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

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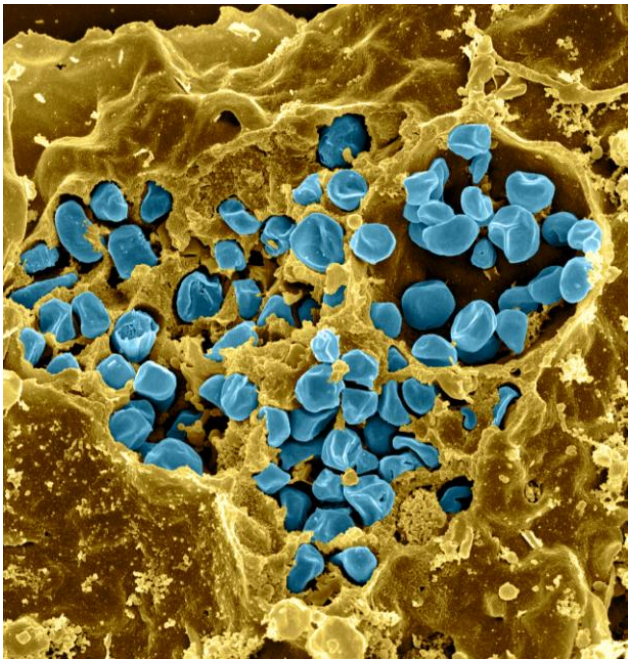
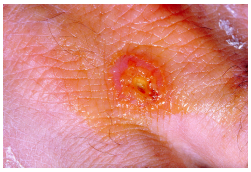
Note, that the R-code is included for learners who are aiming to develop R/markdown scripts to automate their quantitative proteomics data analyses. According to the target audience of the course we either work with a graphical user interface (GUI) in a R/shiny App msqrob2gui (e.g. Proteomics Bioinformatics 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).

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/PDA20/data/quantification/francisella/pept
```

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[["peptideLog"]] <-
 pe[["peptideLog"]][rowData(pe[["peptideLog"]])$Proteins
 %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$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[["peptideLog"]] <- pe[["peptideLog"]][rowData(pe[["peptideLog"]])$Reverse != "+", ]
pe[["peptideLog"]] <- pe[["peptideLog"]][rowData(pe[["peptideLog"]])$
    Contaminant != "+", ]
```

- Drop peptides that were only identified in one sample

```
pe[["peptideLog"]] <- pe[["peptideLog"]][rowData(pe[["peptideLog"]])$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.

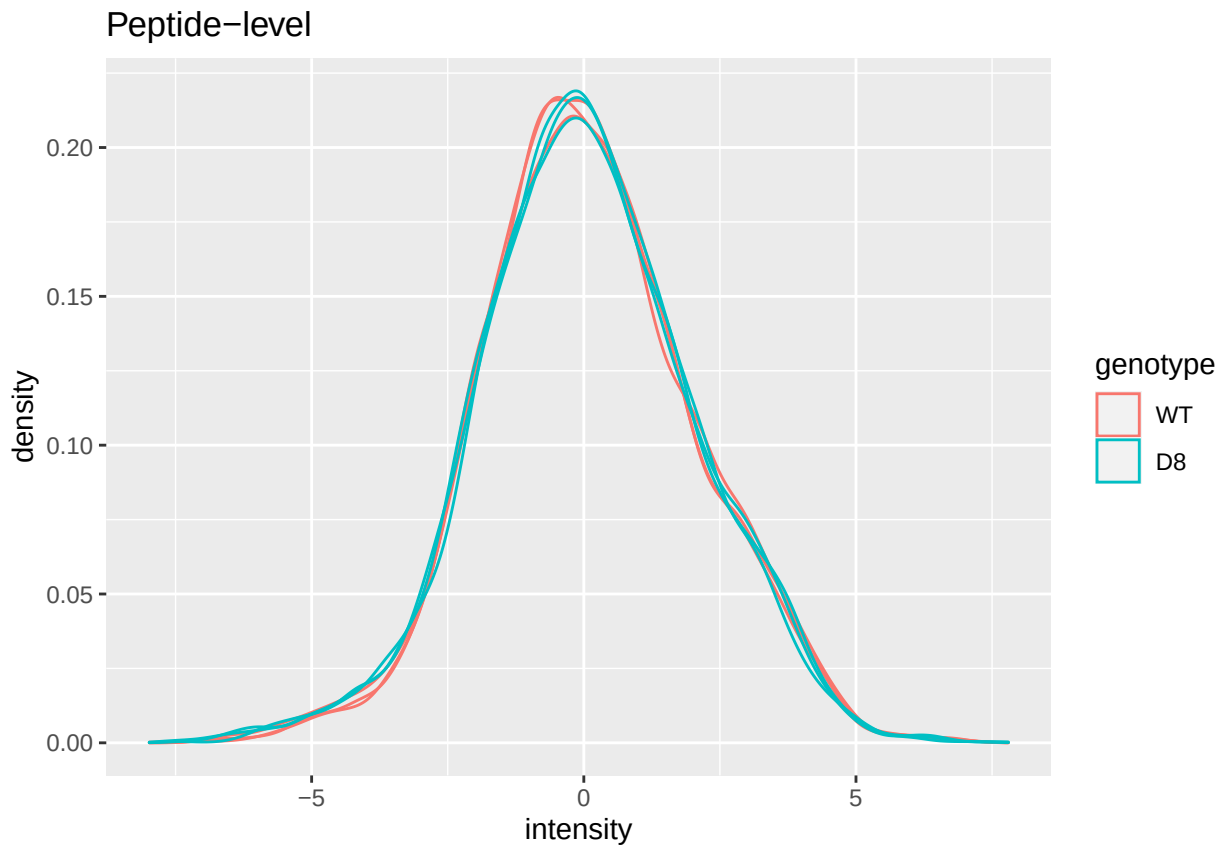
```
pe <- aggregateFeatures(pe,
    i = "peptideNorm",
    fcol = "Proteins",
    na.rm = TRUE,
    name = "protein")
```

```
## 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

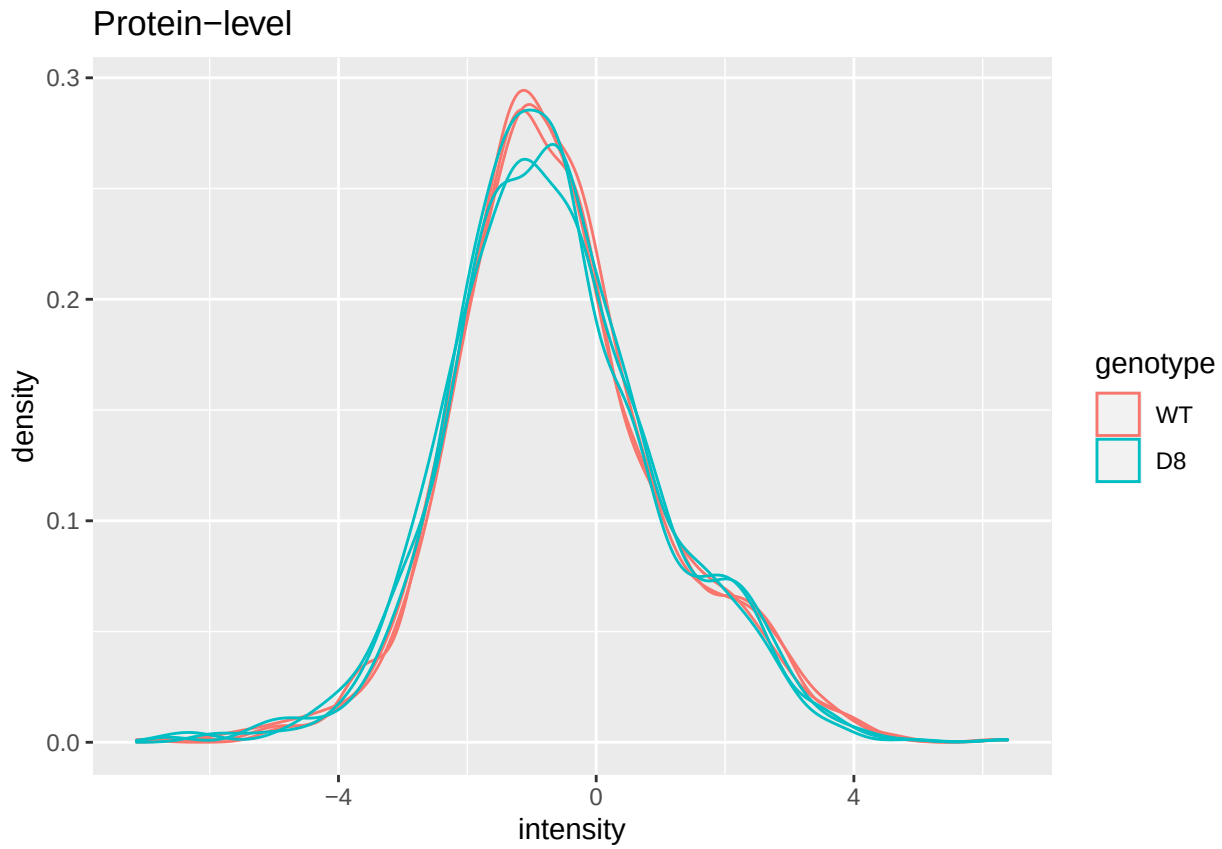
```
pe[["peptideNorm"]] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(genotype = colData(pe)[sample,"genotype"]) %>%
  ggplot(aes(x = intensity,group = sample,color = genotype)) +
    geom_density() +
    ggtitle("Peptide-level")
```

```
## 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

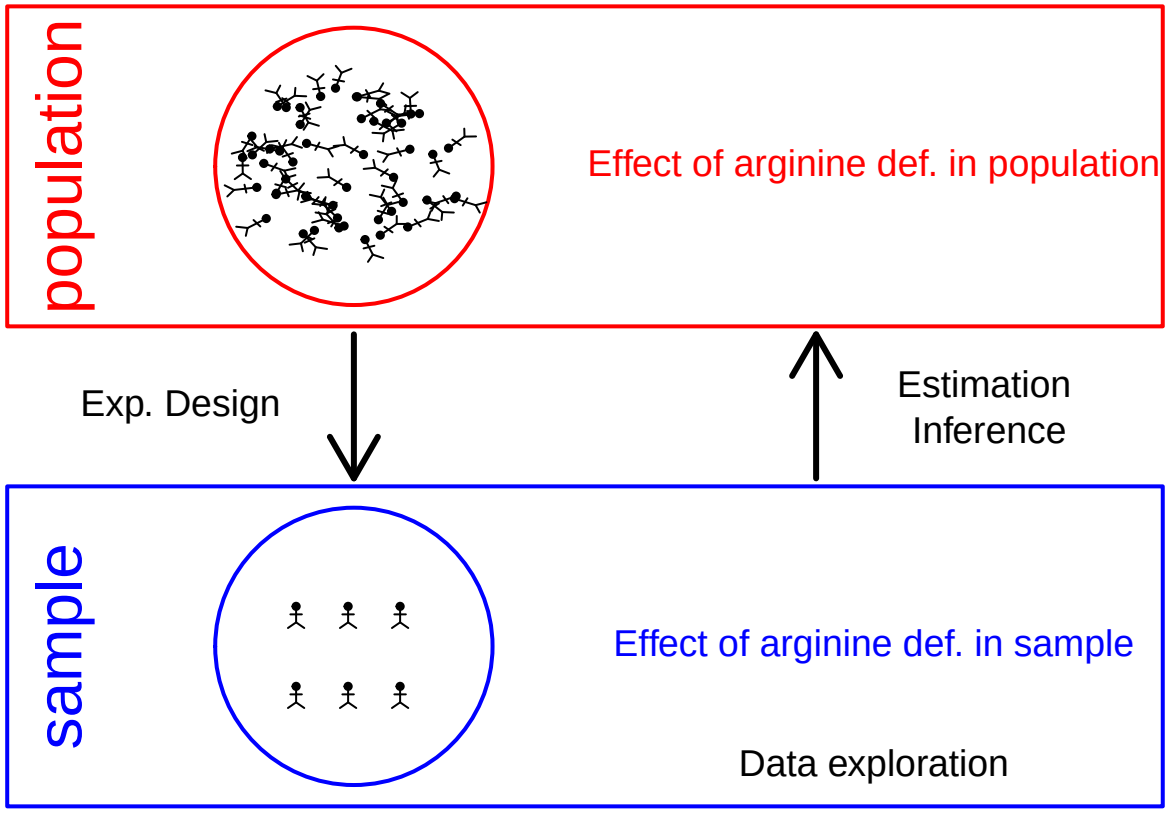
1.3.2 Summarized intensity matrix

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

	Intensity.1WT_20__2h__n1__3	Intensity.1WT_20__2h__n3__3	Intensity.1WT_20__2h__n4__3	Intensity.3D8_20__2h__n3__3	Intensity.3D8_20__2h__n4__3	Intensity.3D8_20__2h__n5__3
WP_003013732	7.2748775	-0.0856247	0.1595370	-0.2809009	0.0035526	0.0567110
WP_003013860	NA	NA	-0.2512039	NA	NA	-0.4865646
WP_003013908	9.0851118	-0.8161658	-0.7557906	-0.4591476	-0.5449424	-0.4962482
WP_003014068	6.6495386	0.8522239	1.1344852	0.5459176	0.9187714	0.5974741
WP_003014272	8.2630863	-1.0430741	-0.8091715	-1.1743951	-1.1924725	-1.2565893
WP_003014328	9.2051672	-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

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

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

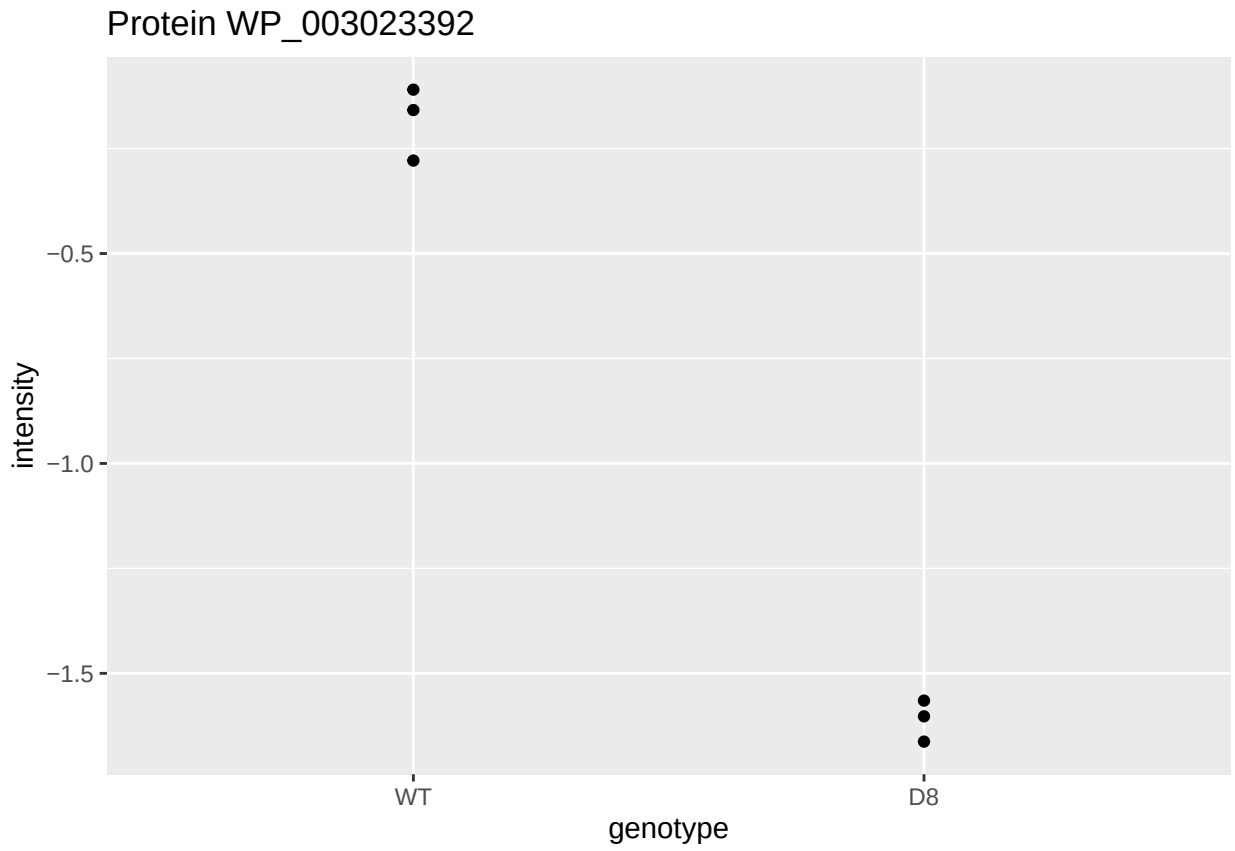
$$T_g = \frac{\widehat{\text{signal}}}{\widehat{\text{Noise}}}$$

If we can assume equal variance in both treatment groups:

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

