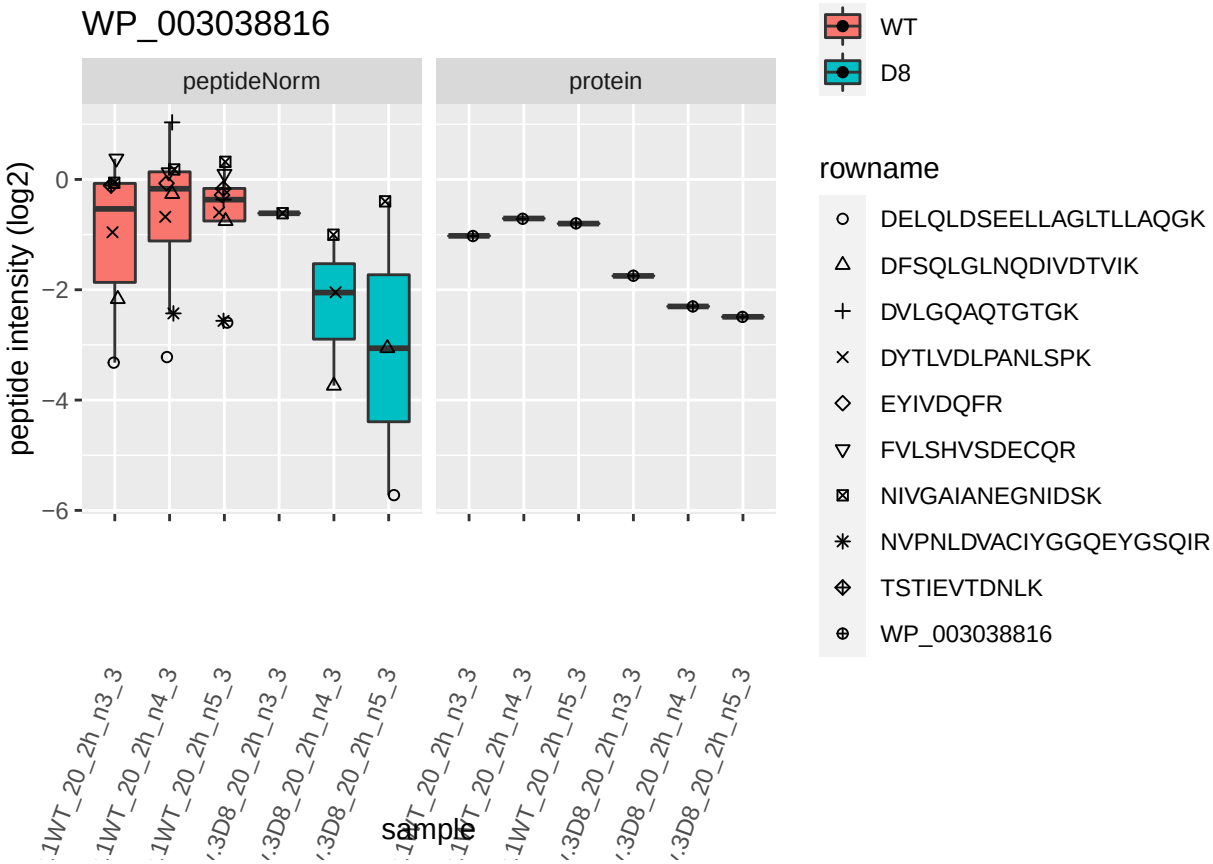
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sample
WT
D8
rowname
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DVLGQAQTGTGK
DYTLVDLPANLSPK
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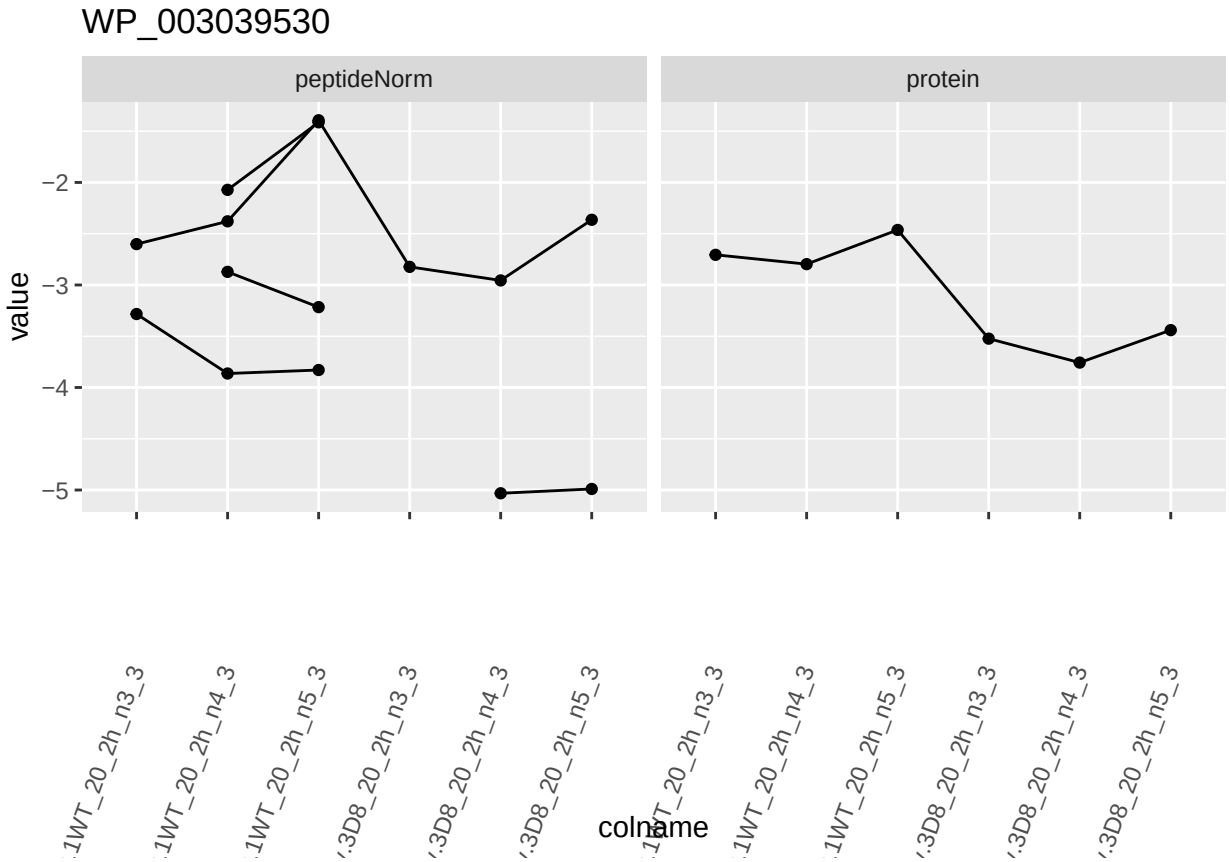
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value
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−4
