Quantitative proteomics data analysis

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Contents

Learning outcomes

1. Be able to aggregate peptide level data to obtain protein-level quantification.
2. Be able to normalise proteomics data and understand why we perform normalistion.
3. Perform and understand statistical tests to identify changes in protein abundances.
4. Perform a functional analysis to identify the perturbed biological processes

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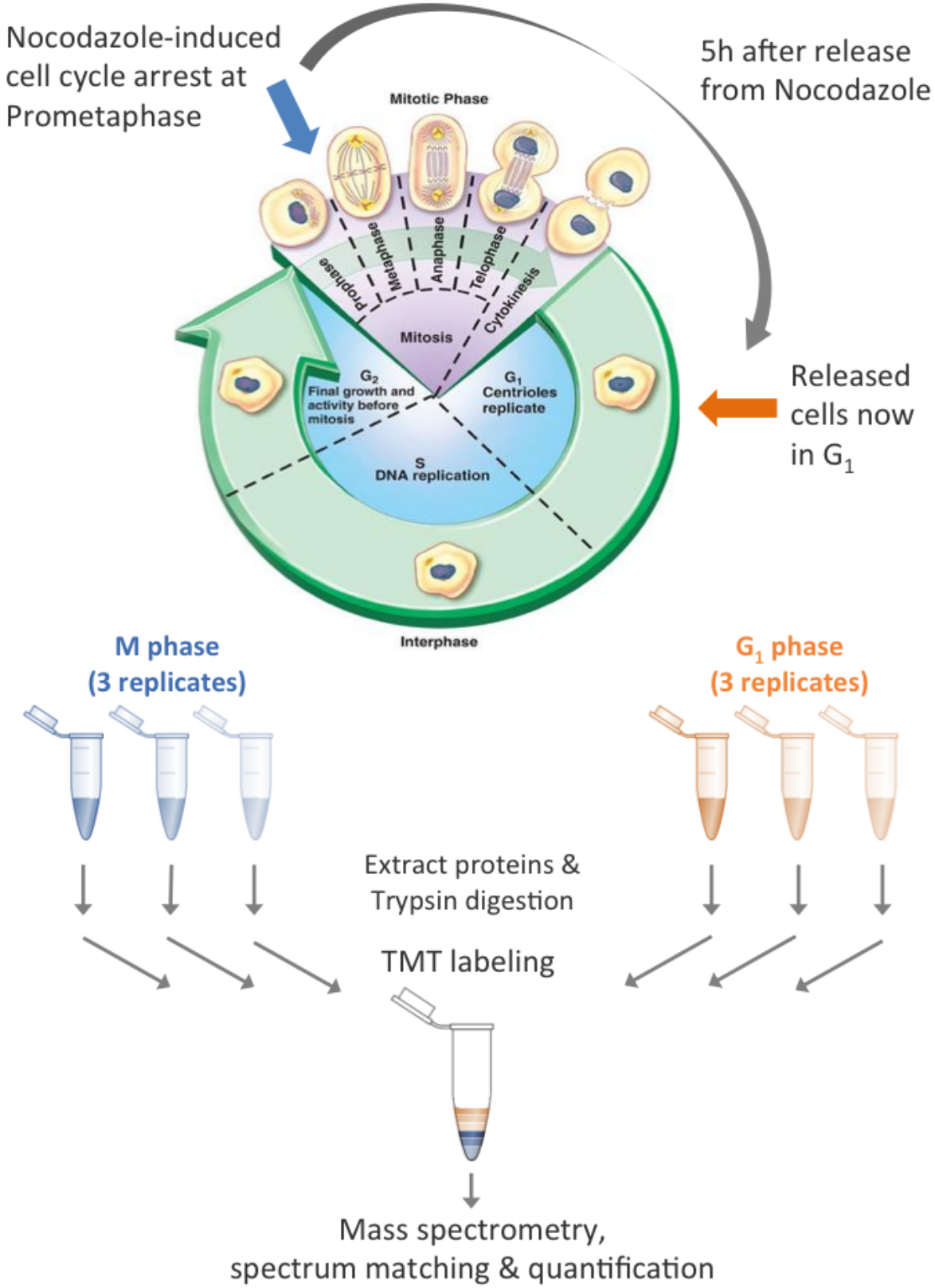
1. Introduction

This practical analyses data from an experiment to examine proteins involved in the cell cycle (See diagram below). Cells from a human cell line are treated with Nocodazole to inhibit polymerisation of microtubules and block transition beyond the prometaphase stage of M-phase.