```
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")
```



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

- 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 α we reject the null hypothesis. *We control the probability on a false positive result at the α-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 α? → 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 $1066 \times 0.05 = 53$.

→ 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 α:
$$p_{\text{adj}} = \min\left(p \times m, 1\right)$$

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 α

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):

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

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
```

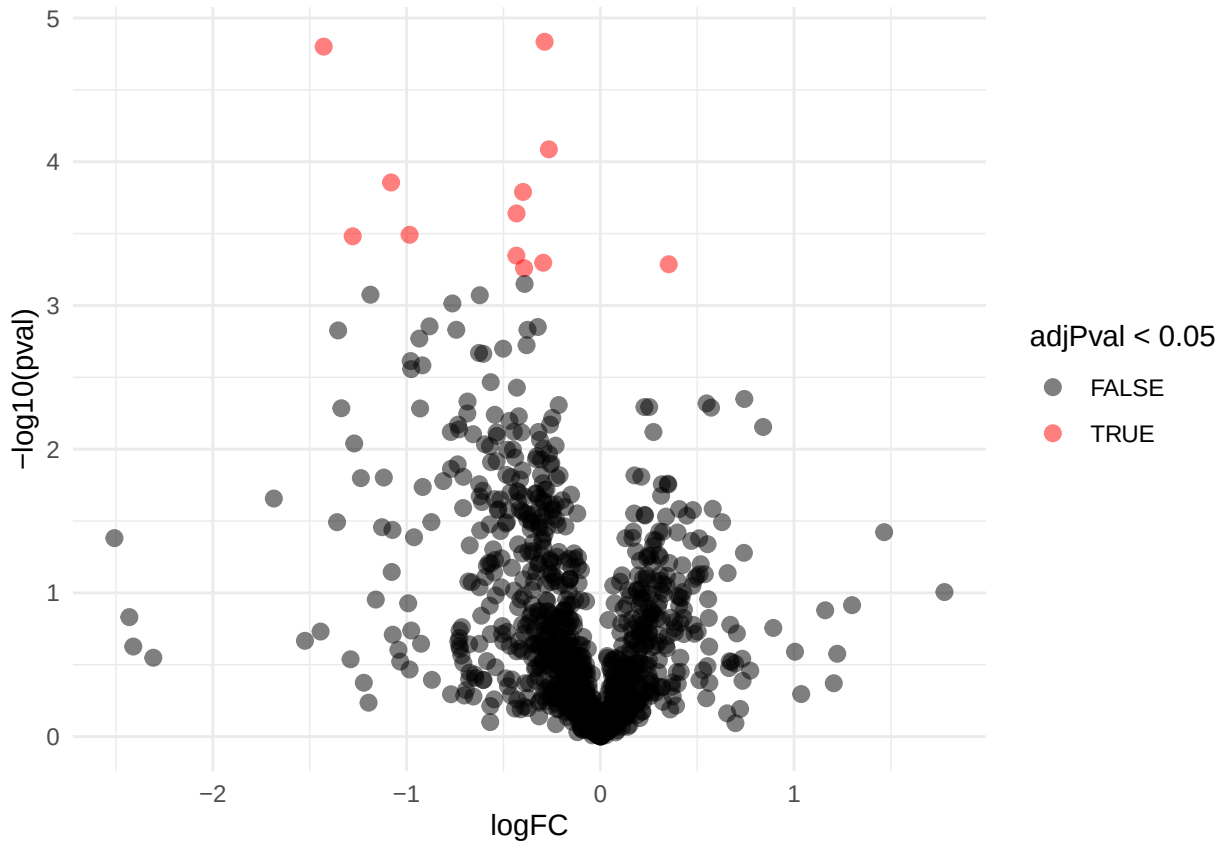
FWER: Bonferroni method:$\alpha_{\text{adj}} = \alpha/m = 0.05/1066 = 5 \times 10^{-5}$

	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
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

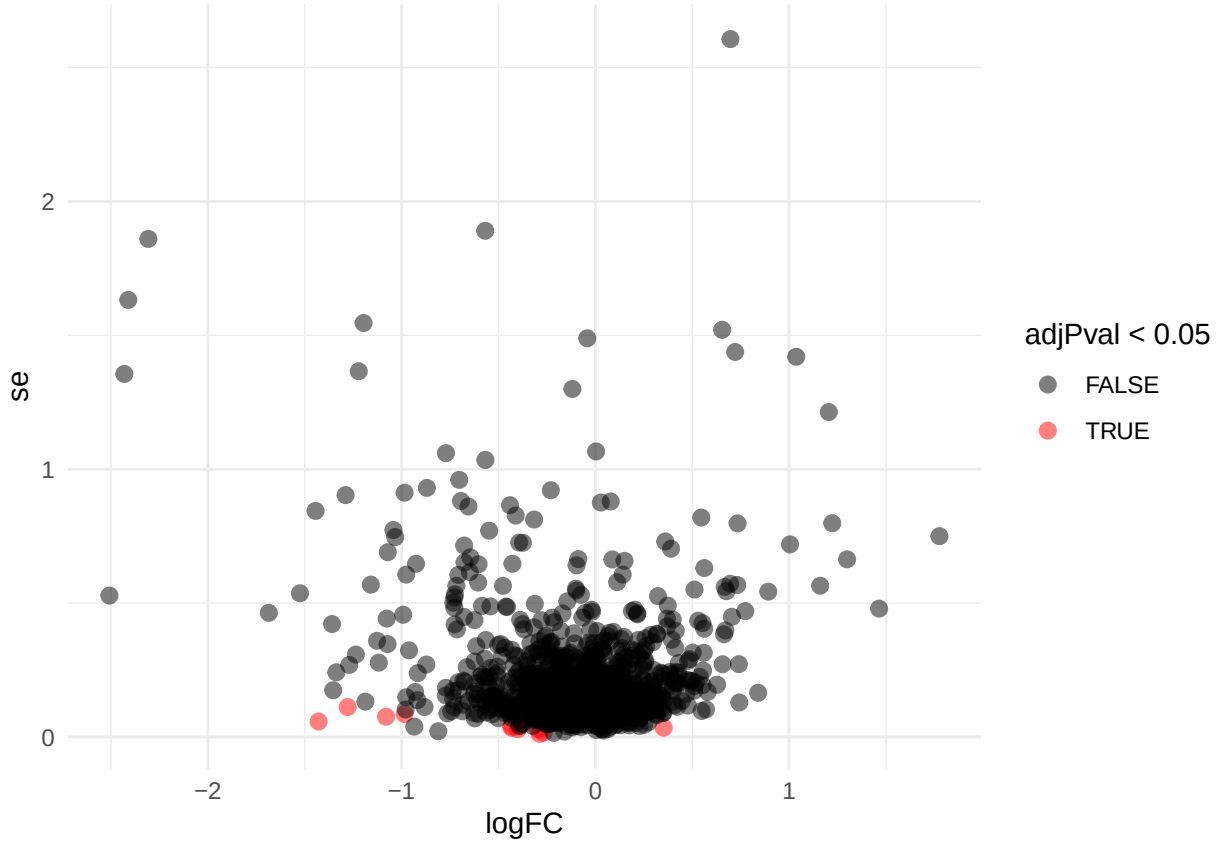
```
problemPlots <- list()
problemPlots[[1]] <- res %>%
  ggplot(aes(x = logFC, y = se, color = adjPval < 0.05)) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal()

for (i in 2:3)
{
problemPlots[[i]] <- colData(pe) %>%
```

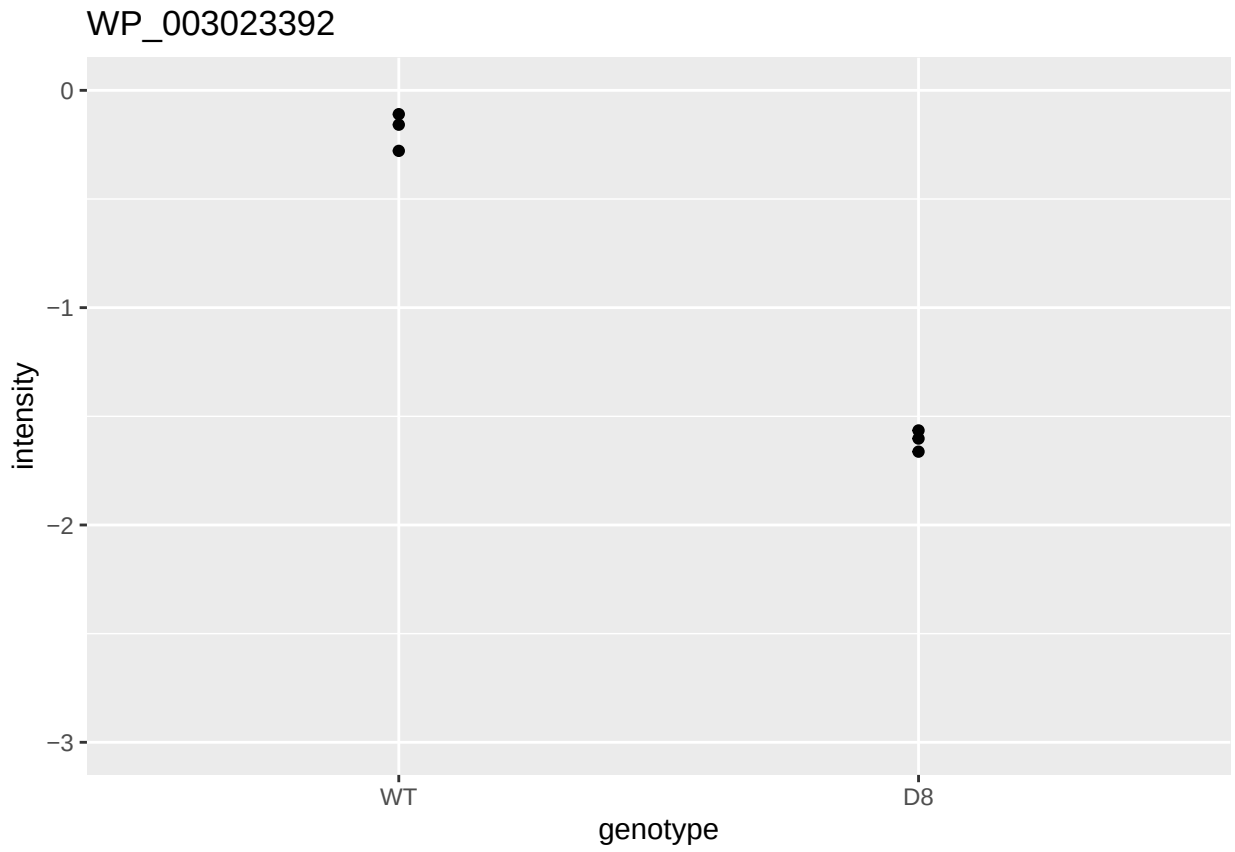
```
    as.data.frame %>%
    mutate(intensity = pe[["protein"]][rownames(res)[i],] %>%
             assay %>%
             c) %>%
    ggplot(aes(x=genotype,y=intensity)) +
    geom_point() +
    ylim(-3,0) +
    ggtitle(rownames(res)[i])
}
```

```
problemPlots
```

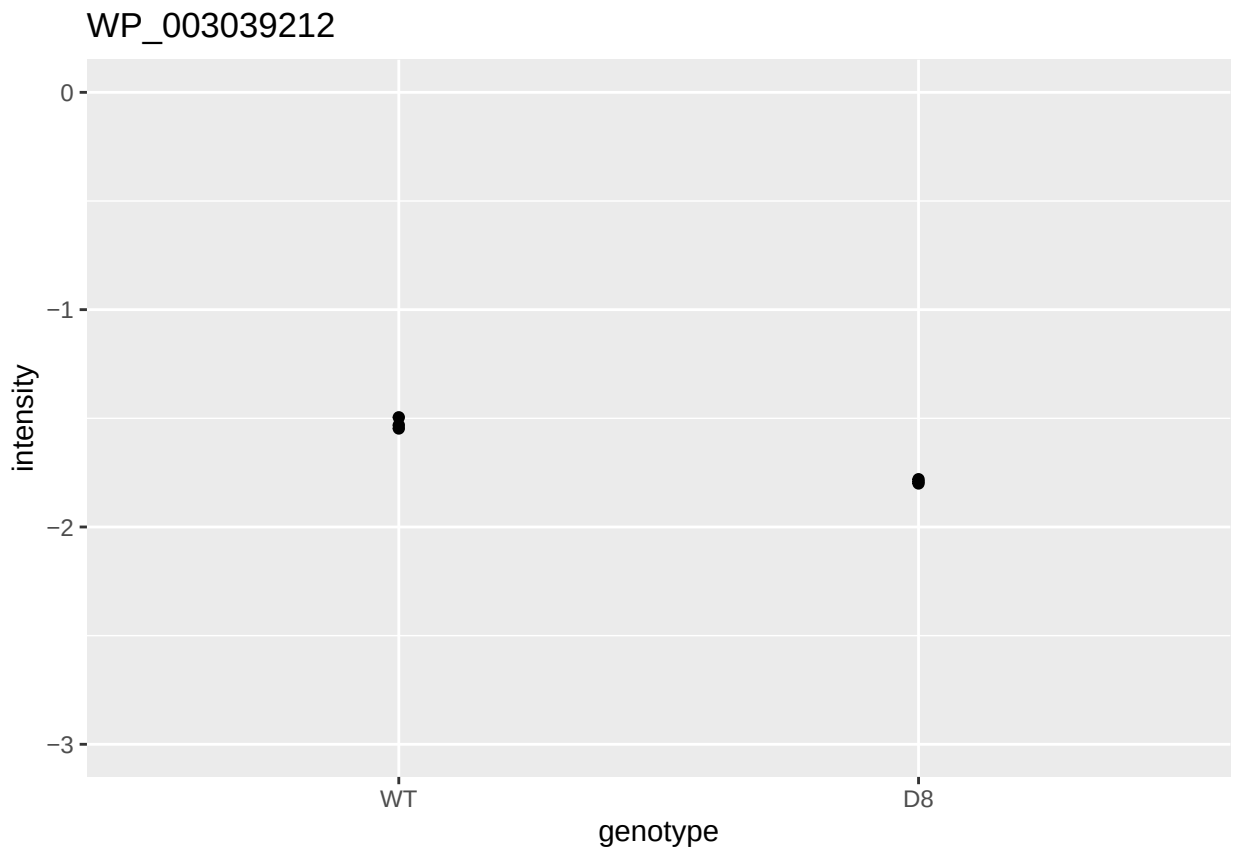
```
## [[1]]
```




```
##
## [[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.

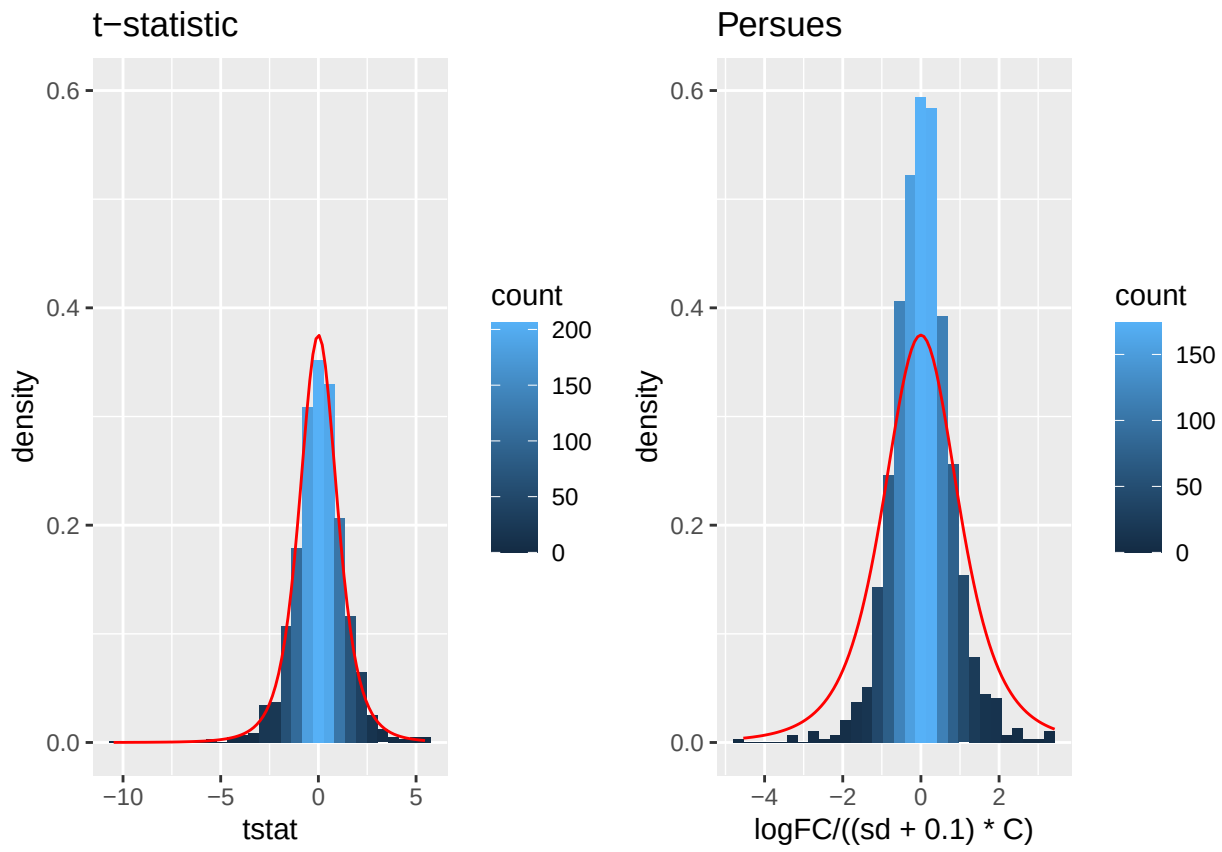
Click to see code