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colname
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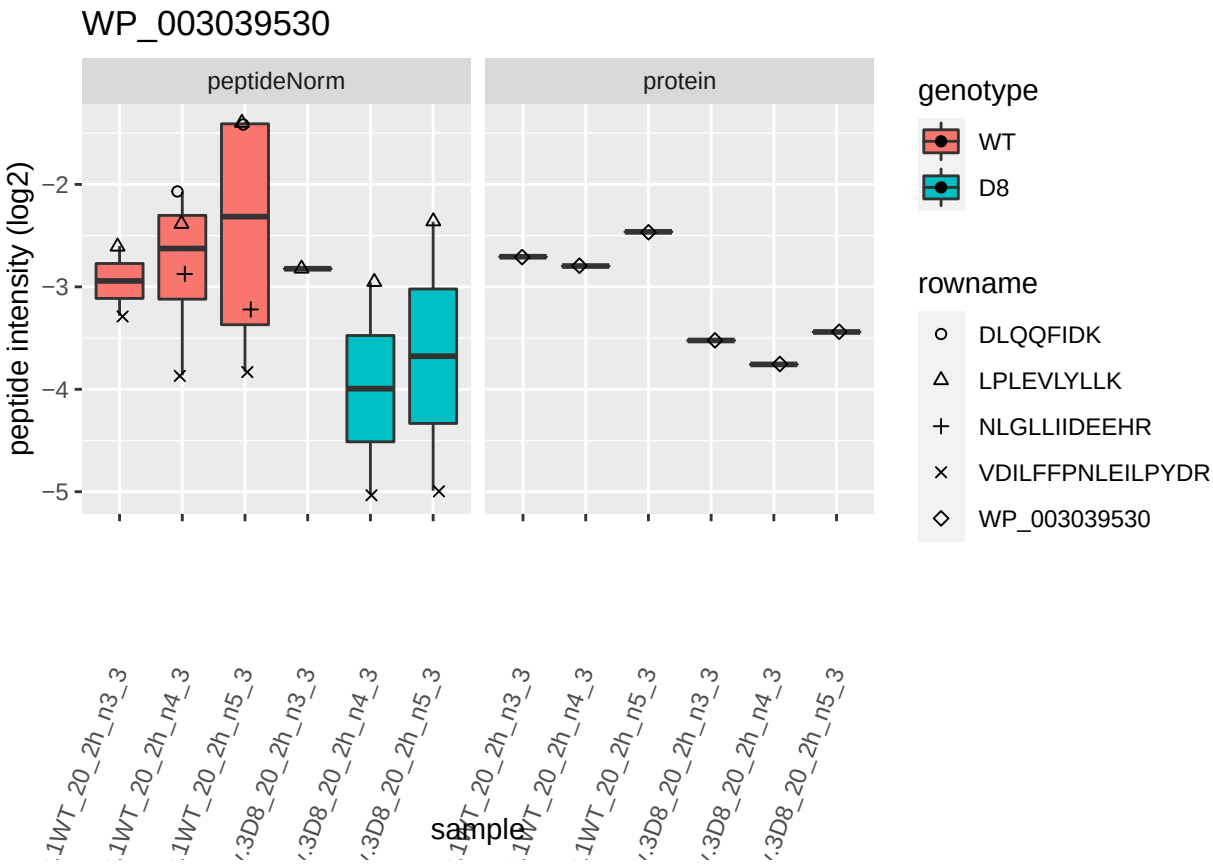
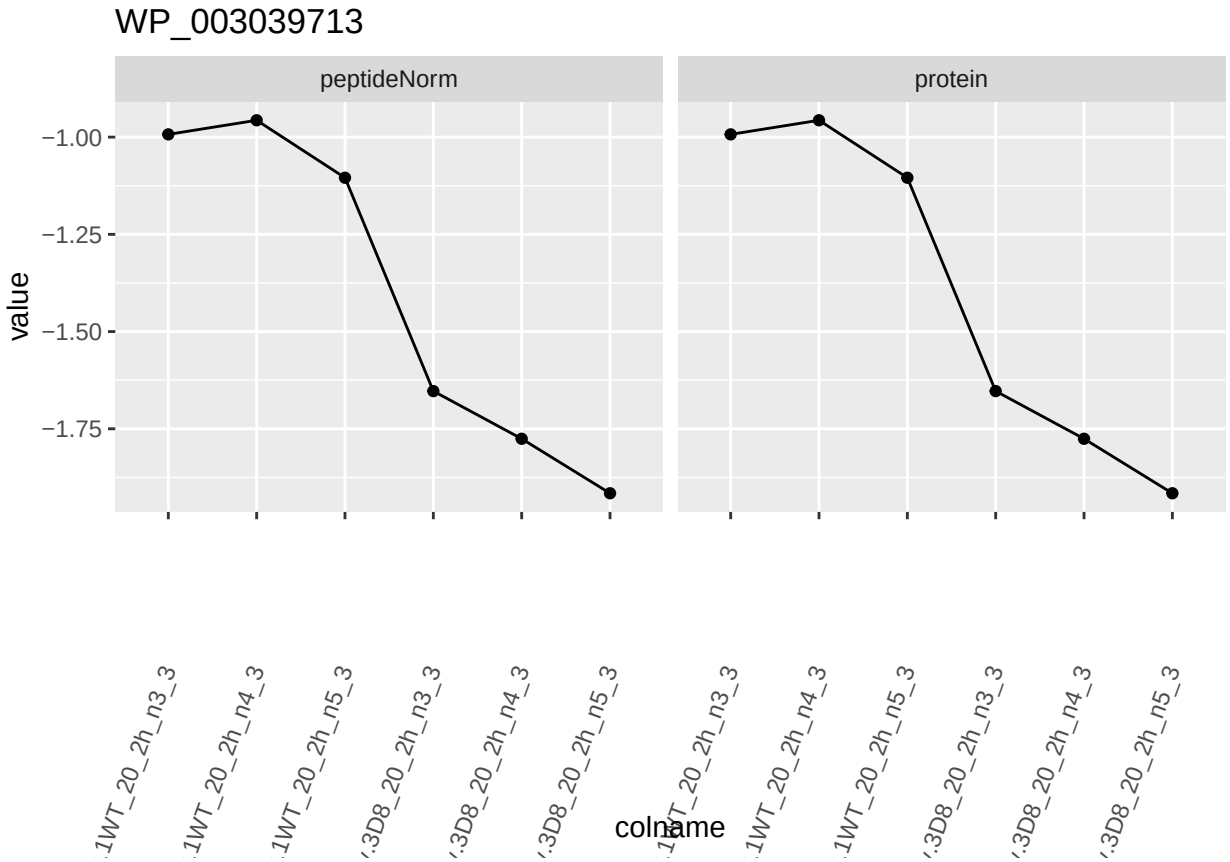
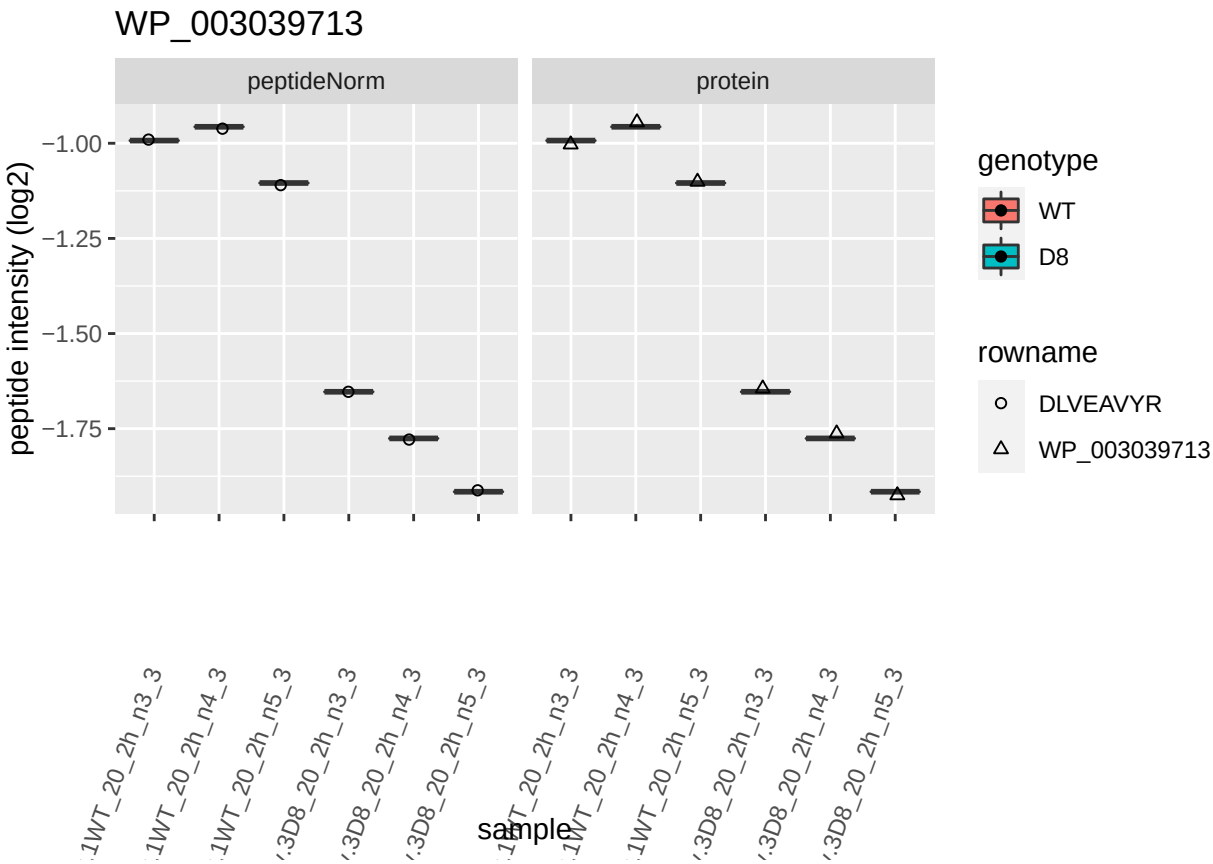
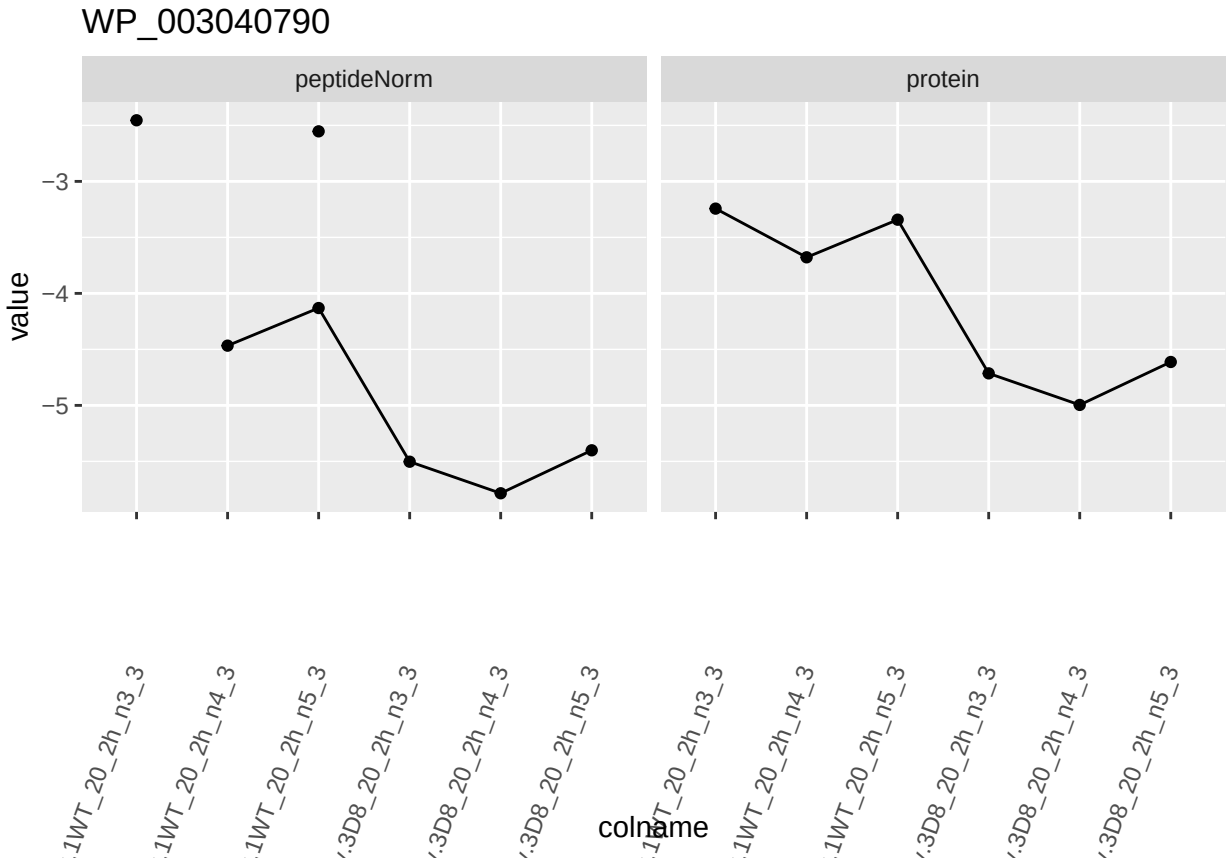
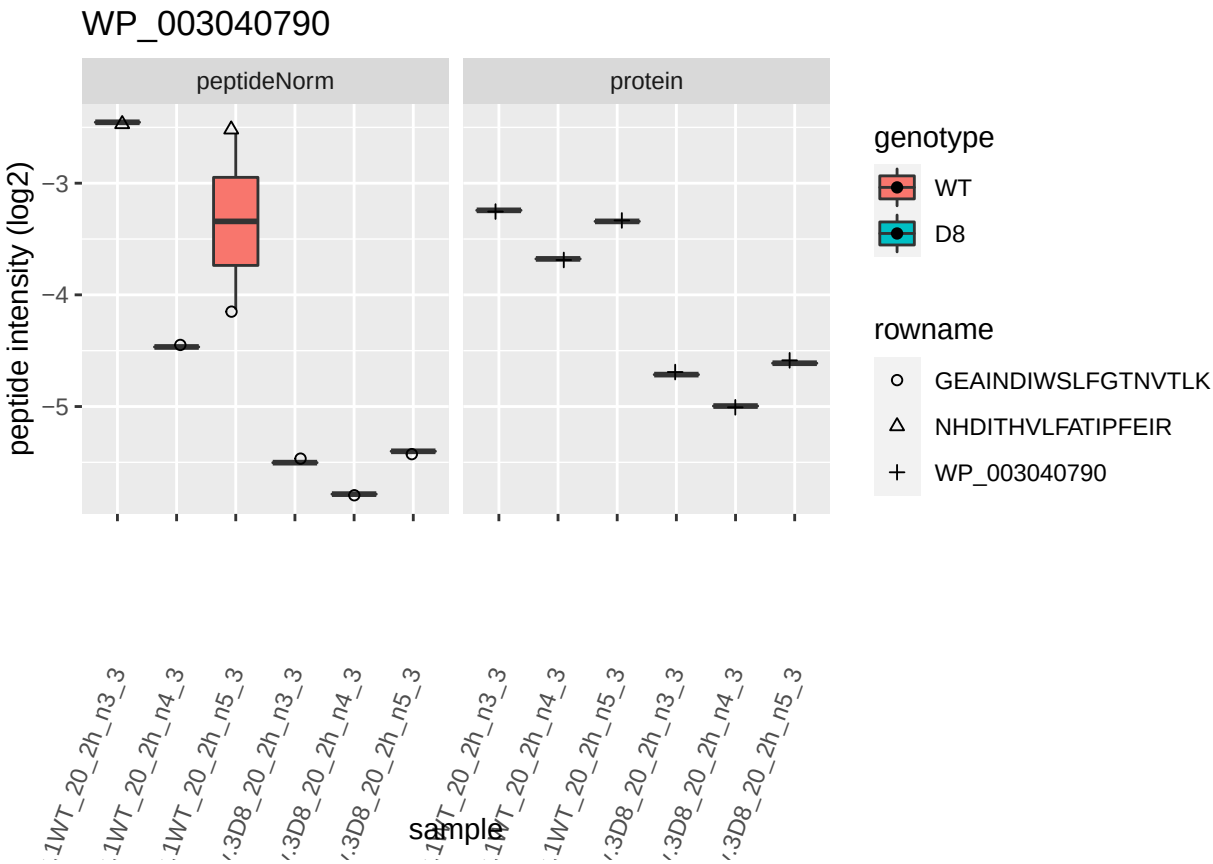
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3