Cells are then released from M-phase by withdrawing Nocodazole. Samples are taken in triplicate at 0 hours and at 5 hours after release from M-phase. This process generates 3 x M-phase and 3 x G1-phase samples from 6 separate batches of cells.

To measure protein abundances, proteins are first digested to peptides with trypsin. The same quantity of peptides from each sample was then labelled using Tandem Mass Tags (TMT). This allows all the samples to be combined into a single MS run to yield quantification over the same peptides in each sample.

The peptide spectrum matching and quantification steps have already been completed and the peptide-level quantification is provided to you.


Nocodazole-induced cell cycle arrest at Prometaphase
5h after release from Nocodazole
Mitotic Phase
Prophase
Metaphase
Anaphase
Telophase
Cytokinesis
Mitosis
G2 Final growth and activity before mitosis
G1 Centrioles replicate
S DNA replication
Interphase
Released cells now in G1
M phase (3 replicates)
G1 phase (3 replicates)
Extract proteins & Trypsin digestion
TMT labeling
Mass spectrometry, spectrum matching & quantification

Check your knowledge

1. Why does inhibiting polymerisation of microtubles block transition beyond prometaphase?

During prometaphase, kinetochore microtubules form and attach to the chromatids kinetochores. In the following phase (metaphase), these kinetochore microtubules organise the sister chromatids along the equatorial plane prior to their separation. If microtubule polymerisation is inhibited, the cell cannot form the kinetochore microtubles and gets stuck in prometaphase

2. What else might inhibiting polymerisation of microtubules do to the cells?

Microtubules have diverse functions in the cell, including the maintainance of cell shape and facilitating intracellular transport. For example, mitochondria need microtubules to move within the cell (https://pubmed.ncbi.nlm.nih.gov/11054698/) and mitochondrial fission is regulated by contact with microtubules (https://www.jbc.org/content/early/2019/01/02/jbc.RA118.006799).

3. Why do we take experimental triplicates?

We need repeated measurements to estimate the variability of our measurement. More replicates will always be better from a statistical point of view, though there are always practical reasons to limit replication. Triplicates is usually seen as the minimum to estimate variance.

4. Why do we digest using trypsin? At which residues does trypsin cleave?

Proteins have a very diverse physicochemical landscape. Consider the difference between a large multi-spanning membrane protein with lots of hydrophobic residues and a small soluble enzyme. By digesting the proteins, we reduce the size of the landscape considerably, thus simplifying the analysis. We use trypsin because it cleaves at specific residues, has high proteolytic activity, and is very stable. The peptides generated by trypsin tend to fall into a suitable size range for MS. Native trypsin is rarely used, but rather recombinant forms which are not autocatalytic.

Trypsin cleaves on the carboxyl terminal side of arginine and lysine amino acid residues, except when either is followed by proline.

5. What is a tandem mass tag? Chemical tags which can be added to peptides amino groups of Lysine or Arginine residues. TMTs have the same nominal mass (i.e., are isobaric) and a chemical structure composed of an amine-reactive NHS ester group, a spacer arm (mass normalizer), and a mass reporter which has a different mass for each tag. Labelling peptides from different samples with TMT thus causes the same mass shift in the peptide for each sample. During MS fragmentation, the mass reporter tags are liberated, and their relative intensities represent the relative sample abundances.

https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-mass-spectrometry-analysis/protein-quantitation-mass-spectrometry/tandem-mass-tag-systems.html

6. Why do we process all samples in the same MS run? To reduce technical variance and run-to-run variability, whereby some peptides may be quantified in just a subset of samples.

7. What is peptide spectrum matching? The process of matching the MS2 ions observed with a peptide sequence that would be expected to produce this set of ions.