```
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("Persues")
```

```
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

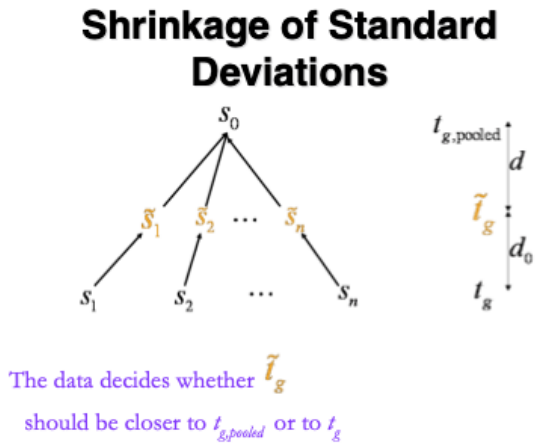


Figure courtesy to Rafael Irizarry

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

- **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

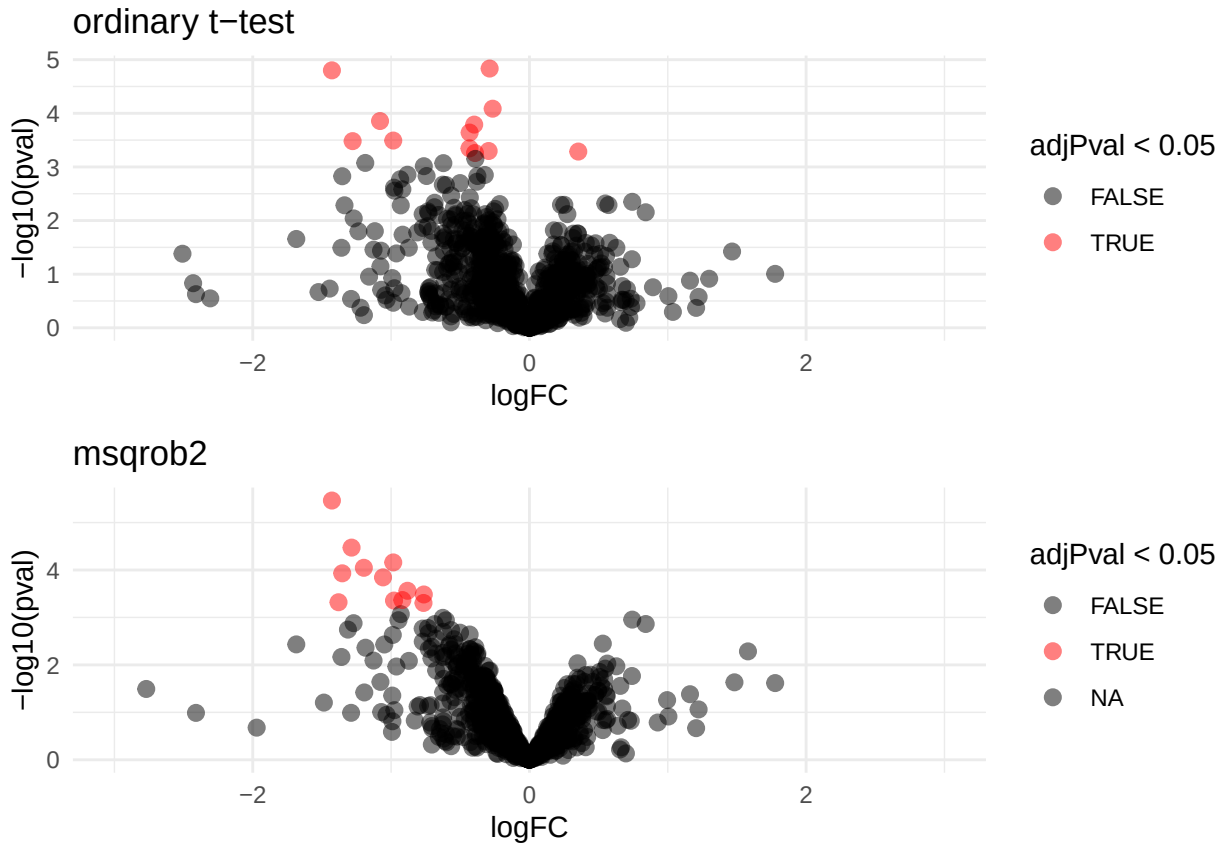
```
pe <- msqrob(object = pe, i = "protein", formula = ~genotype)
L <- makeContrast("genotypeD8 = 0", parameterNames = c("genotypeD8"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L)
```

```
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).
```

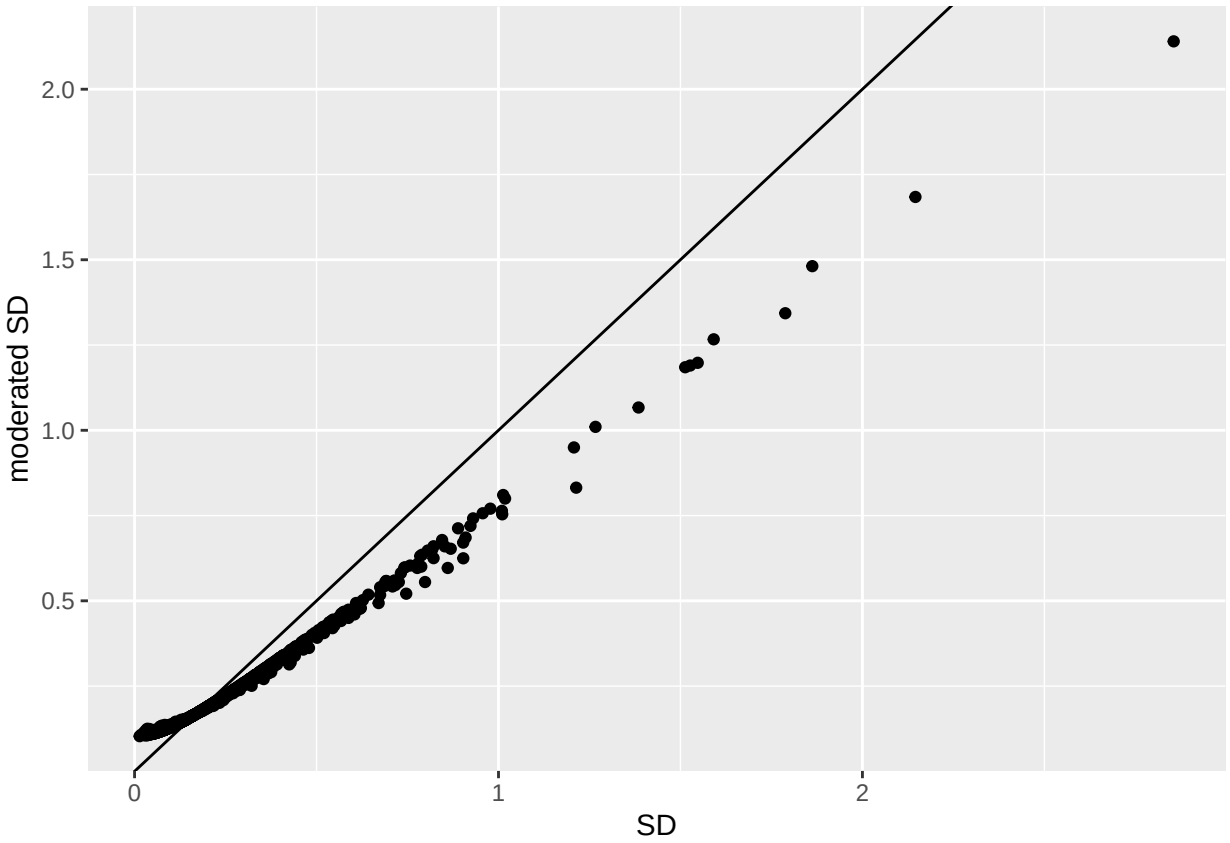


- The volcano plot opens up when using the EB variance estimator
- Borrowing strength to estimate the variance using empirical Bayes solves the issue of returning proteins with a low fold change as significant due to a low variance.

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 = )
```

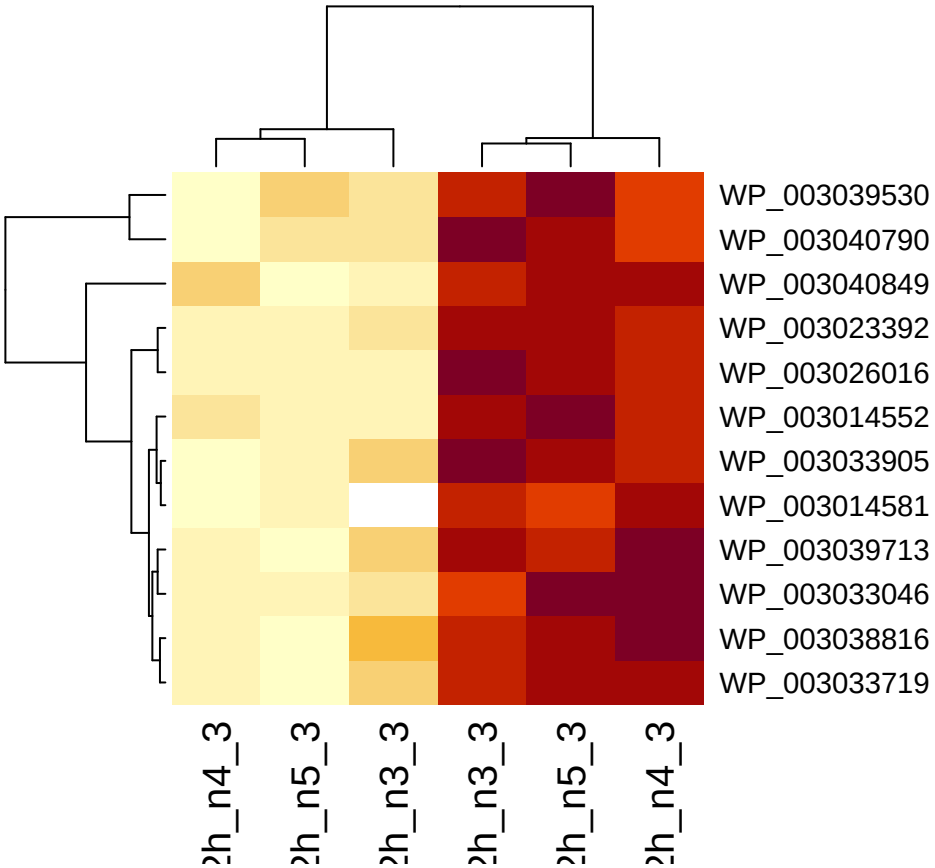
```
## Warning: Removed 109 rows containing missing values (geom_point).
```



- 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, ])
```