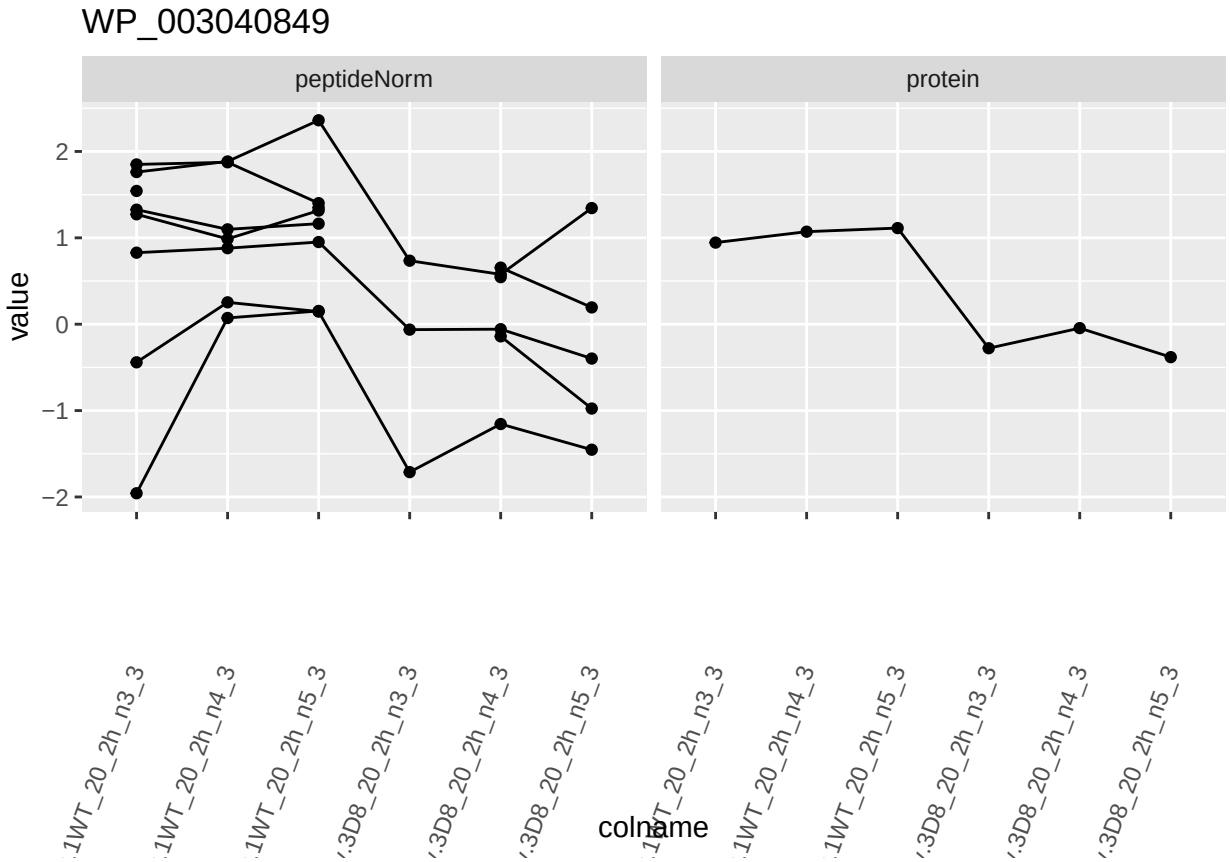

WP_003039530
peptideNorm
protein
peptide intensity (log2)
−2
−3
−4
−5
sample
genotype
WT
D8
rowname
DLQQFIDK
LPLEVLYLLK
NLGLLIIDEEHR
VDILFFPNLEILPYDR
WP_003039530