First, we load the required libraries. If you get a message saying `Error in library(sds) : there is no package called ...`,

temporarily uncomment the neccessary lines to install the package

```
#install.packages("tidyr")
#install.packages("dplyr")
#install.packages("ggplot2")

suppressMessages(library(tidyr))
```

```
suppressMessages(library(dplyr))
suppressMessages(library(ggplot2))
```

2. Preparing the data

2a. Loading and reading in the peptide-level quantification

Usually your data is provided to you in some format such as a text file, excel file or some other format. We would like to load this data into R so we can analyse it.

For the purposes of this practical, peptides which come from proteins in the cRAP database of common proteomics contaminates, e.g keratin, have been removed. Peptides which cannot be uniquely assigned to a single protein have also been removed.

The data is provided here in a tab-separated text file `"data/peptide_data.tsv"`, which is located in the repository. We start by reading the peptide-level quantification data into a `dataframe`. The code chunk below loads the data from the .tsv file into an R dataframe called `peptides_quant`.

```
peptides_quant <- read.table("data/peptide_data.tsv", sep="\t", header=T)
```

If we take a look at the column names (R function `colnames()`) of the `peptides_quant` dataframe, we can see we have 9 columns. The first 3 columns describe the sequence of the peptide, the modifications which were detected and the protein which the peptide has been assigned to. The next 6 columns provide the quantification values for the 3 M-phase and 3 G1-phase samples.

```
print(colnames(peptides_quant))
```

```
## [1] "Sequence"       "Modifications"  "master_protein" "M_1"
## [5] "M_2"            "M_3"            "G1_1"           "G1_2"
## [9] "G1_3"
```

```
print(head(peptides_quant, 5))
```

```
##           Sequence                                  Modifications master_protein
## 1     EESTSSGNVSNR                          1xTMT6plex [N-Term]         P23193
## 2   TSNERPGSGQGQGR                          1xTMT6plex [N-Term]         Q8TAD8
## 3 AQHSNAAQTQTGEANR                          1xTMT6plex [N-Term]         P34897
## 4     QQQPPGAQGCPR 1xTMT6plex [N-Term]; 1xCarbamidomethyl [C10]         A8MYA2
## 5   TVSTSSQPEENVDR                          1xTMT6plex [N-Term]         P42684
##   M_1 M_2 M_3 G1_1 G1_2 G1_3
## 1 1.4  NA  NA   NA   NA   NA
## 2 2.7  NA  NA  3.5  1.6  3.5
## 3 1.7  NA 1.7  2.0  1.6  2.0
## 4 6.8 2.1 7.6  8.5  6.3  8.5
## 5 2.7  NA 6.2  2.7  1.0  2.7
```

```
print(dim(peptides_quant))
```

```
## [1] 20065     9
```

You'll notice that some of the quantification values of the peptides are "NA".

We can also see that some of the peptides have the same sequence but different modifications. Below, we inspect one such peptide sequence

```
filter(peptides_quant, Sequence == 'AAAEELQEAAGAGDGATENGVQPPK')
```

```
##                     Sequence                                      Modifications
## 1 AAAEELQEAAGAGDGATENGVQPPK 2xTMT6plex [N-Term; K25]; 2xDeamidated [Q/N]
```

```
## 2 AAAEELQEAAGAGDGATENGVQPPK 2xTMT6plex [N-Term; K25]; 1xDeamidated [N19]
##   master_protein  M_1  M_2  M_3 G1_1 G1_2 G1_3
## 1         Q9H9Y2 30.5 21.2 32.6 27.1 29.7 27.1
## 2         Q9H9Y2 23.6 12.3 27.0 30.3 25.4 30.3
```