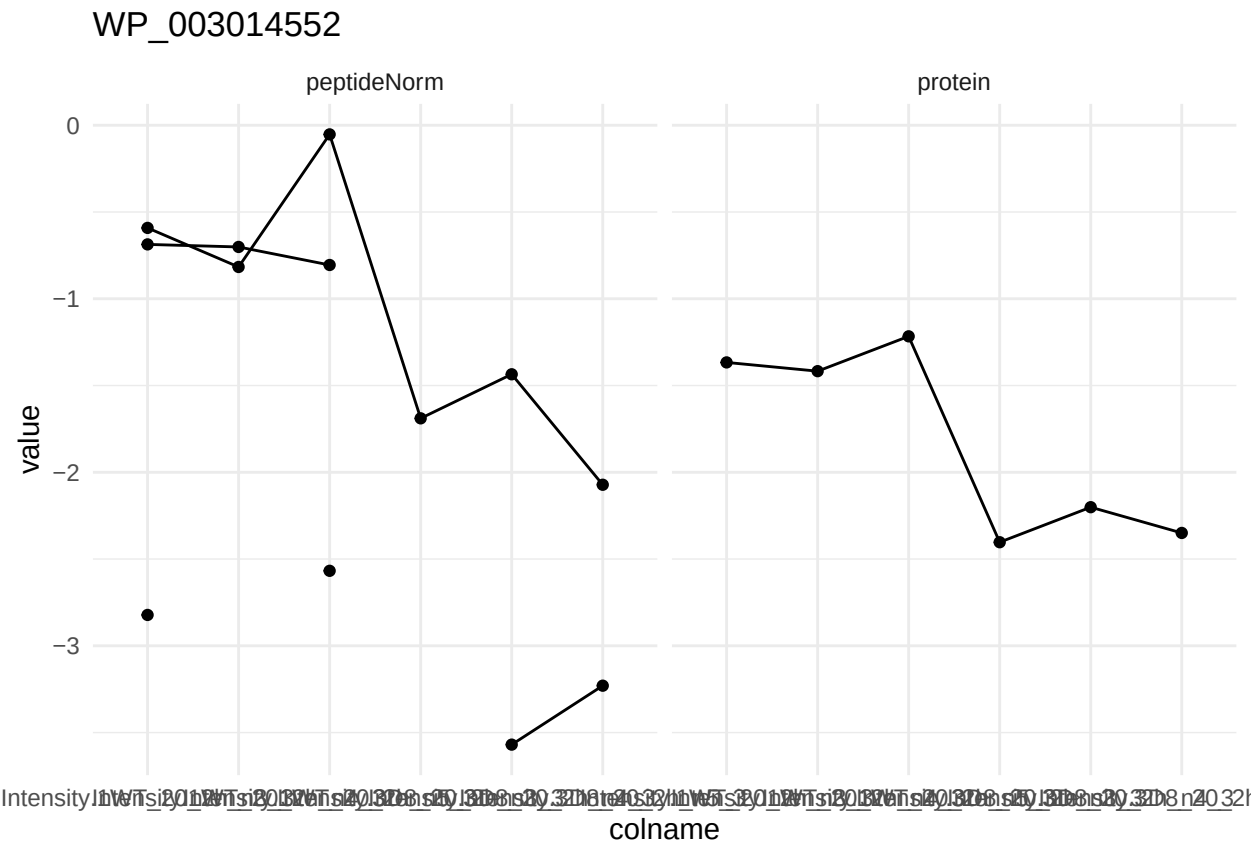


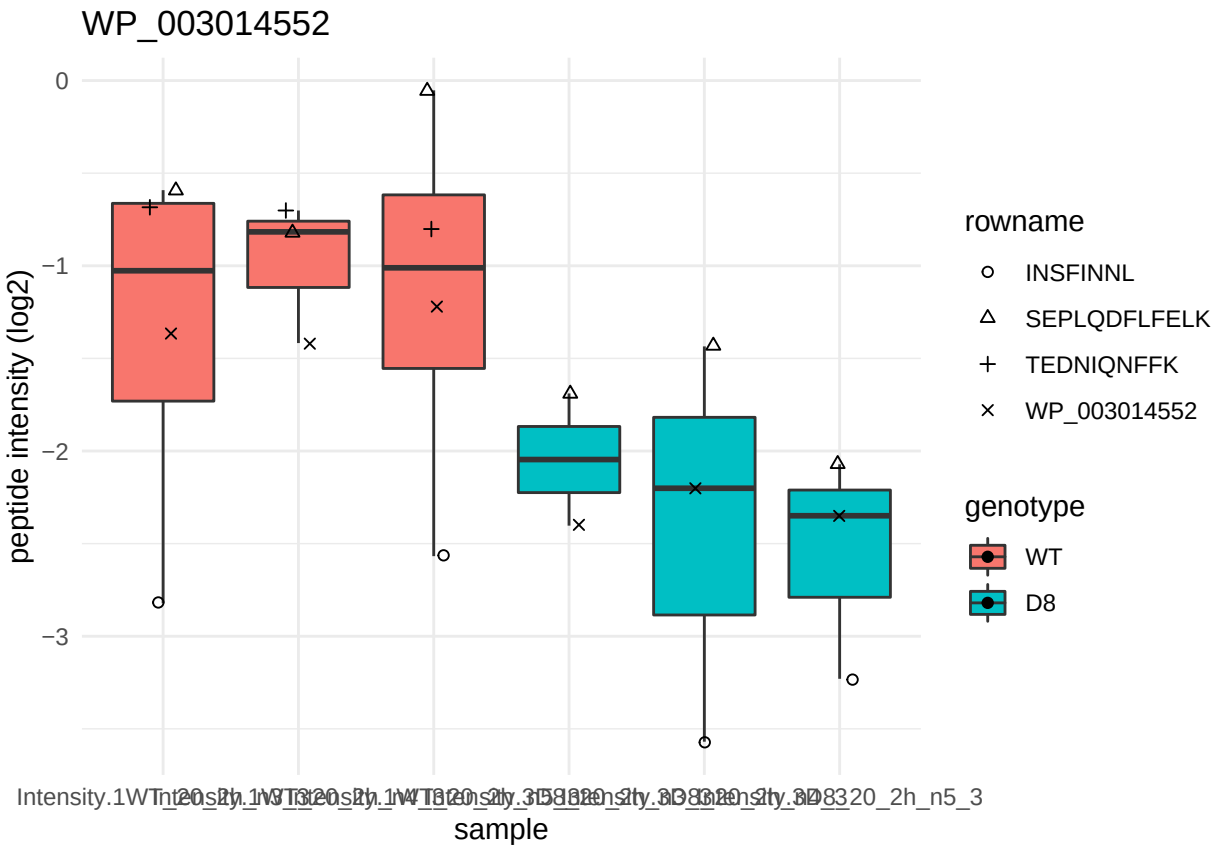

```
for (protName in sigNames)
    {
        pePlot <- pe[protName, , c("peptideNorm", "protein")]
        pePlotDf <- data.frame(longFormat(pePlot))
        pePlotDf$assay <- factor(pePlotDf$assay,
            levels = c("peptideNorm", "protein")
        )
        pePlotDf$genotype <- as.factor(colData(pePlot)[pePlotDf$colname,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

        # plotting
        p1 <- ggplot(
            data = pePlotDf,
            aes(x = colname, y = value, group = rowname)
        ) +
            geom_line() +
            geom_point() +
            theme_minimal() +
            facet_grid(~assay) +
            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_minimal()
        facet_grid(~assay)
        print(p2)
}
```