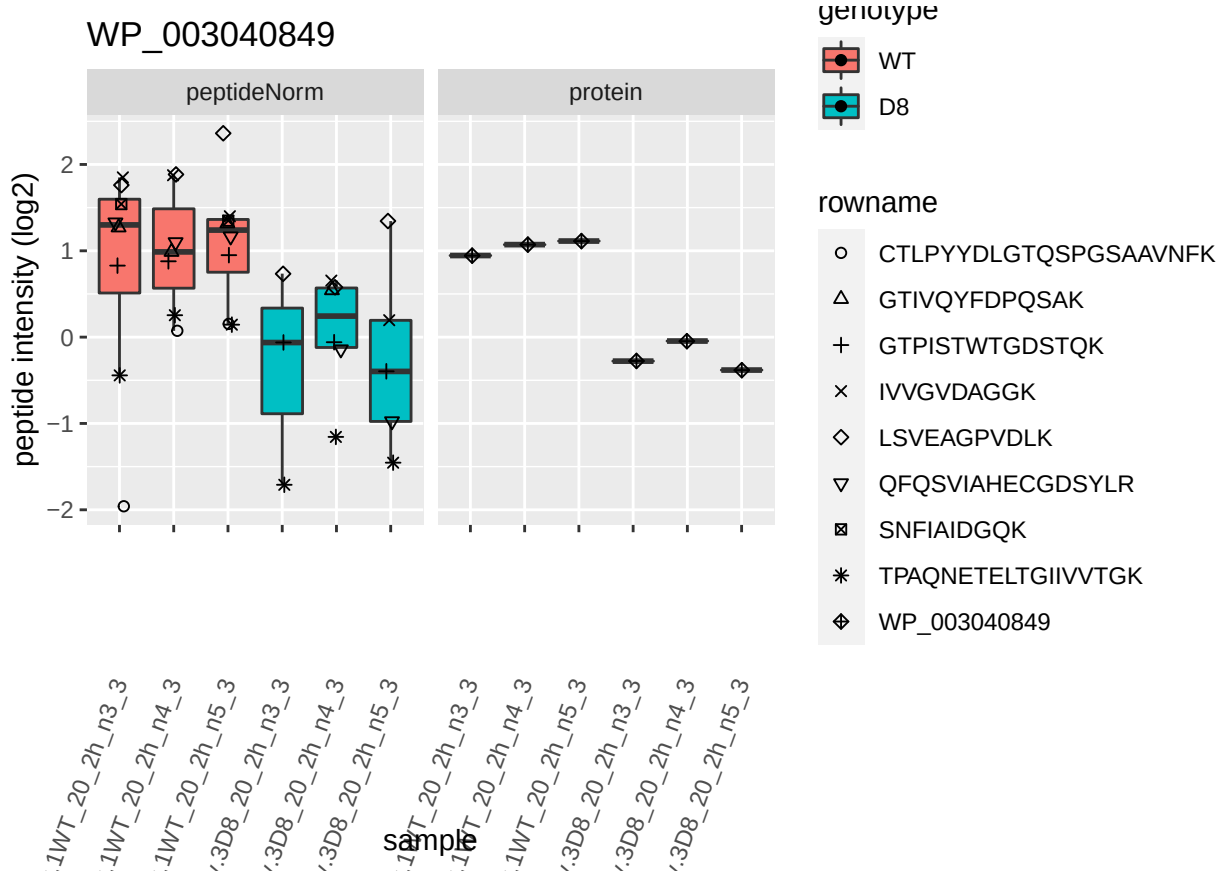

WP_003039713
peptideNorm
protein
value
−1.00
−1.25
−1.50
−1.75
colname
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3


WP_003039713
peptideNorm
protein
peptide intensity (log2)
−1.00
−1.25
−1.50
−1.75
genotype
WT
D8
rowname
DLVEAVYR
WP_003039713
sample


WP_003040790
peptideNorm
protein
value
−3
−4
−5
colname
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3


WP_003040790
peptideNorm
protein
peptide intensity (log2)
−3
−4
−5
sample
genotype
WT
D8
rowname
GEAINDIWSLFGTNVTLK
NHDITHVLFATIPFEIR
WP_003040790


WP_003040849
peptideNorm
protein
value
2
1
0
−1
−2
colname
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3




# 2 Experimental Design

## 2.1 Sample size

$$\log_2 \mathrm{FC} = \bar{y}_{p1} - \bar{y}_{p2}$$

$$T_g = \frac{\log_2 \mathrm{FC}}{\mathrm{se}_{\log_2 \mathrm{FC}}}$$

$$T_g = \frac{\widehat{\text{signal}}}{\widehat{\text{Noise}}}$$

If we can assume equal variance in both treatment groups:

$$\mathrm{se}_{\log_2 \mathrm{FC}} = \mathrm{SD}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

→ if number of bio-repeats increases we have a higher power!

- cfr. Study of tamoxifen treated Estrogen Recepter (ER) positive breast cancer patients

## 2.2 Randomized complete block designs

$$\sigma^2 = \sigma^2_{bio} + \sigma^2_{\text{lab}} + \sigma^2_{\text{extraction}} + \sigma^2_{\text{run}} + \dots$$

- Biological: fluctuations in protein level between mice, fluctations in protein level between cells, ...
- Technical: cage effect, lab effect, week effect, plasma extraction, MS-run, ...

## 2.3 Nature methods: Points of significance - Blocking

https://www.nature.com/articles/nmeth.3005.pdf

### 2.3.1 Mouse example

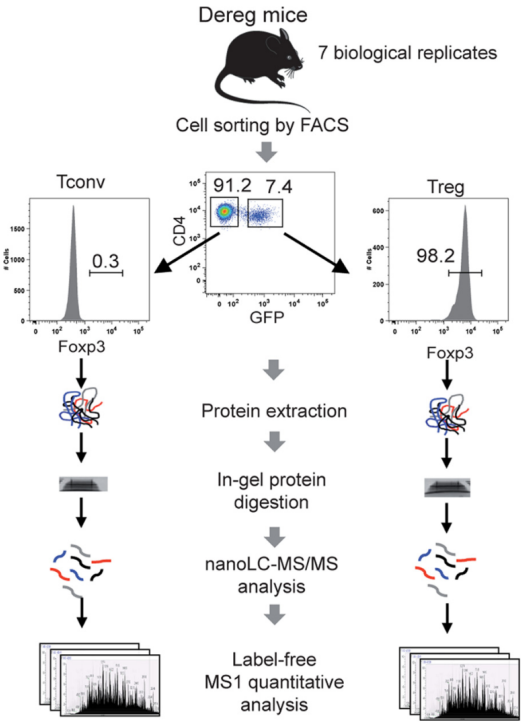



FIG. 1. **Label-free quantitative analysis of conventional and regulatory T cell proteomes.** General analytical workflow based on cell sorting by flow cytometry using the DEREG mouse model and parallel proteomic analysis of Tconv and Treg cell populations by nanoLC-MS/MS and label-free relative quantification.

Duguet et al. (2017) MCP 16(8):1416-1432. doi: 10.1074/mcp.m116.062745

- All treatments of interest are present within block!
- We can estimate the effect of the treatment within block!

To illustrate the power of blocking we have subsetted the data of Duguet et al. in a

- completely randomized design with
  - four mice for which we only have measurements on the ordinary T-cells
  - four mice for which we only have measurements on the regulatory T-cells

- randomized complete block design with four mice for which we both have
  - measurements on ordinary T-cells as well as
  - measurements on regulatory T-cells

### 2.3.2 Data

Click to see code

```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
library(plotly)
library(gridExtra)

peptidesFile <- "https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTcell/peptid
peptidesFile2 <- "https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTcell/pept
peptidesFile3 <- "https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTcell/pept

ecols <- grep("Intensity\\.", names(read.delim(peptidesFile)))
pe <- readQFeatures(
  table = peptidesFile,
  fnames = 1,
  ecol = ecols,
  name = "peptideRaw", sep="\t")

ecols2 <- grep("Intensity\\.", names(read.delim(peptidesFile2)))
pe2 <- readQFeatures(
  table = peptidesFile2,
  fnames = 1,
  ecol = ecols2,
  name = "peptideRaw", sep="\t")

ecols3 <- grep("Intensity\\.", names(read.delim(peptidesFile3)))
pe3 <- readQFeatures(
  table = peptidesFile3,
  fnames = 1,
  ecol = ecols3,
  name = "peptideRaw", sep="\t")

### Design
colData(pe)$celltype <- substr(
  colnames(pe[["peptideRaw"]]),
  11,
  14) %>%
  unlist %>%
  as.factor

colData(pe)$mouse <- pe[[1]] %>%
  colnames %>%
  strsplit(split="[.]")  %>%
  sapply(function(x) x[3]) %>%
```

```
  as.factor

colData(pe2)$celltype <- substr(
  colnames(pe2[["peptideRaw"]]),
  11,
  14) %>%
  unlist %>%
  as.factor

colData(pe2)$mouse <- pe2[[1]] %>%
  colnames %>%
  strsplit(split="[.]")  %>%
  sapply(function(x) x[3]) %>%
  as.factor

colData(pe3)$celltype <- substr(
  colnames(pe3[["peptideRaw"]]),
  11,
  14) %>%
  unlist %>%
  as.factor

colData(pe3)$mouse <- pe3[[1]] %>%
  colnames %>%
  strsplit(split="[.]")  %>%
  sapply(function(x) x[3]) %>%
  as.factor
```

### 2.3.3 Preprocessing

#### 2.3.3.1 Log-transform

Click to see code to log-transfrom the data

- We calculate how many non zero intensities we have for each peptide and this can be useful for filtering.

```
rowData(pe[["peptideRaw"]])$nNonZero <- rowSums(assay(pe[["peptideRaw"]]) > 0)

rowData(pe2[["peptideRaw"]])$nNonZero <- rowSums(assay(pe2[["peptideRaw"]]) > 0)

rowData(pe3[["peptideRaw"]])$nNonZero <- rowSums(assay(pe3[["peptideRaw"]]) > 0)
```

- Peptides with zero intensities are missing peptides and should be represent with a `NA` value rather than `0`.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA

pe2 <- zeroIsNA(pe2, "peptideRaw") # convert 0 to NA

pe3 <- zeroIsNA(pe3, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")

pe2 <- logTransform(pe2, base = 2, i = "peptideRaw", name = "peptideLog")

pe3 <- logTransform(pe3, base = 2, i = "peptideRaw", name = "peptideLog")
```

#### 2.3.3.2 Filtering Click to see details on filtering

1. Handling overlapping protein groups

In our approach a peptide can map to multiple proteins, as long as there is none of these proteins present in a smaller subgroup.