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−2
−3
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rowname
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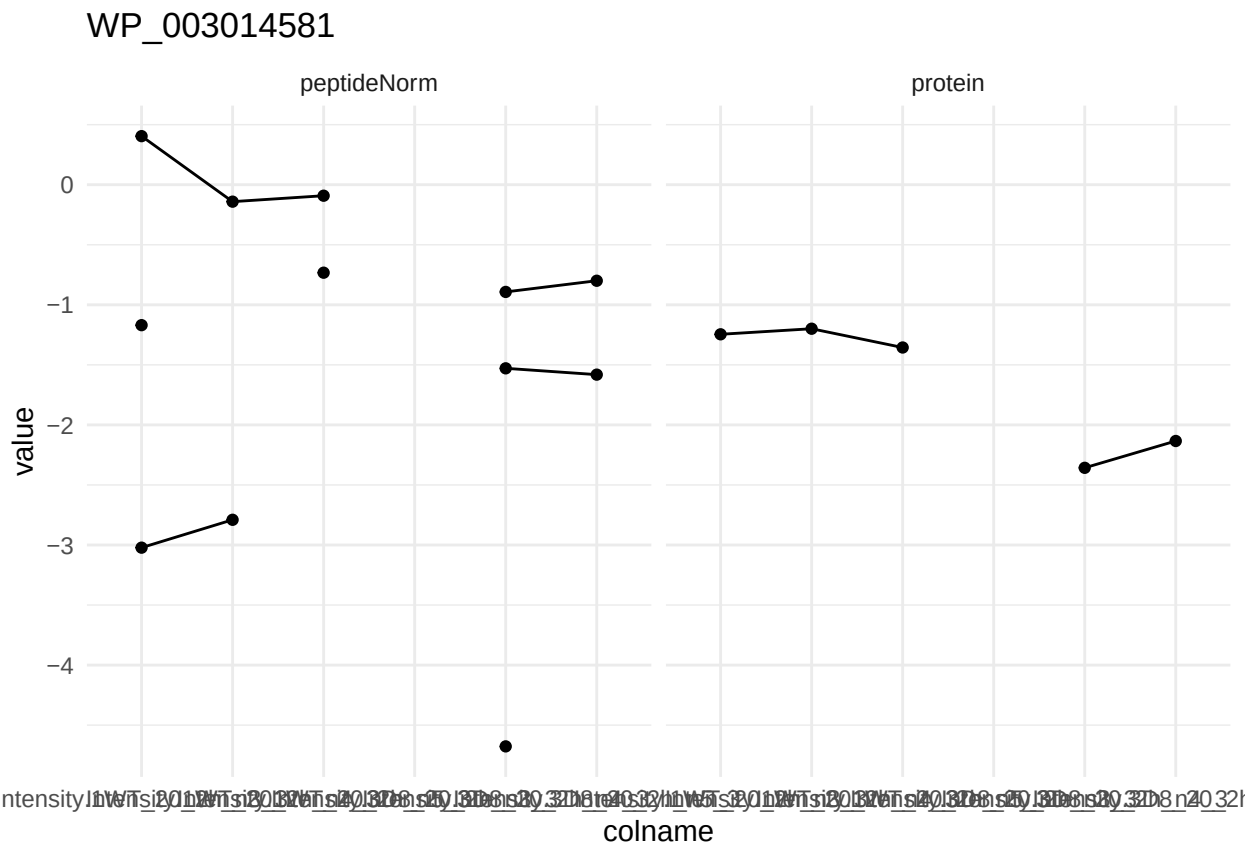
SEPLQDFLFELK
TEDNIQNFFK
WP_003014552
genotype
WT
D8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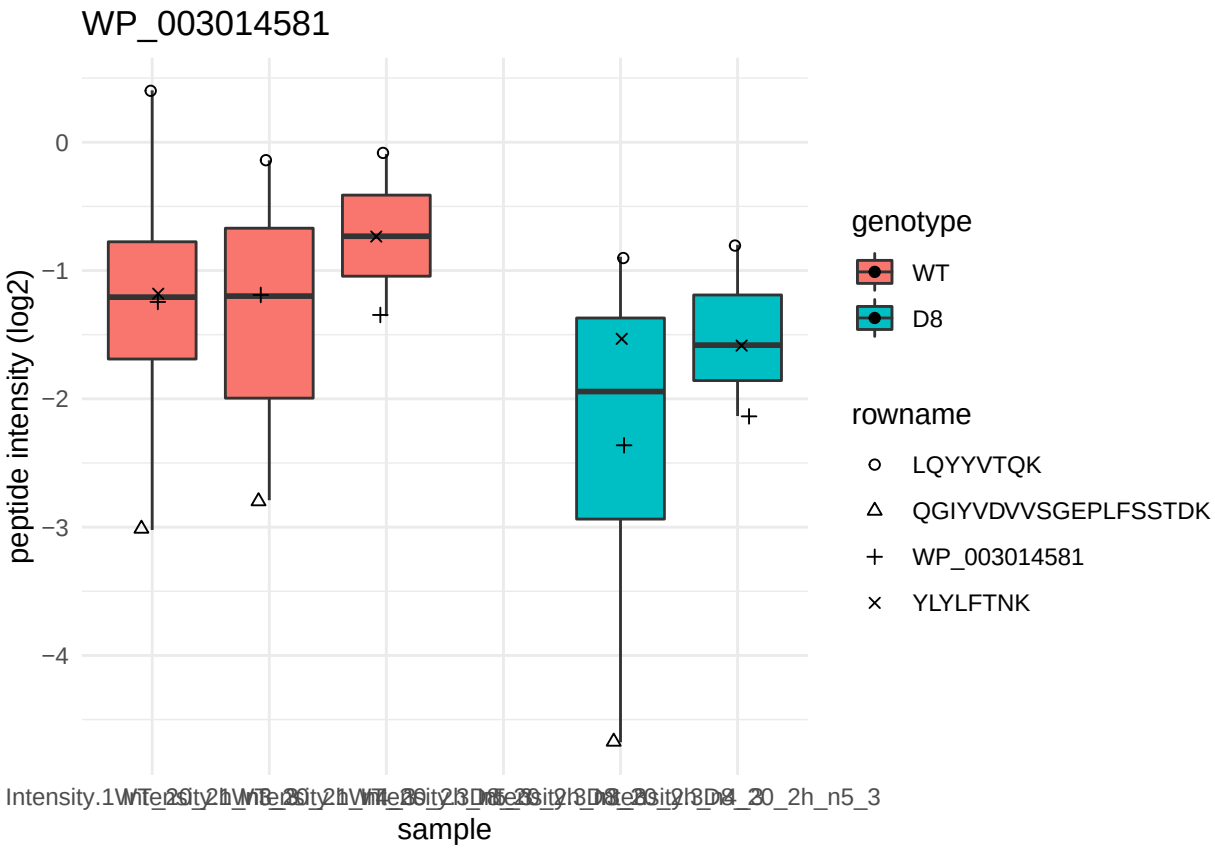
colname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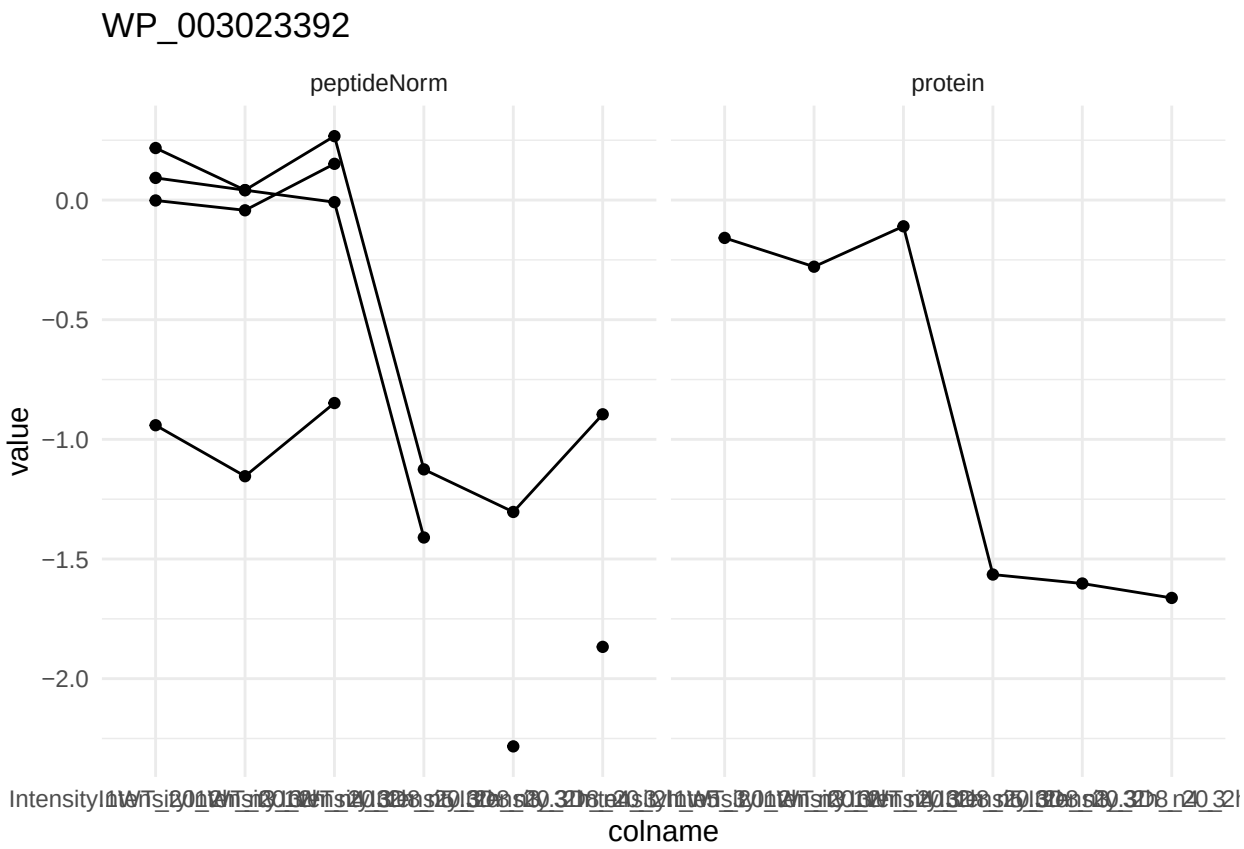
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−1.0
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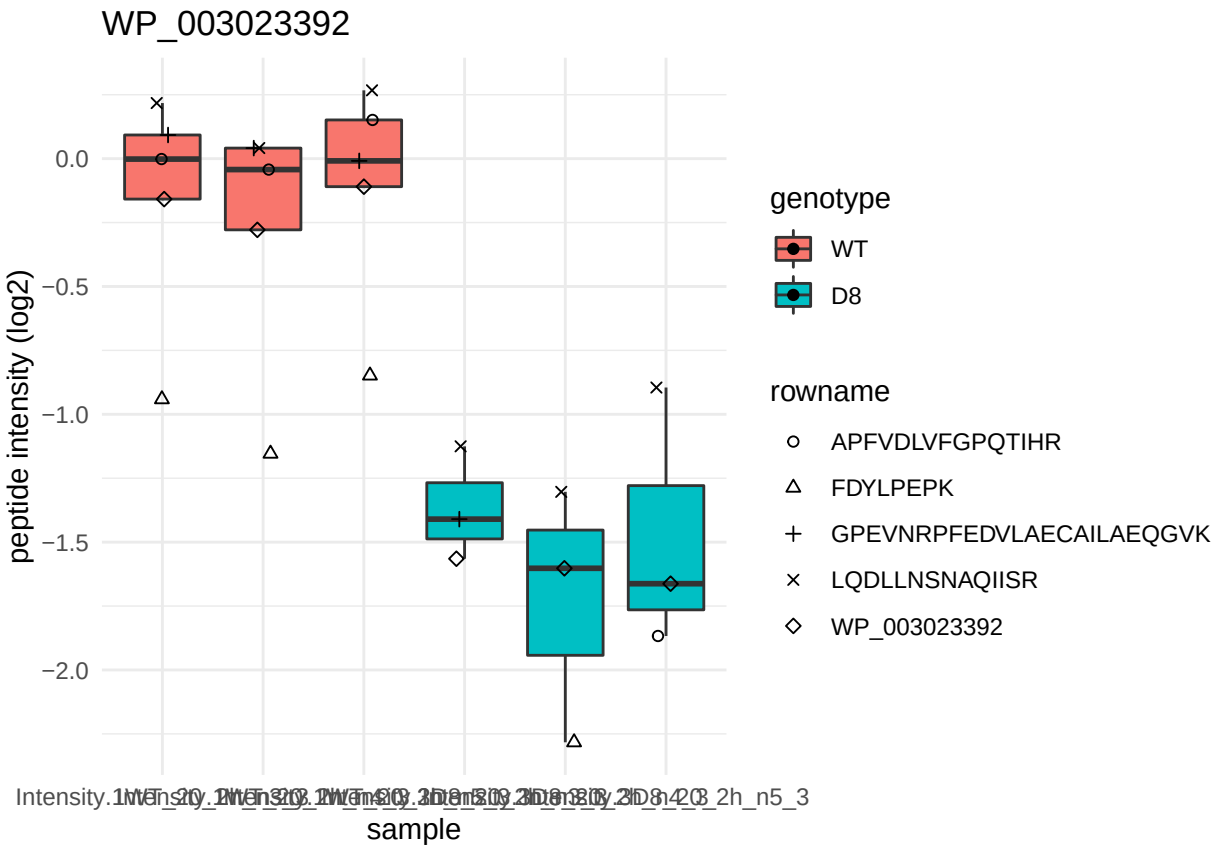
−2.0
value
colname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sample
genotype
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D8
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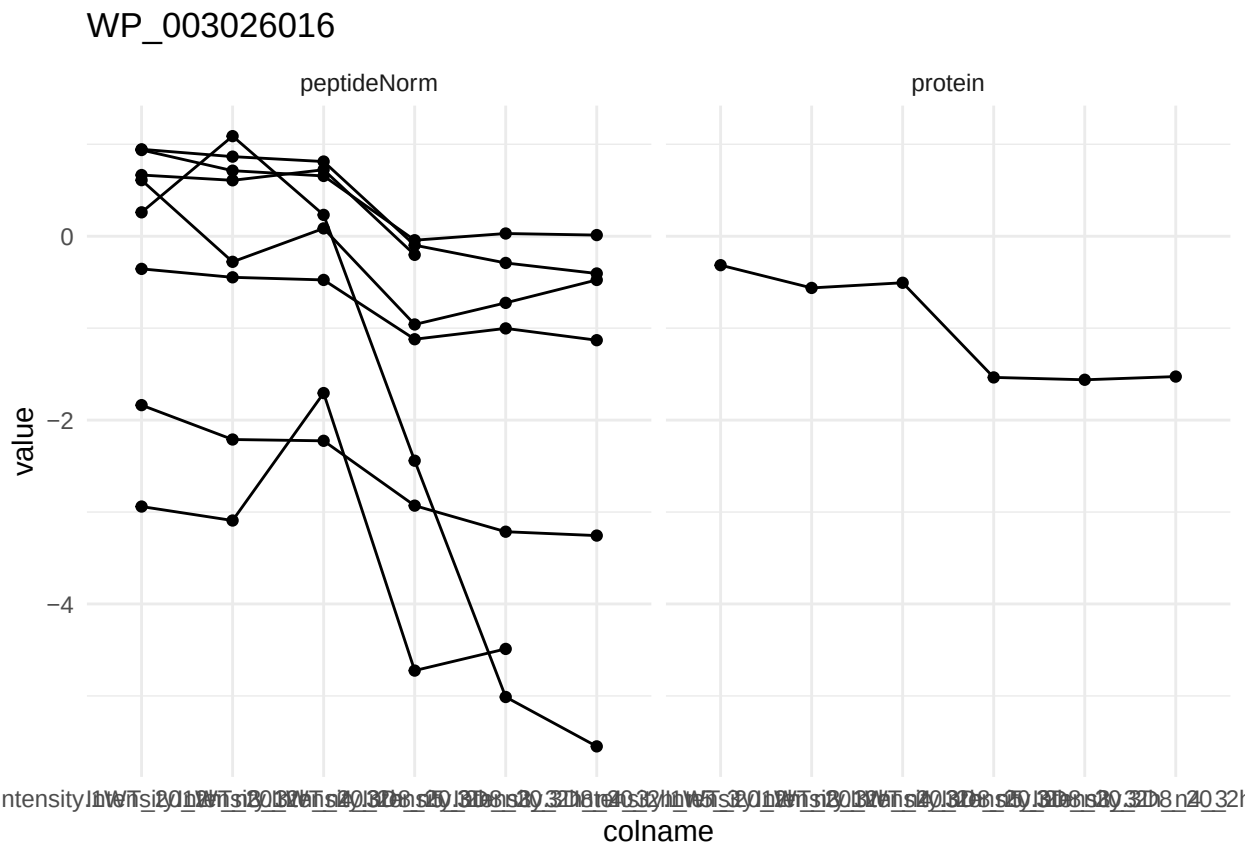
GPEVNRPFEDVLAECAILAEQGVK
LQDLLNSNAQIISR
WP_003023392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protein
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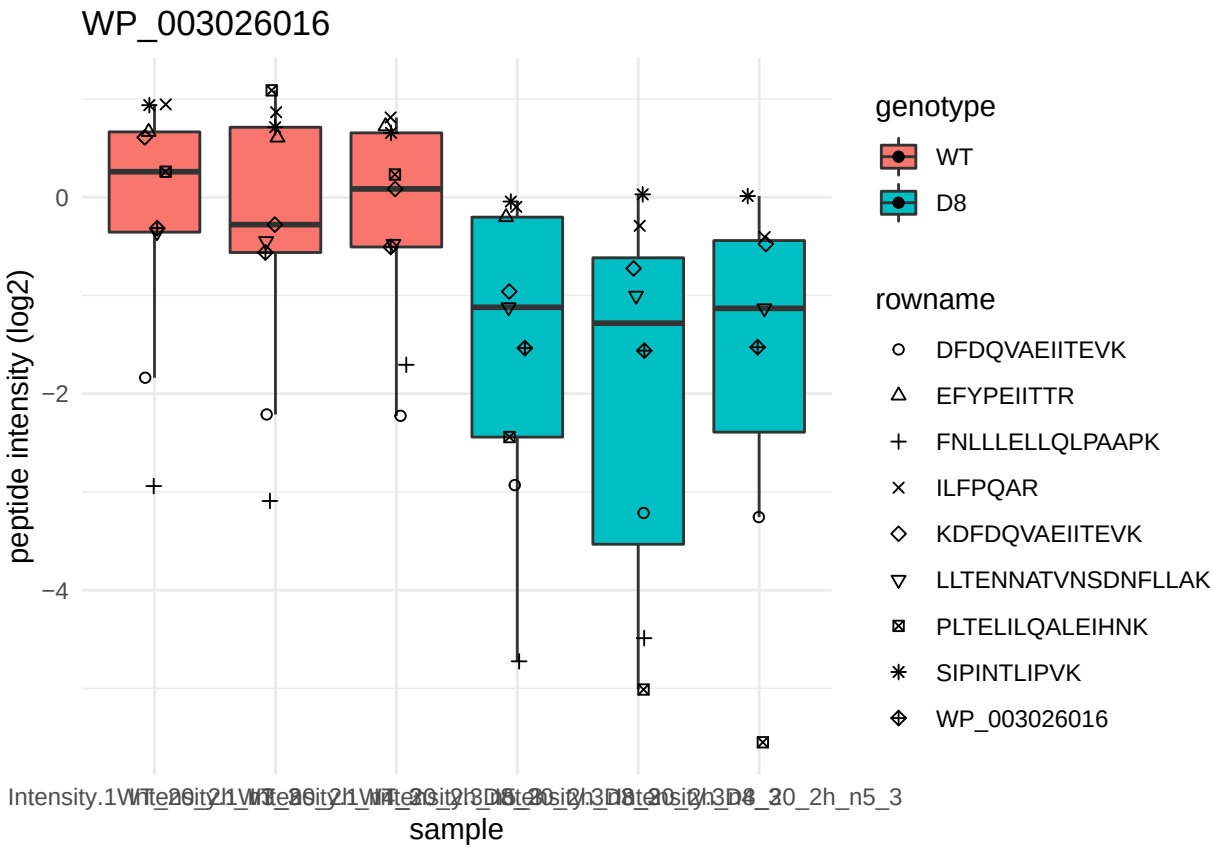
value
colname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−2
−4
sample
genotype
WT
D8
rowname
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EFYPEIITTR
FNLLLELLQLPAAPK
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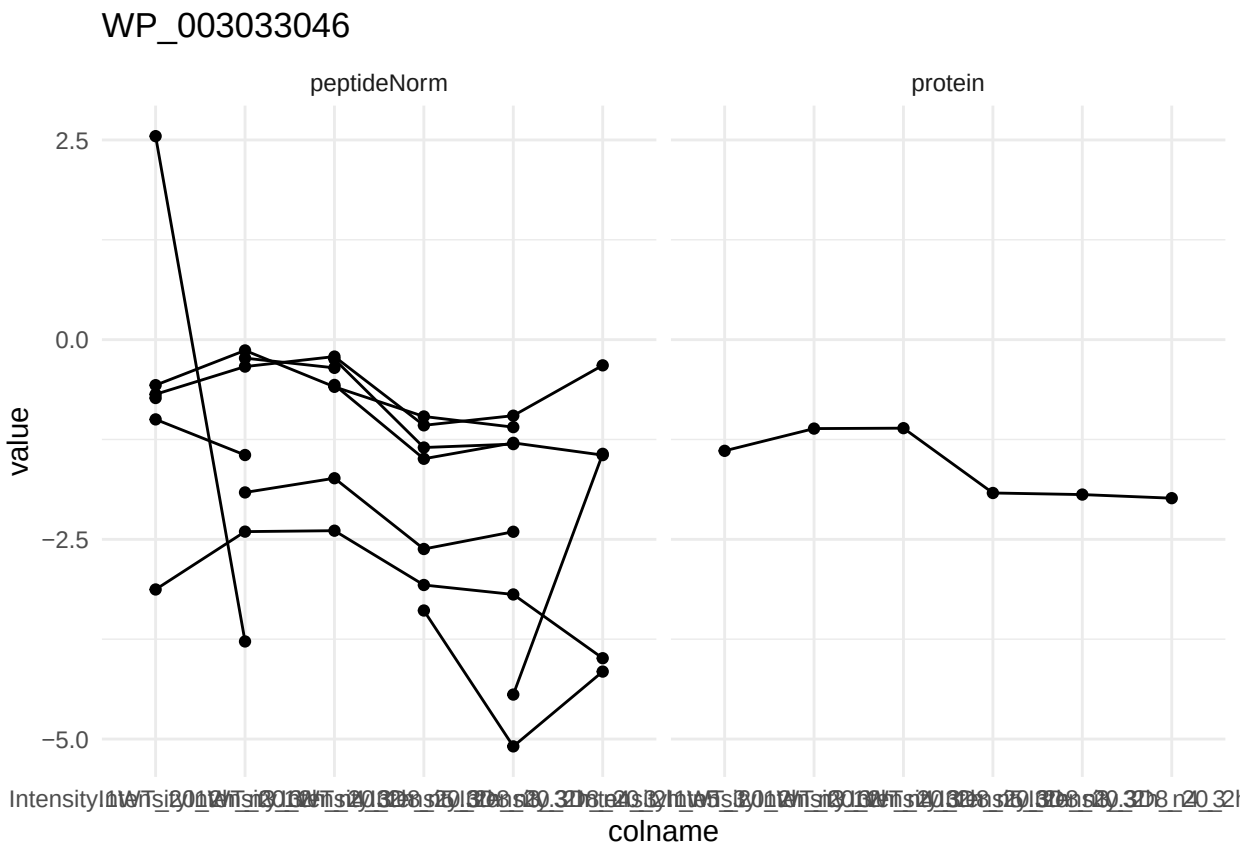
KDFDQVAEIITEVK
LLTENNATVNSDNFLLAK
PLTELILQALEIHNK
SIPINTLIPVK
WP_003026016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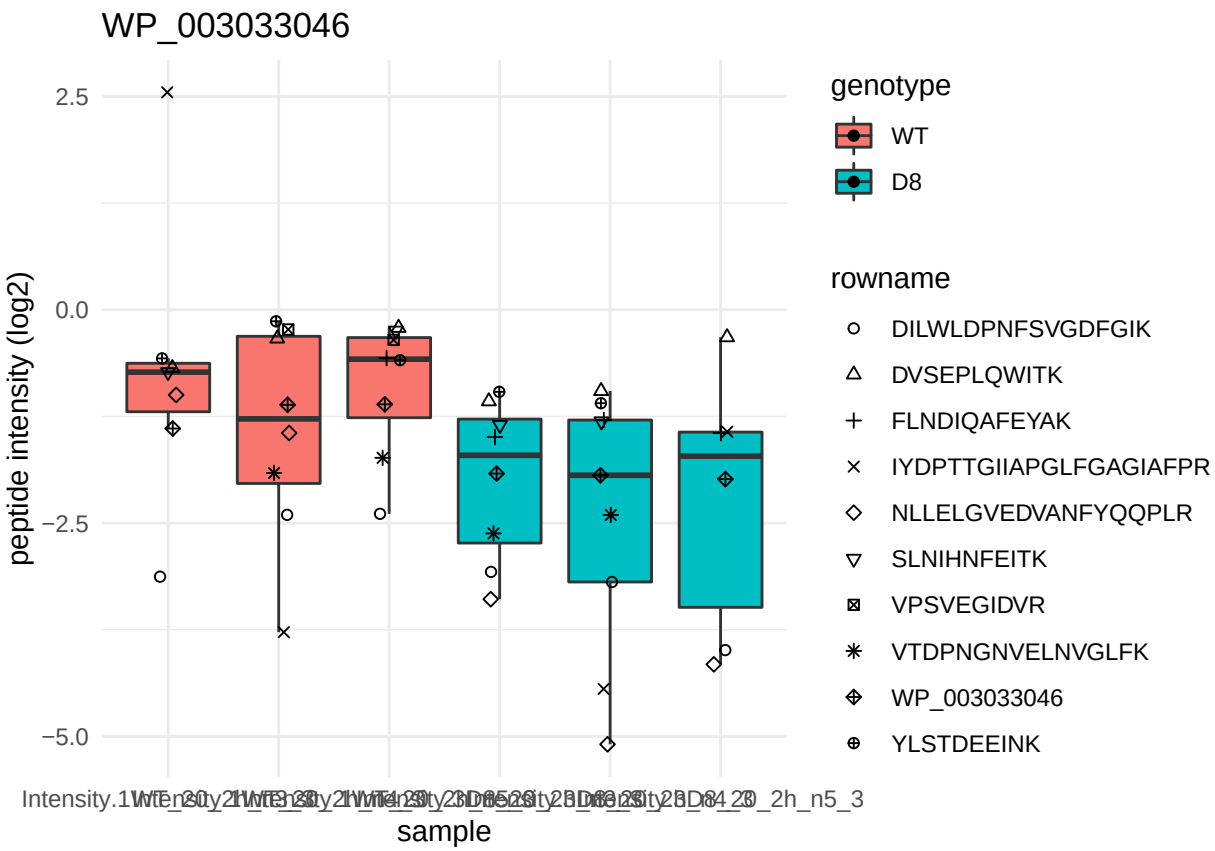
value
colname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−2.5
−5.0
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sample
genotype
WT
D8
rowname
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DVSEPLQWITK
FLNDIQAFEYAK
IYDPTTGIIAPGLFGAGIAFPR
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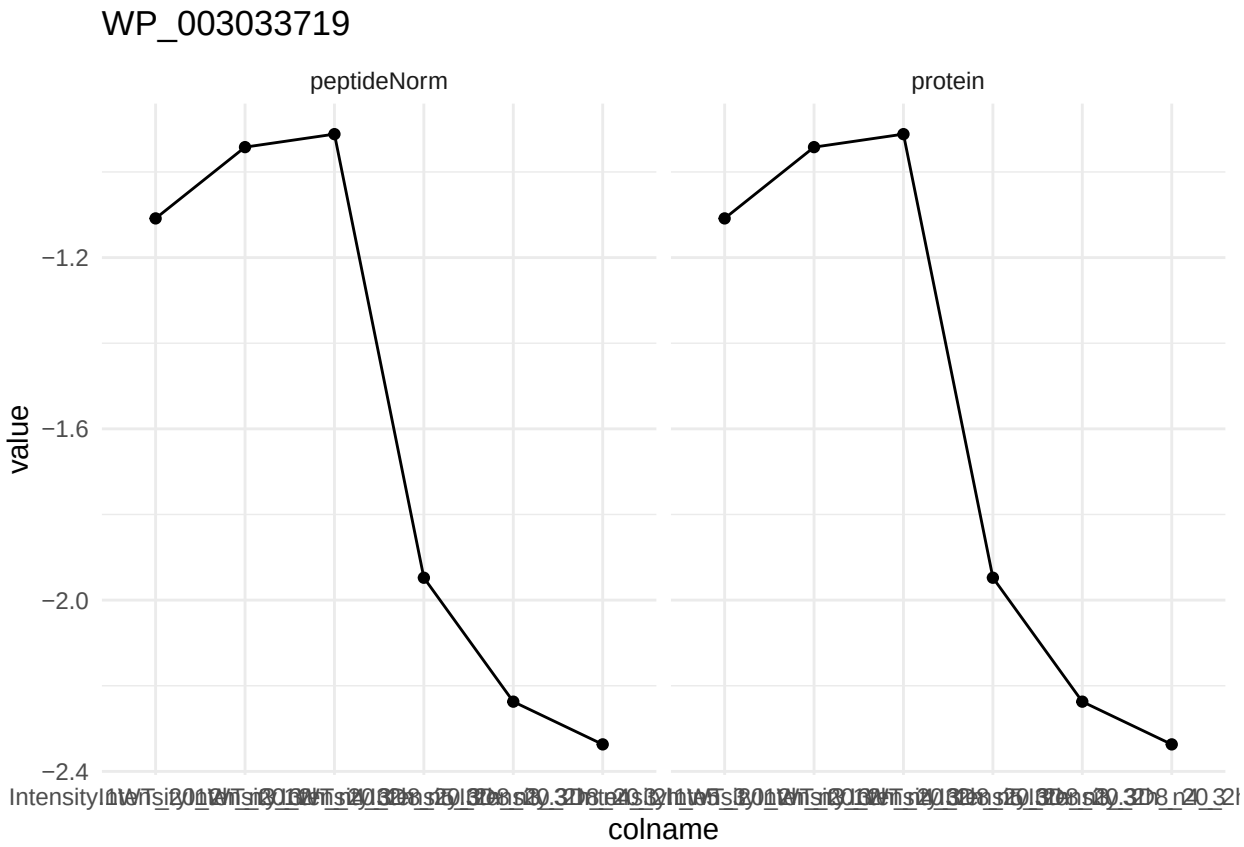
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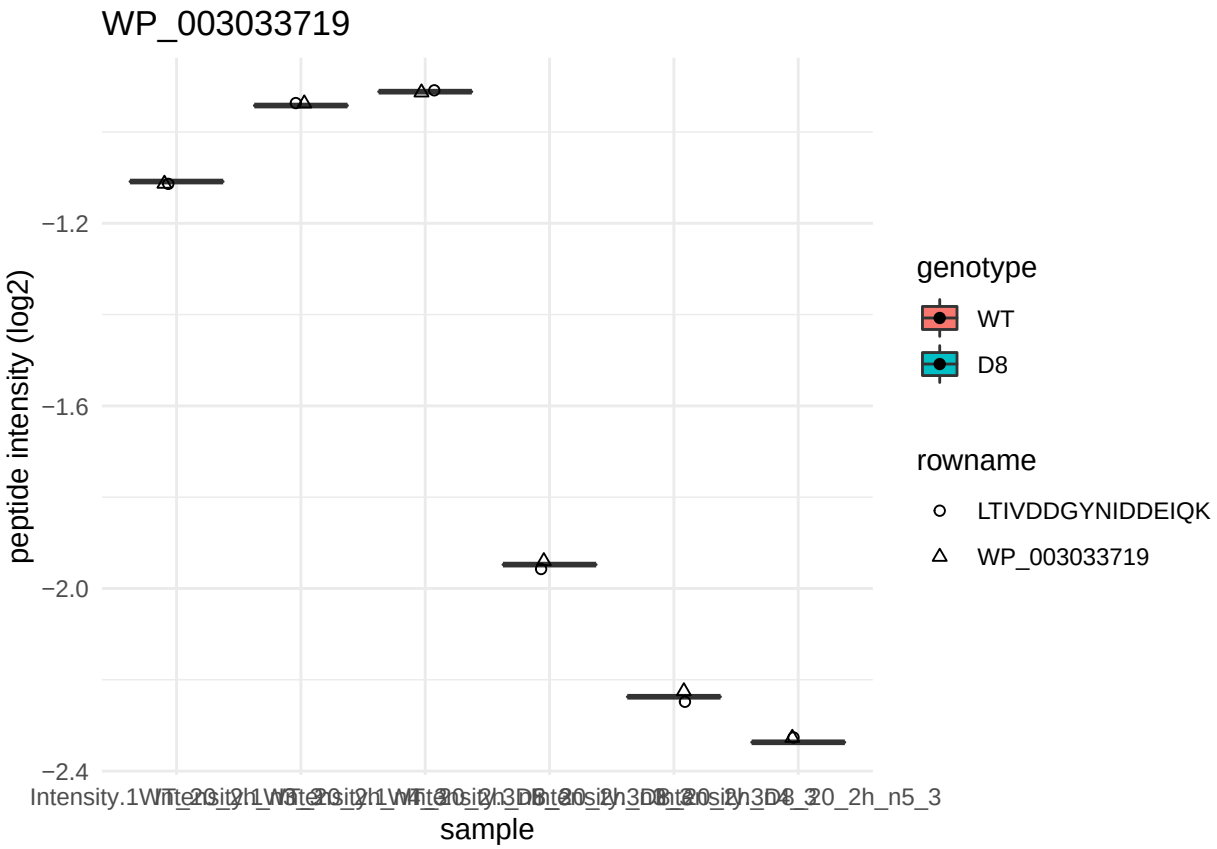
colname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genotype
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D8
rowname
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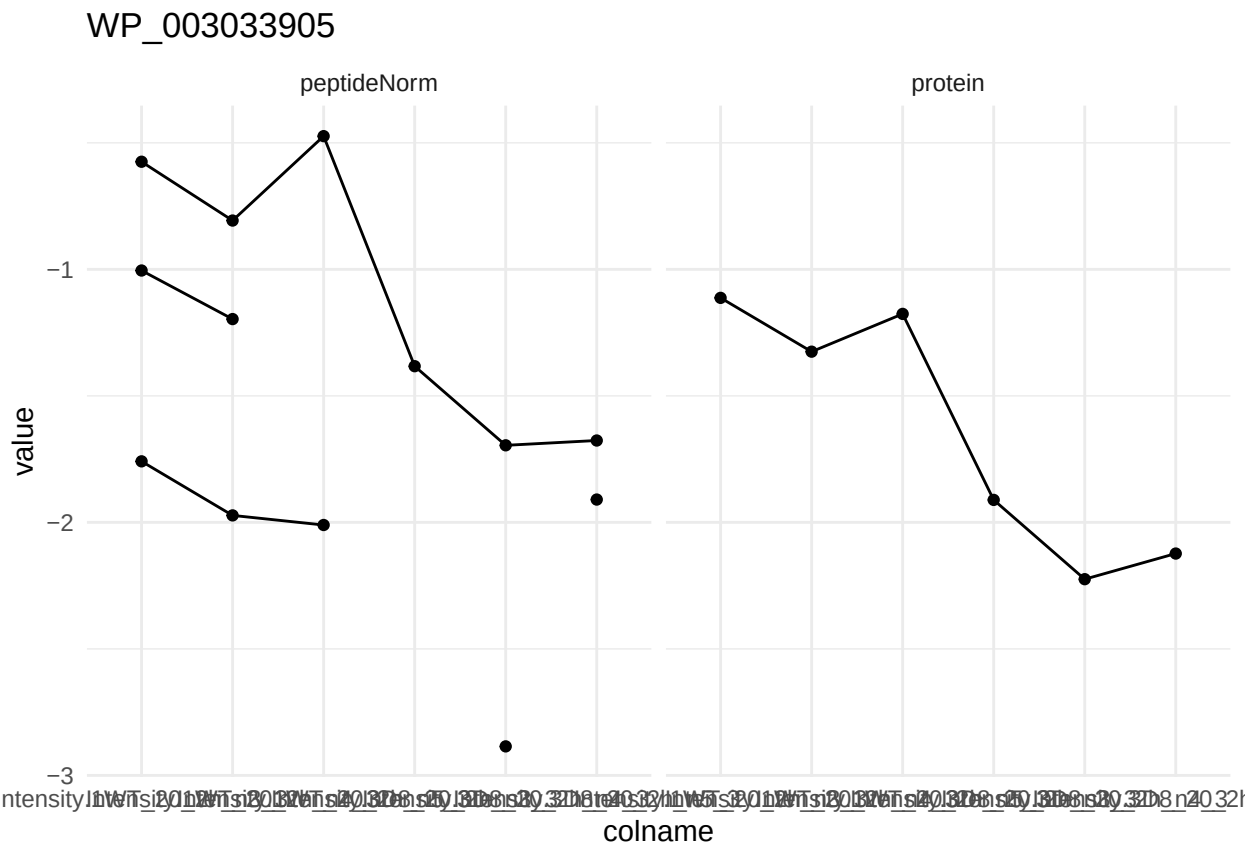
WP_003033719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protein
value
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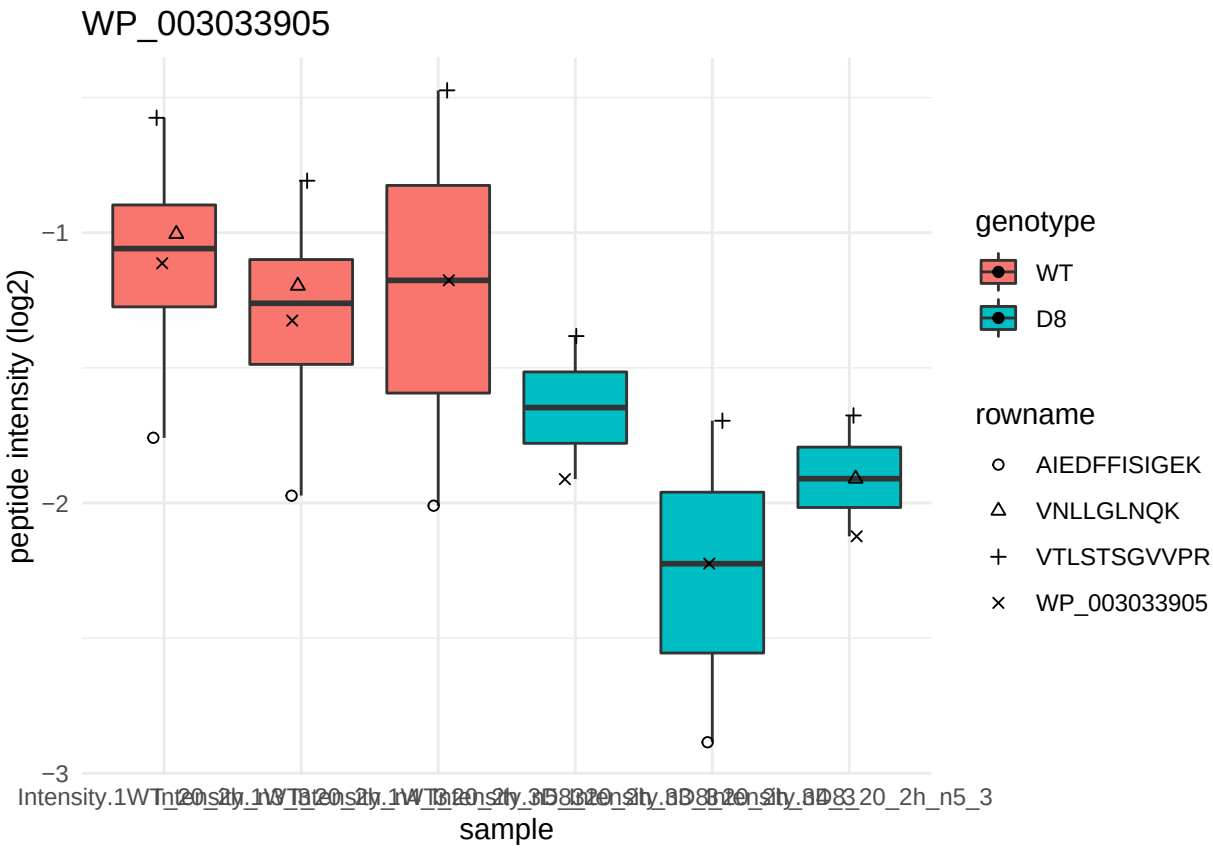
−3
colname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D8
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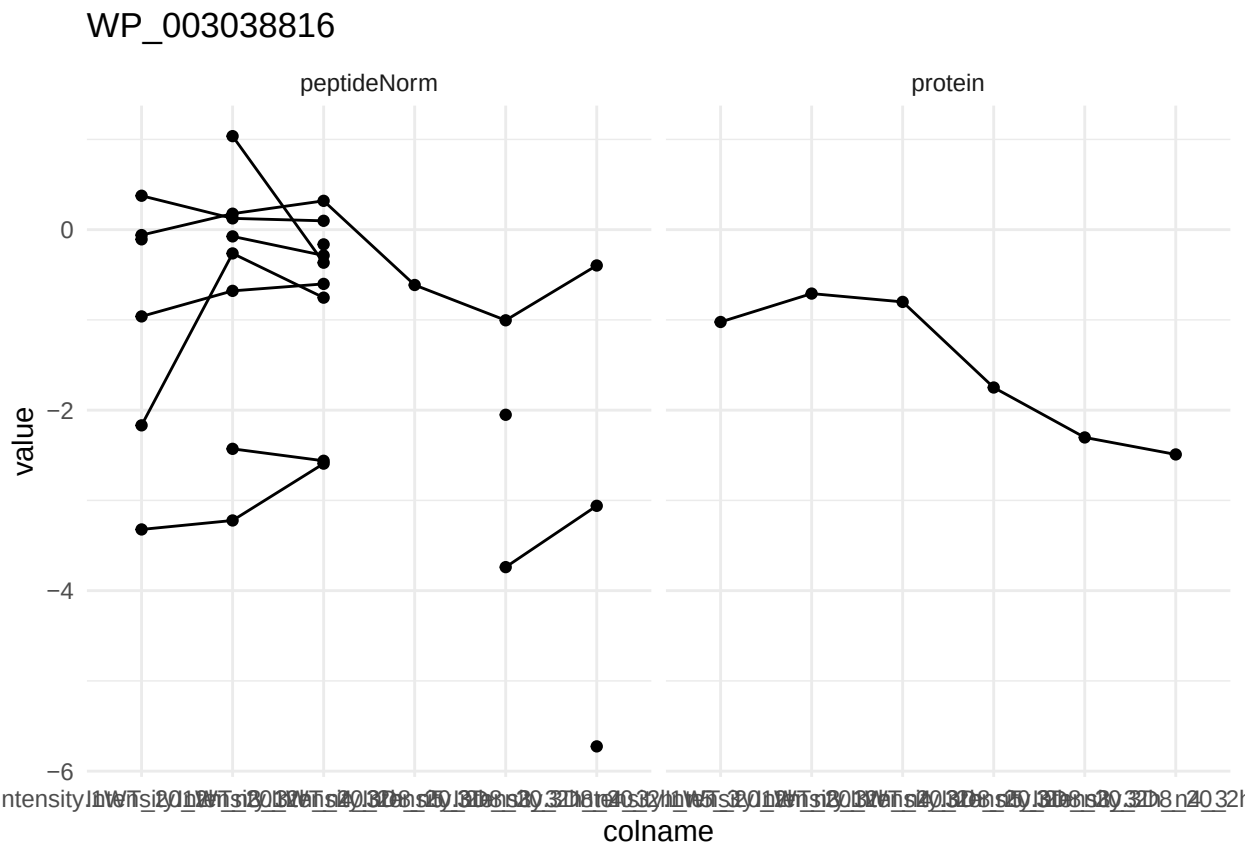