```
pe <- filterFeatures(pe, ~ Proteins %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$Proteins))

pe2 <- filterFeatures(pe2, ~ Proteins %in% smallestUniqueGroups(rowData(pe2[["peptideLog"]])$Proteins))

pe3 <- filterFeatures(pe3, ~ Proteins %in% smallestUniqueGroups(rowData(pe3[["peptideLog"]])$Proteins))
```

2. Remove reverse sequences (decoys) and contaminants

We now remove the contaminants, peptides that map to decoy sequences, and proteins which were only identified by peptides with modifications.

```
pe <- filterFeatures(pe,~Reverse != "+")
pe <- filterFeatures(pe,~ Potential.contaminant != "+")

pe2 <- filterFeatures(pe2,~Reverse != "+")
pe2 <- filterFeatures(pe2,~ Potential.contaminant != "+")

pe3 <- filterFeatures(pe3,~Reverse != "+")
pe3 <- filterFeatures(pe3,~ Potential.contaminant != "+")
```

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at last twice.

```
pe <- filterFeatures(pe,~ nNonZero >=2)
nrow(pe[["peptideLog"]])
```

```
## [1] 44449
```

```
pe2 <- filterFeatures(pe2,~ nNonZero >=2)
nrow(pe2[["peptideLog"]])
```

```
## [1] 43401
```

```
pe3 <- filterFeatures(pe3,~ nNonZero >=2)
nrow(pe3[["peptideLog"]])
```

```
## [1] 47431
```

#### 2.3.3.3 Normalization Click to see code to normalize the data

```
pe <- normalize(pe,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")

pe2 <- normalize(pe2,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")


pe3 <- normalize(pe3,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")
```

#### 2.3.3.4 Summarization Click to see code to summarize the data

```
pe <- aggregateFeatures(pe,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
pe2 <- aggregateFeatures(pe2,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
pe3 <- aggregateFeatures(pe3,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

### 2.3.4 Data Exploration: what is impact of blocking?

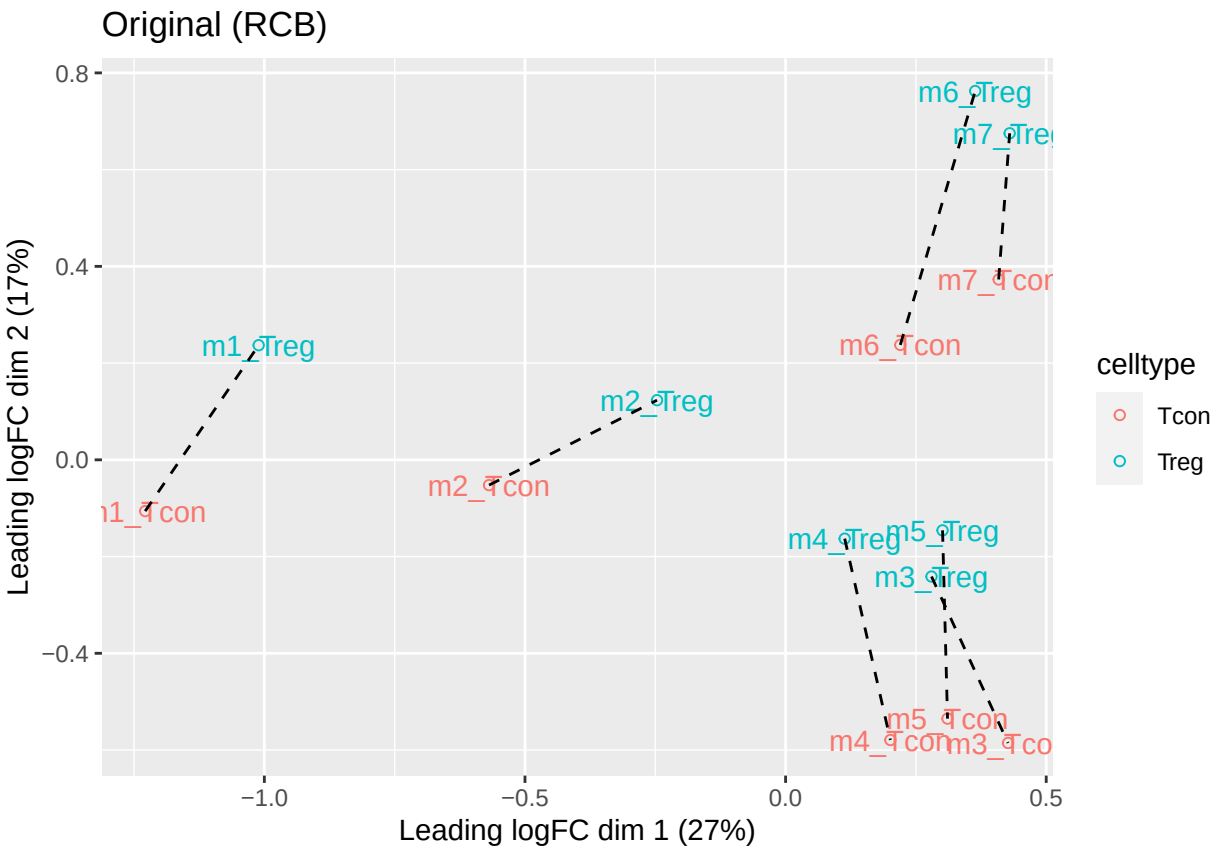
Click to see code

```
levels(colData(pe3)$mouse) <- paste0("m",1:7)
mdsObj3 <- plotMDS(assay(pe3[["protein"]]), plot = FALSE)
mdsOrig <- colData(pe3) %>%
  as.data.frame %>%
  mutate(mds1 = mdsObj3$x,
         mds2 = mdsObj3$y,
         lab = paste(mouse,celltype,sep="_")) %>%
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  geom_line(color = "black", linetype = "dashed") +
  xlab(
    paste0(
      mdsObj3$axislabel,
      " ",
      1,
      " (",
      paste0(
        round(mdsObj3$var.explained[1] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ylab(
    paste0(
      mdsObj3$axislabel,
      " ",
      2,
      " (",
      paste0(
        round(mdsObj3$var.explained[2] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ggtitle("Original (RCB)")

levels(colData(pe)$mouse) <- paste0("m",1:4)
mdsObj <- plotMDS(assay(pe[["protein"]]), plot = FALSE)
mdsRCB <- colData(pe) %>%
  as.data.frame %>%
  mutate(mds1 = mdsObj$x,
         mds2 = mdsObj$y,
```