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protein
value
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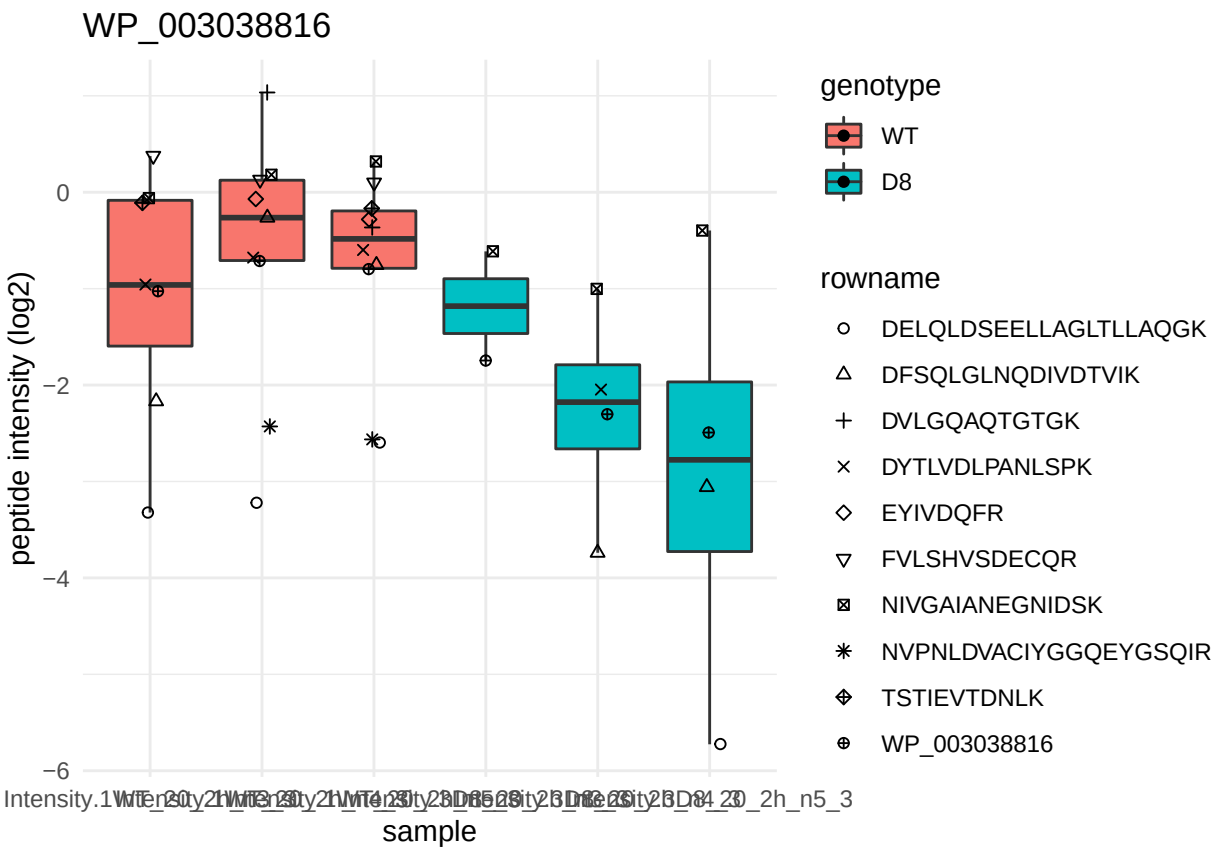
−4
−6
colname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−4
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genotype
WT
D8
rowname
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DFSQLGLNQDIVDTVIK
DVLGQAQTGTGK
DYTLVDLPANLSPK
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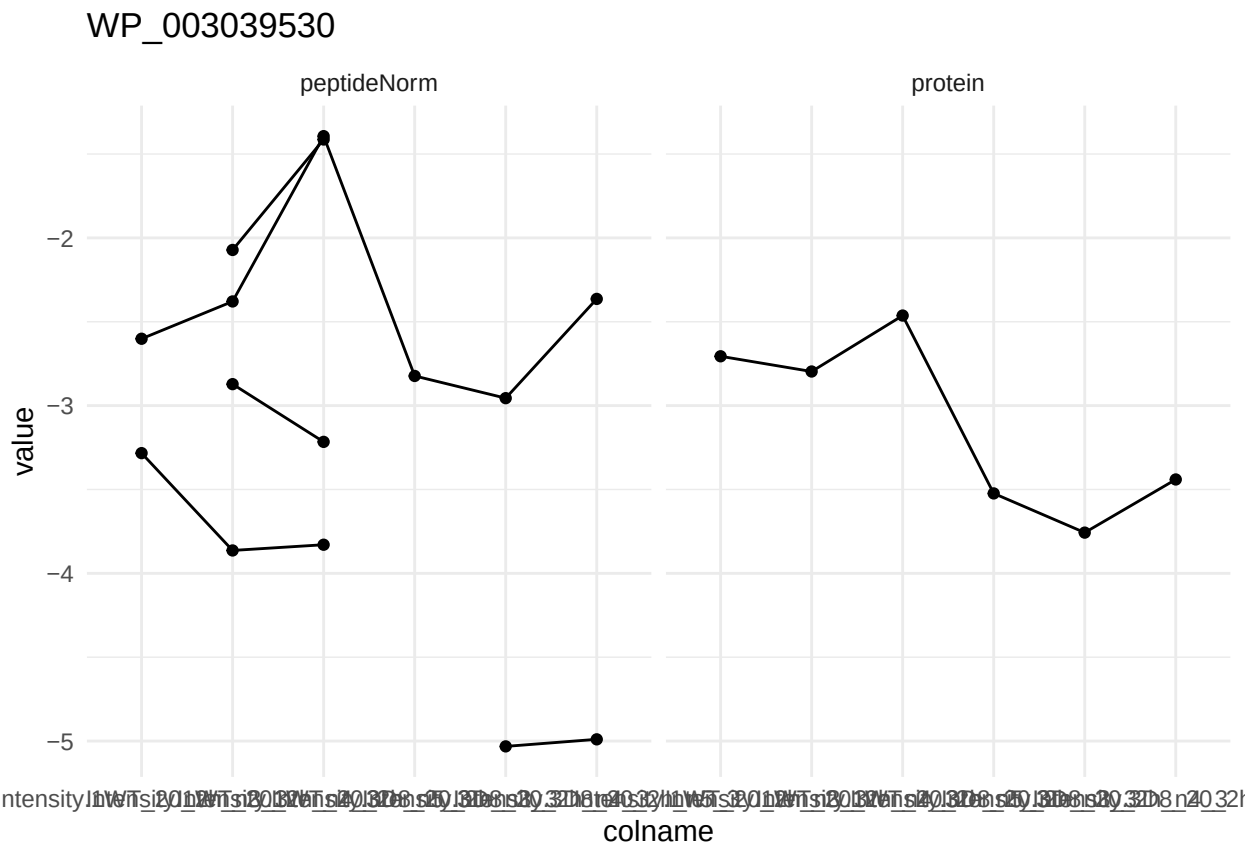
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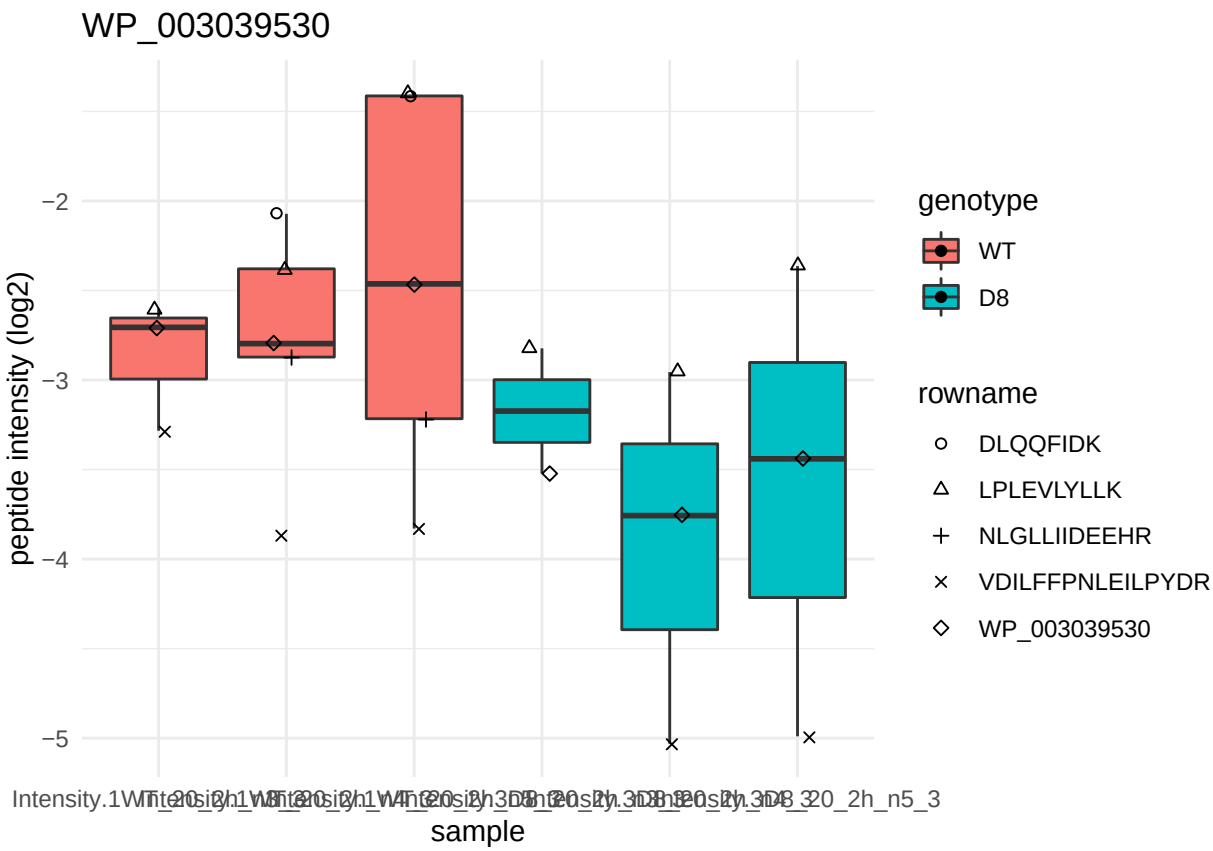
colname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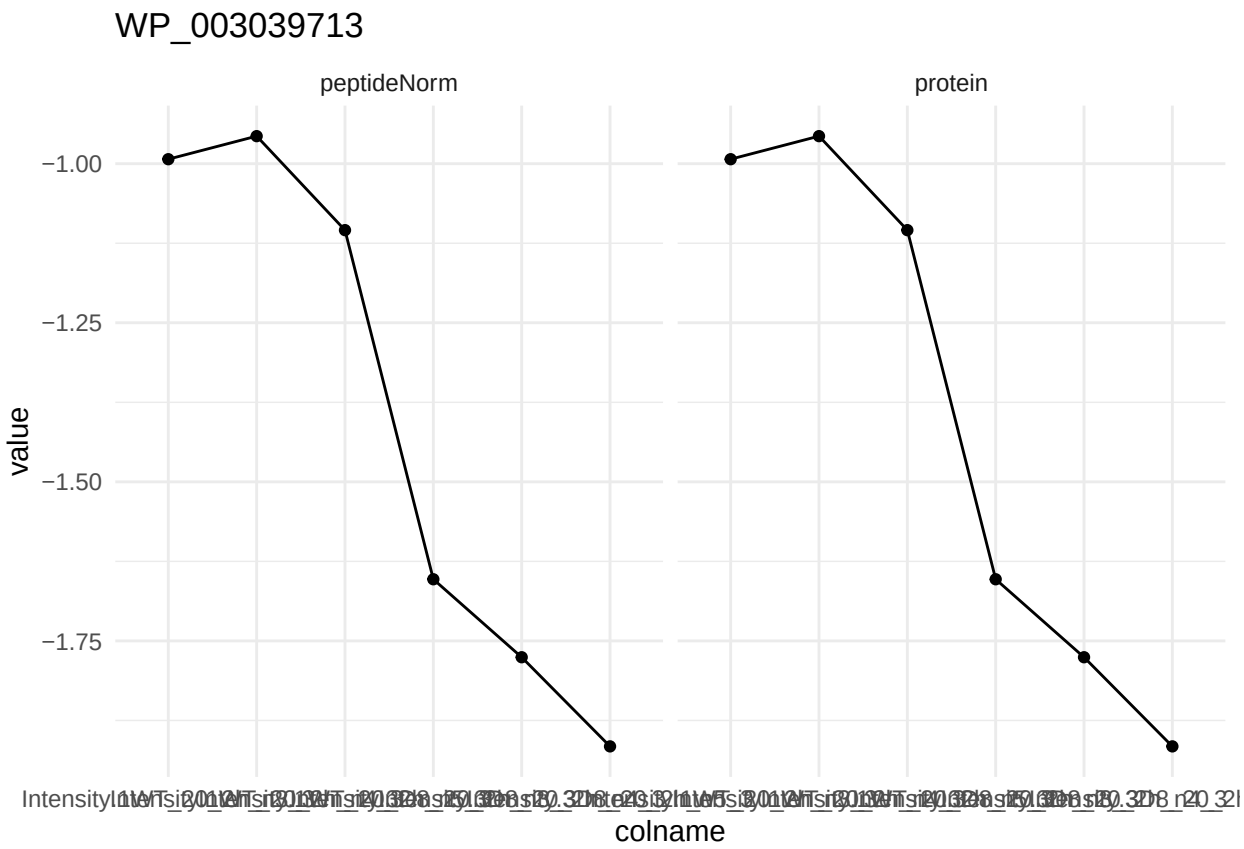
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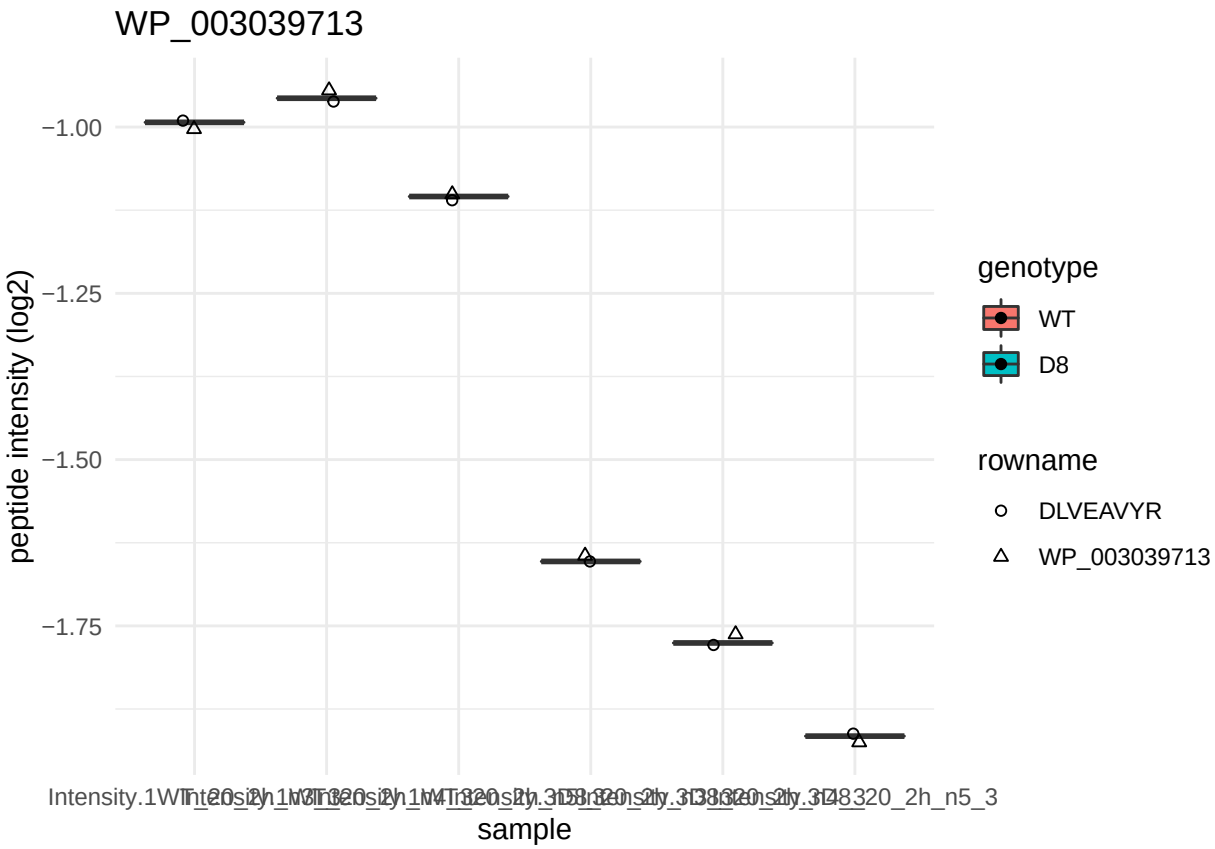
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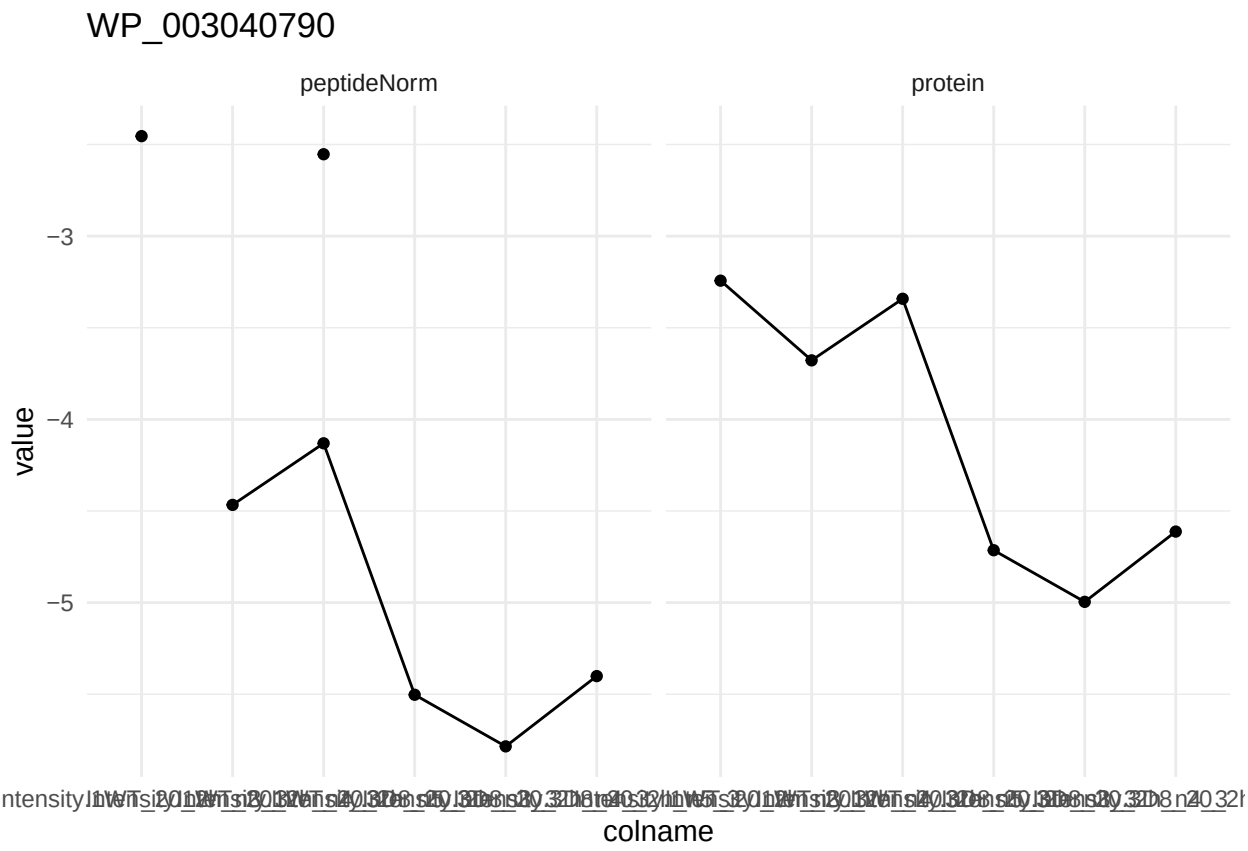
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protein
value
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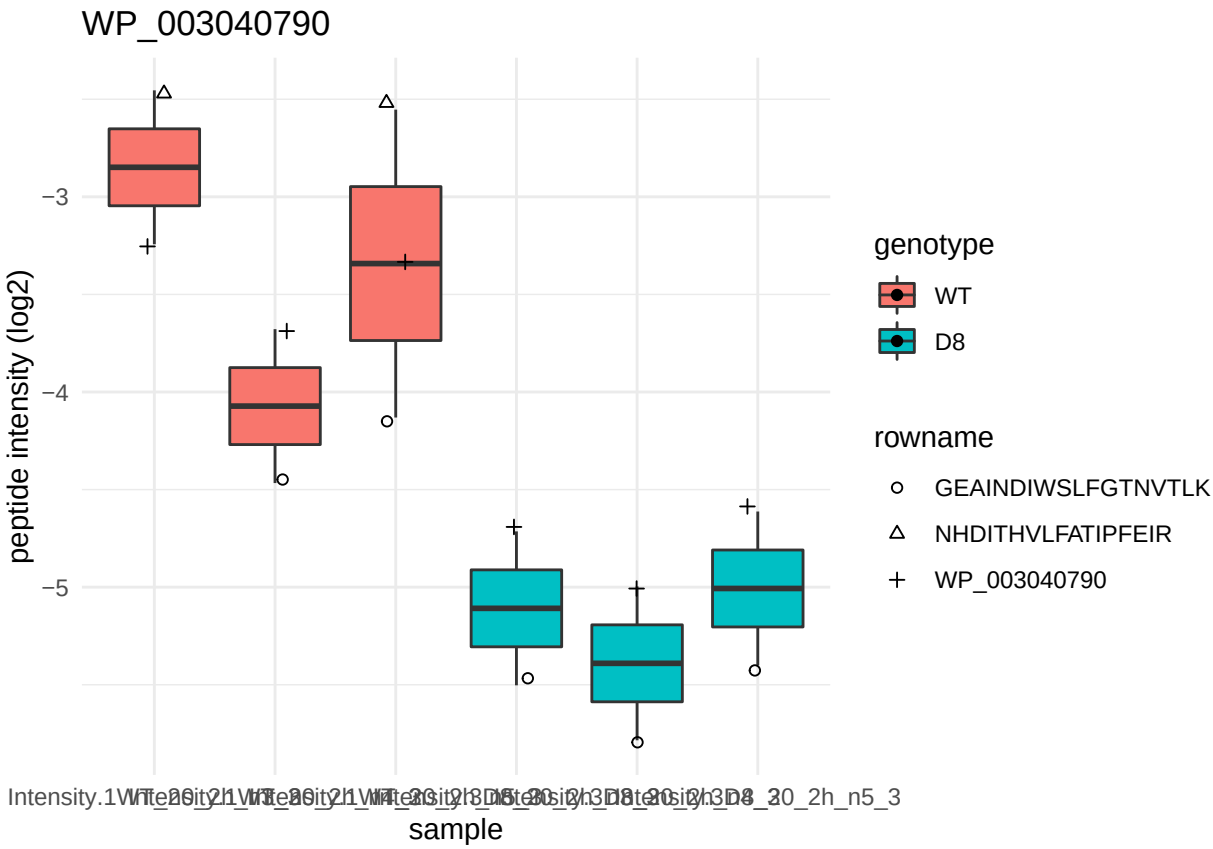
colname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D8
rowname
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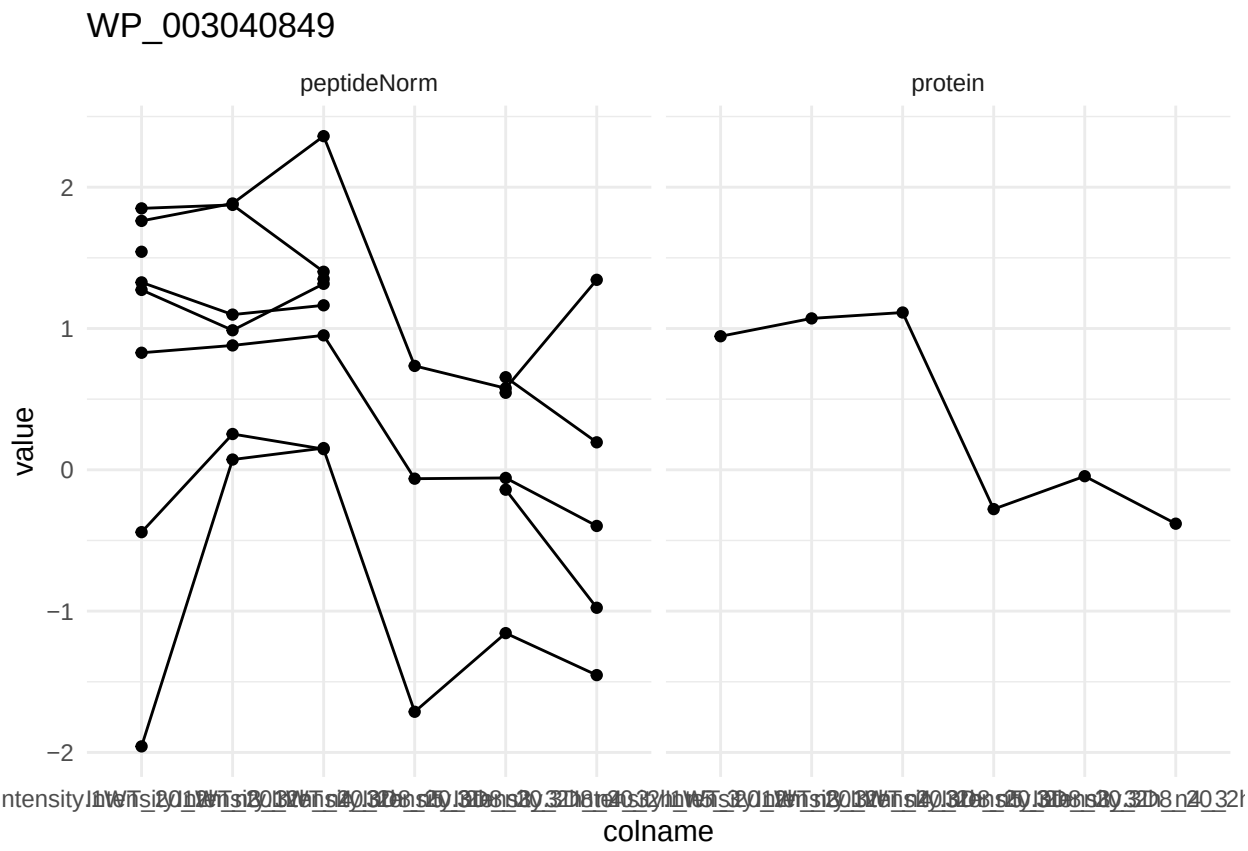
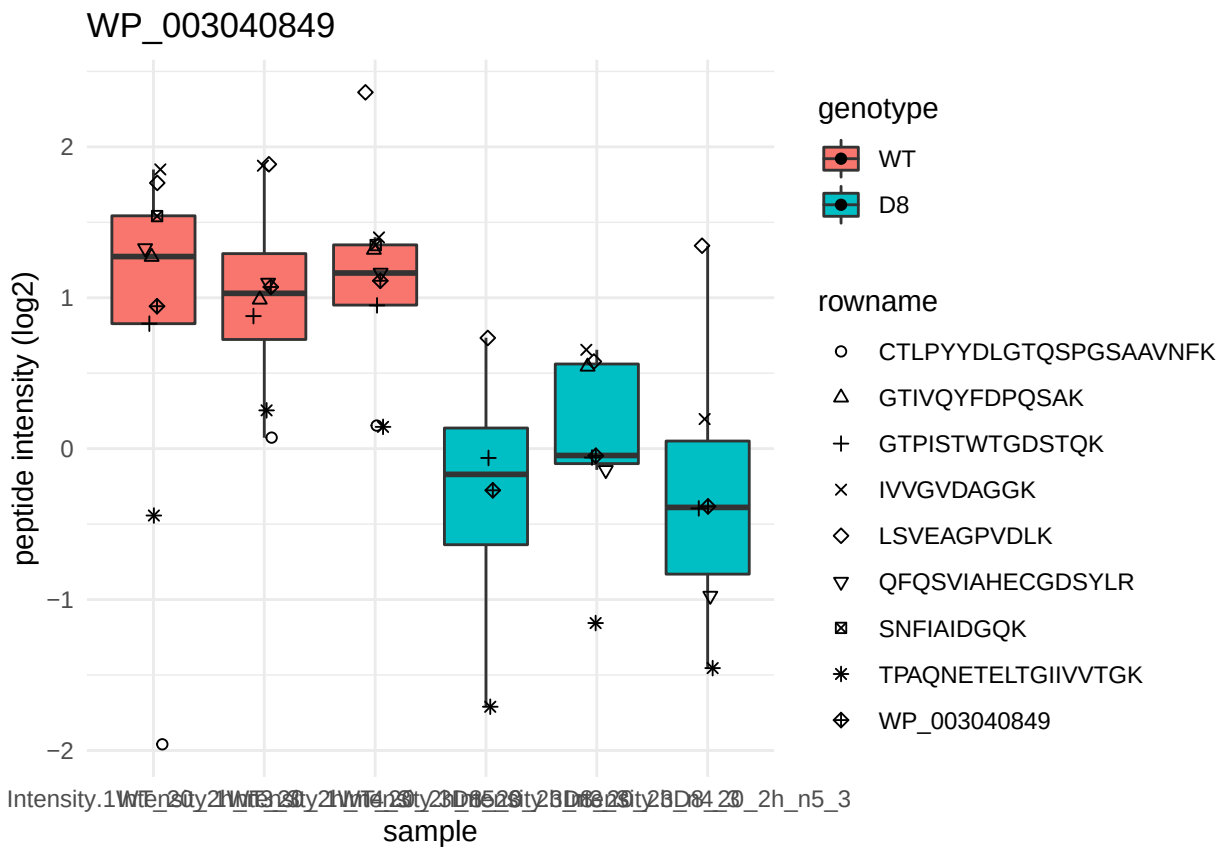