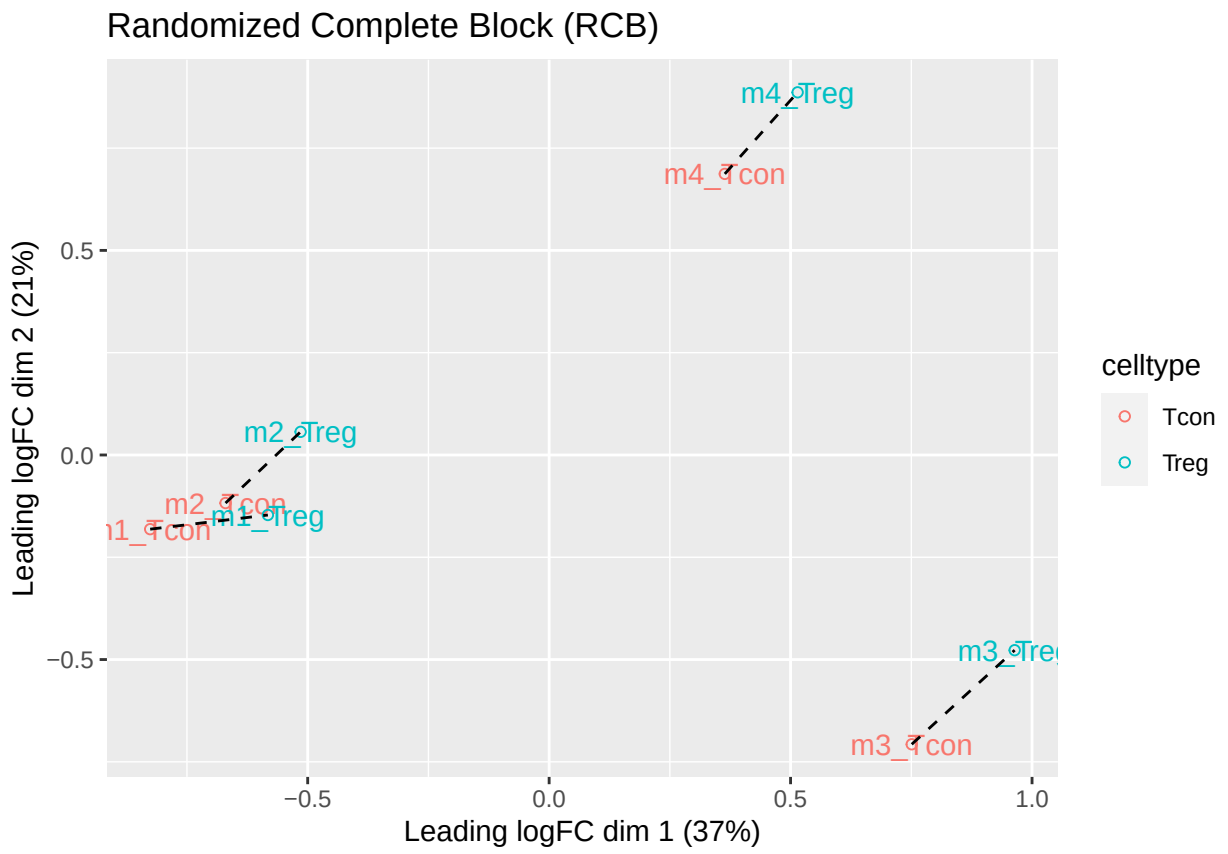
```
        lab = paste(mouse,celltype,sep="_")) %>%
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  geom_line(color = "black", linetype = "dashed") +
  xlab(
    paste0(
      mdsObj$axislabel,
      " ",
      1,
      " (",
      paste0(
        round(mdsObj$var.explained[1] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ylab(
    paste0(
      mdsObj$axislabel,
      " ",
      2,
      " (",
      paste0(
        round(mdsObj$var.explained[2] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ggtitle("Randomized Complete Block (RCB)")


levels(colData(pe2)$mouse) <- paste0("m",1:8)
mdsObj2 <- plotMDS(assay(pe2[["protein"]]), plot = FALSE)
mdsCRD <- colData(pe2) %>%
  as.data.frame %>%
  mutate(mds1 = mdsObj2$x,
         mds2 = mdsObj2$y,
         lab = paste(mouse,celltype,sep="_")) %>%
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  xlab(
    paste0(
      mdsObj$axislabel,
      " ",
      1,
      " (",
      paste0(
        round(mdsObj2$var.explained[1] *100,0),
        "%"
```

```
      ),
    ")"
    )
  ) +
ylab(
  paste0(
    mdsObj$axislabel,
    " ",
    2,
    " (",
    paste0(
      round(mdsObj2$var.explained[2] *100,0),
      "%"
      ),
    ")"
    )
  ) +
ggtitle("Completely Randomized Design (CRD)")
```

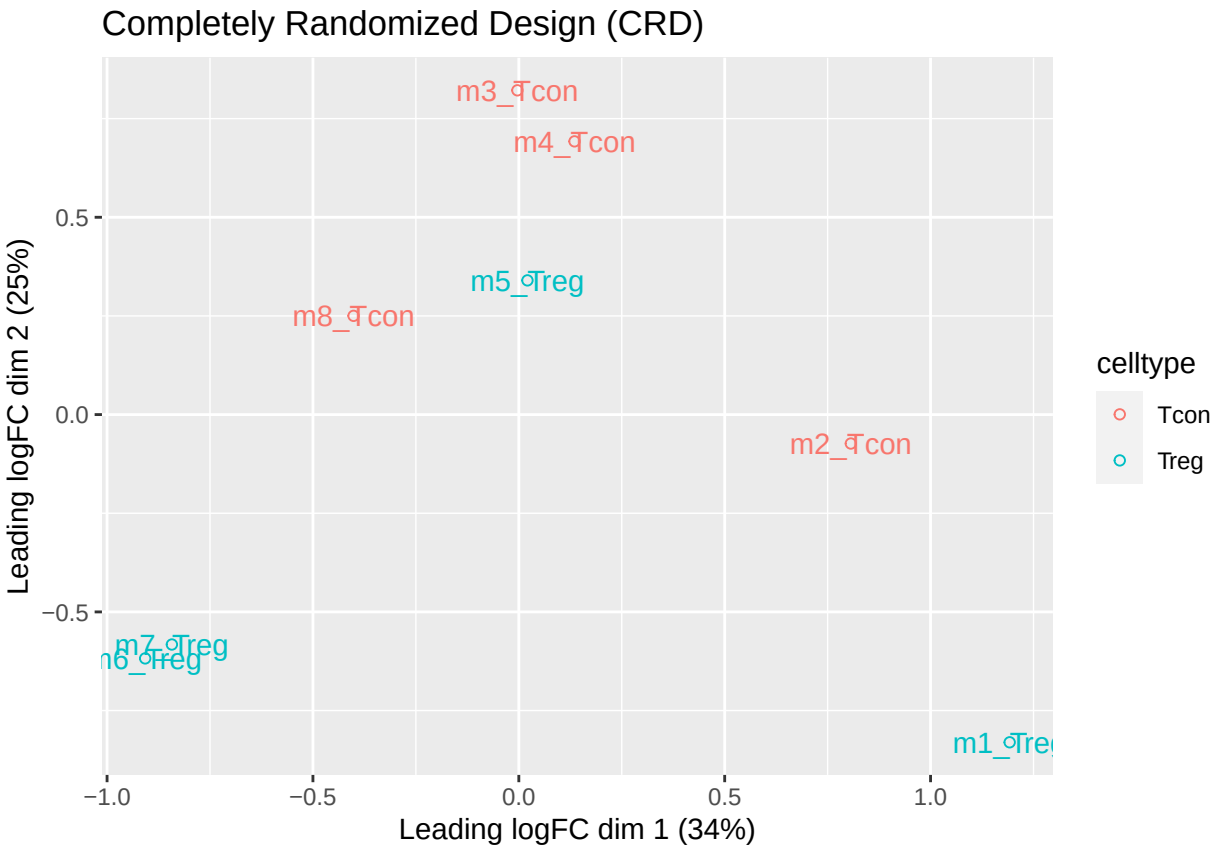
```
mdsOrig
```




```
mdsRCB
```


Randomized Complete Block (RCB)
m4_Treg
m4_Tcon
m2_Treg
m2_Tcon
m1_Treg
m3_Treg
m3_Tcon
0.5
0.0
−0.5
Leading logFC dim 2 (21%)
−0.5
0.0
0.5
1.0
Leading logFC dim 1 (37%)
celltype
Tcon
Treg

```
mdsCRD
```




- We observe that the leading fold change is according to mouse
- In the second dimension we see a separation according to cell-type
- With the Randomized Complete Block design (RCB) we can remove the mouse effect from the analysis!
- We can isolate the between block variability from the analysis using linear model:
  - Formula in R
    $$y \sim \text{celltype} + \text{mouse}$$
  - Formula

$$y_i = \beta_0 + \beta_{\text{Treg}} x_{i,\text{Treg}} + \beta_{m2} x_{i,m2} + \beta_{m3} x_{i,m3} + \beta_{m4} x_{i,m4} + \epsilon_i$$

with

- $x_{i,Treg} = \begin{cases} 1 & \text{Treg} \\ 0 & \text{Tcon} \end{cases}$
- $x_{i,m2} = \begin{cases} 1 & \text{m2} \\ 0 & \text{otherwise} \end{cases}$
- $x_{i,m3} = \begin{cases} 1 & \text{m3} \\ 0 & \text{otherwise} \end{cases}$

- $x_{i,m4} = \begin{cases} 1 & \text{m4} \\ 0 & \text{otherwise} \end{cases}$
- Possible in msqrob2 and MSstats but not possible with Perseus!

## 2.4 Modeling and inference

### 2.4.1 RCB analysis

```
pe <- msqrob(
  object = pe,
  i = "protein",
  formula = ~ celltype + mouse)
```

### 2.4.2 CRD analysis

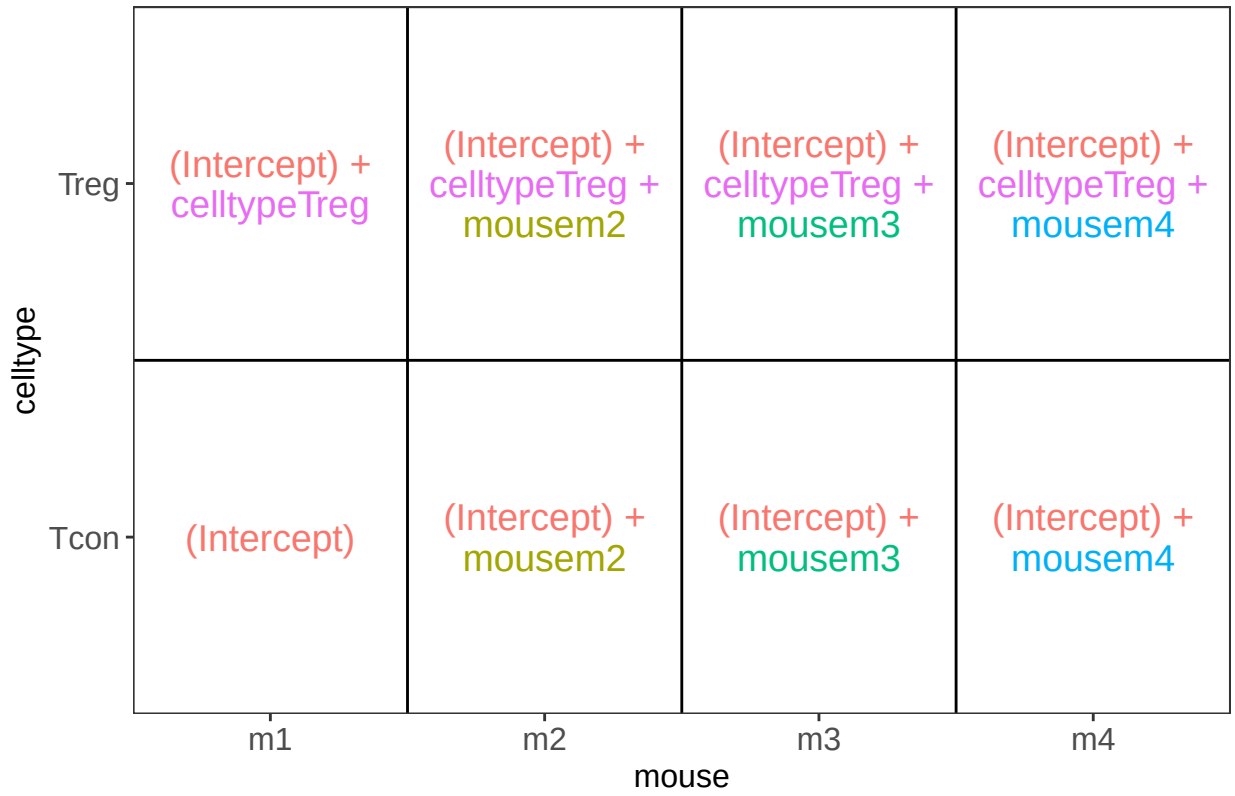
```
pe2 <- msqrob(
  object = pe2,
  i = "protein",
  formula = ~ celltype)
```

### 2.4.3 Estimation, effect size and inference

Effect size in RCB

```
library(ExploreModelMatrix)
VisualizeDesign(colData(pe),~ celltype + mouse)$plotlist
```