WP_003040849
peptideNorm
protein
value
2
1
0
−1
−2
colname



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 Blocking

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

- 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

2.4 Mouse example

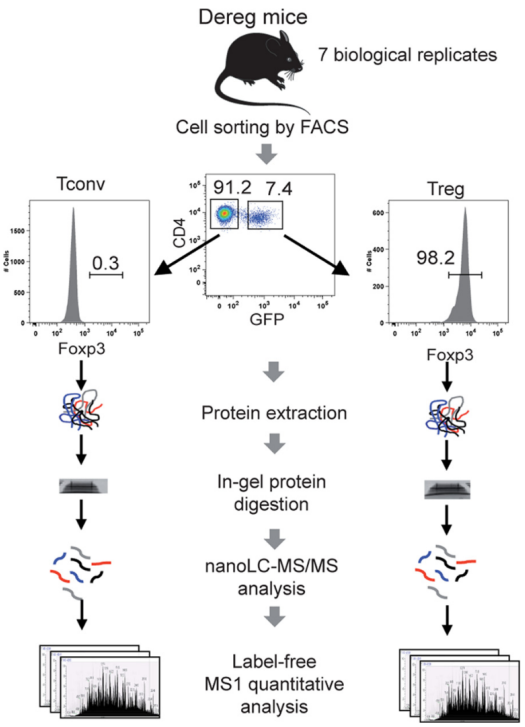


FIG. 1. **Label-free quantitative analysis of conventional and regulatory T cell proteomes.** General analytical workflow based on cell sorting by flow cytometry using the DEREG mouse model and parallel proteomic analysis of Tconv and Treg cell populations by nanoLC-MS/MS and label-free relative quantification.

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!
- We can isolate the between block variability from the analysis using linear model:

$$y \sim \text{type} + \text{mouse}$$

- Not possible with Perseus!

2.4.1 Assess the impact of blocking in the tutorial session!

- Completely randomized design with only one cell type per mouse (Treg and Tconv)

- Randomized complete block design assessing Treg and Tconv on each mouse