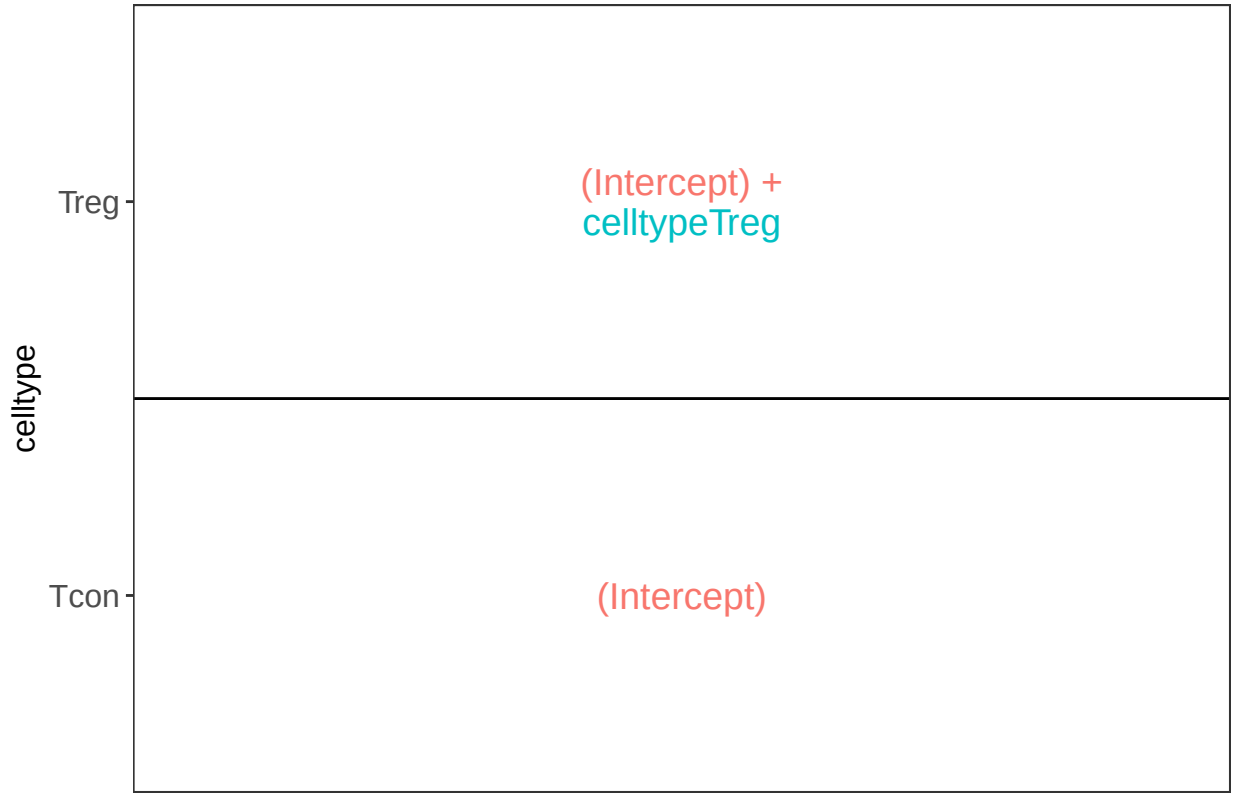
```
## [[1]]
```




Effect size in CRD

```
VisualizeDesign(colData(pe2),~ celltype)$plotlist
```

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

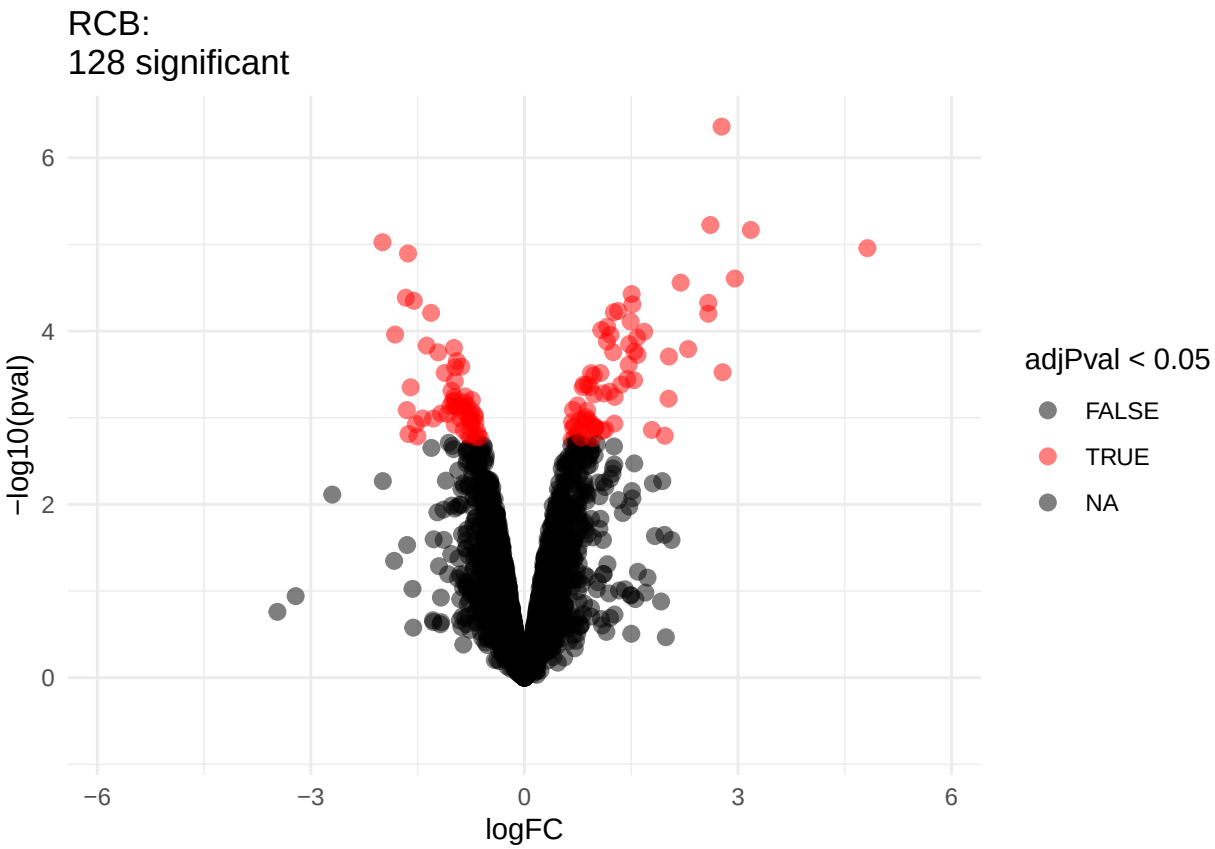


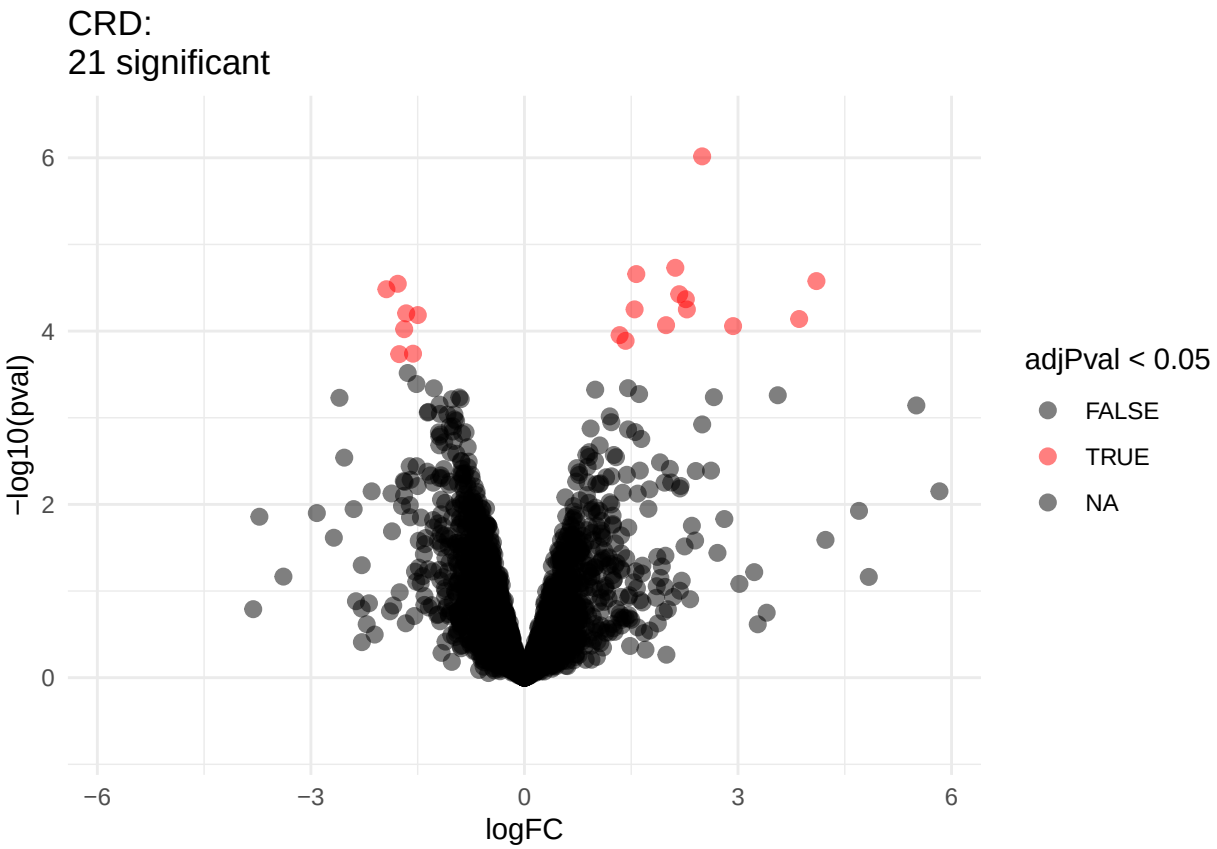

Click to see code for statistical inference

```
L <- makeContrast("celltypeTreg = 0", parameterNames = c("celltypeTreg"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L)
pe2 <- hypothesisTest(object = pe2, i = "protein", contrast = L)
```

#### 2.4.4 Comparison of results

Click to see code


RCB:
128 significant
6
4
2
0
−log10(pval)
−6
−3
0
3
6
logFC
adjPval < 0.05
FALSE
TRUE
NA



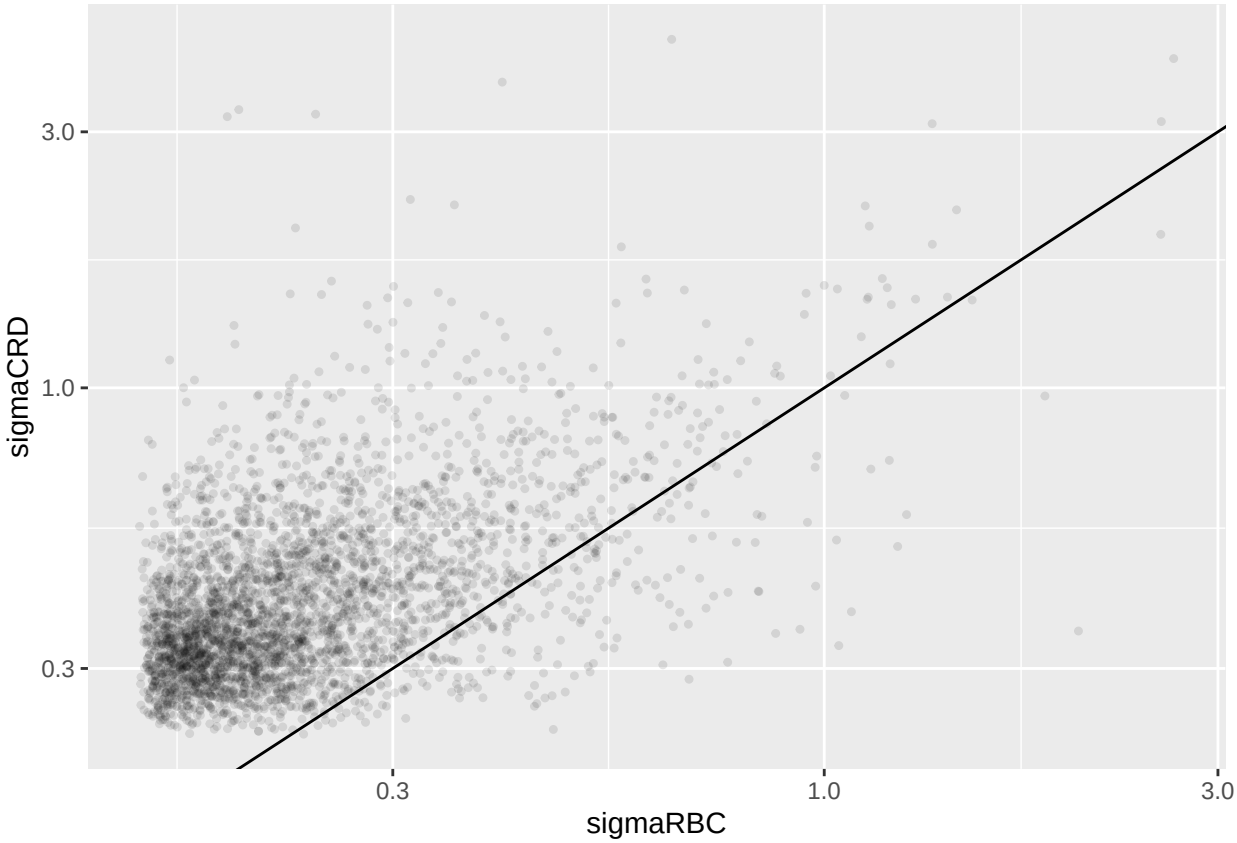

### 2.4.5 Comparison of standard deviation

Click to see code

```
accessions <- rownames(pe[["protein"]])[rownames(pe[["protein"]])%in%rownames(pe2[["protein"]])]
dat <- data.frame(
sigmaRBC = sapply(rowData(pe[["protein"]])$msqrobModels[accessions], getSigmaPosterior),
sigmaCRD <- sapply(rowData(pe2[["protein"]])$msqrobModels[accessions], getSigmaPosterior)
)

plotRBCvsCRD <- ggplot(data = dat, aes(sigmaRBC, sigmaCRD)) +
    geom_point(alpha = 0.1, shape = 20) +
    scale_x_log10() +
    scale_y_log10() +
    geom_abline(intercept=0,slope=1)
```

```
  plotRBCvsCRD
```

```
## Warning: Removed 743 rows containing missing values (geom_point).
```




- We clearly observe that the standard deviation of the protein expression in the RCB is smaller for the majority of the proteins than that obtained with the CRD
- Why are some of the standard deviations for the RCB with the correct analysis larger than than of the RCB with the incorrect analysis that ignored the mouse blocking factor?
- Can you think of a reason why it would not be useful to block on a particular factor?

# 3 Software & code

- Our R/Bioconductor package msqrob2 can be used in R markdown scripts or with a GUI/shinyApp in the msqrob2gui package.
- The GUI is intended as a introduction to the key concepts of proteomics data analysis for users who have no experience in R.
- However, learning how to code data analyses in R markdown scripts is key for open en reproducible science and for reporting your proteomics data analyses and interpretation in a reproducible way.
- More information on our tools can be found in our papers (L. J. Goeminne, Gevaert, and Clement 2016), (L. J. E. Goeminne et al. 2020) and (Sticker et al. 2020). Please refer to our work when using our tools.
- Clips on the code on importing the data and preprocessing can be found in Part I Preprocessing
- A clip on the code for modelling and statistical inference with msqrob2 is included below

# References

Goeminne, L. J. E., A. Sticker, L. Martens, K. Gevaert, and L. Clement. 2020. "MSqRob Takes the Missing Hurdle: Uniting Intensity- and Count-Based Proteomics." *Anal Chem* 92 (9): 6278–87.

Goeminne, L. J., K. Gevaert, and L. Clement. 2016. "Peptide-level Robust Ridge Regression Improves Estimation, Sensitivity, and Specificity in Data-dependent Quantitative Label-free Shotgun Proteomics." *Mol Cell Proteomics* 15 (2): 657–68.

Sticker, A., L. Goeminne, L. Martens, and L. Clement. 2020. "Robust Summarization and Inference in Proteome-wide Label-free Quantification." *Mol Cell Proteomics* 19 (7): 1209–19.