Check your knowledge

1. Where can I find keratin in daily life? Hair, skin, nails, horns, hooves, feathers, claws etc.
2. Why is keratin a contaminate? (Most of) the above are found in every laboratory. Keratin is also very stable. So stable it can be used to identify the species of birds that have hit and disabled airplane engines. https://investigativegenetics.biomedcentral.com/articles/10.1186/2041-2223-2-16
3. Why do we worry about contaminates? Our interest is the cells/tissue etc that we are studying. Where contaminants could be from the biological material or laboratory contamination, it's important to be aware that we may be quantifying from external nuisance souces. In general, it's much easier to exlcude analysis of proteins such as keratins.
4. What does if mean to say a peptide is 'assigned' to a protein. Why could a peptide be assigned to multiple proteins? A peptide is assigned to a protein when the amino acid sequence of the peptide matches part of the protein amino acid sequence (a `substring`). Since peptides are substrings of a protein, it's quite possible for the same peptide to be part of multiple proteins sequences. Think for example of recent gene duplication events where the protein sequences have diverged enough to be considered separate proteins, but still have stretches of the same amino acid sequence.
5. How many peptides were quantified in this experiment? 20065
6. What do you think NA means in this context? Not Available = Not quantified
7. Why do you think some of the quantification values are NA? Reporter tag intensities fall below the limit of detection. Or tag intensities is literally zero, e.g peptide is not present in sample
8. What do you think we should do with NA? Open question. Here, we remove them because they are relatively low frequency. Where this would mean losing a lot of data, imputation may be more suitable. Or else, if the missing values are predominantly from one sample, it may be justified to remove this sample. Finally, when we come to aggregate peptide intensities to protein intensities, we may wish to keep missing values and use an aggregation method that handles missing values appropriately.
9. Why do peptide 'modifications' represent?

a) The biological explanation: post-translational modifications (PTMs)
b) The technical explanation: sample handling induced modifications such as oxidation.
c) The protocol explanation: we have labeled our peptides with TMT, so every peptide should have 'TMT6plex' modifications In this case, we did not look for (a), so the MS only identified (b) and (c).

10. Why might we have multiple quantification values for the same peptide but with different modifications? Both explanation (b) & (c) above could have multiple variants for a single peptide. For example, there may be more than one residue which can be deamidated, or TMT labelled.

2b. `magrittr` pipes

The `magrittr` package introduced 'pipe' functions to R. Here, we will be using the `%>%` pipe a few times so the next two cells demonstrates how this works in case you haven't come across it before as the syntax can look a little odd. Feel free to skip these cells if you're confident you understand '%>%'.

The `%>%` pipe allows you to use the output from one function to be the input for the next function, much like the `|` pipe in bash. The main advantage of this is to make the code easier to understand. The pipe can be

used to pass any object. However, to keep things simple here, for the example below, we will just consider a simple vector of values, from which we wish to identify the mean of the log2-transformed values.

```
values <- c(1, 4, 100, 21, 34)

# we can wrap the mean and log functions together, but this can get ugly,
# especially if there are lots of functions and arguments.
mean(log(values, base=2))
```

```
## [1] 3.624727
```

```
# with magrittr pipes, we can see the 'flow' from
values %>%  # the values vector, through
  log(base=2) %>%  # the log(base=2) transformation, and finally,
  mean()  # the mean summarisation
```

```
## [1] 3.624727
```

Magrittr pipes become especially useful for piping together complex functions when reformating, summarising and plotting data. They also make it much easier to document each step. If you're wondering how the package got its name, see The_Treachery_of_Images

With that aside done, let's return to the practical.

2c. Aggregating the peptide quantification data to generate protein-level quantification

We can aggregate the quantification values across the multiple instances of the same peptide with difference modifications using the `dyplr` package

```
peptides_quant_no_mods <- peptides_quant %>%
  select(-Modifications) %>% # exclude the Modifications column
  group_by(Sequence, master_protein) %>% # group by the Sequence and master_protein columns
  summarise_all(list(~sum(.))) %>% # aggregate the quantification values using the sum function
  data.frame() # convert back to a data.frame

print(head(peptides_quant_no_mods))
```

```
##                Sequence master_protein   M_1   M_2   M_3  G1_1  G1_2  G1_3
## 1              AAAAAAAK         Q9NYV4  92.2  38.3  66.4  78.9  61.6  78.9
## 2          AAAAAAALQAK         P36578 371.7 182.3 383.8 394.4 315.2 394.4
## 3 AAAAASAAGPGGLVAGKEEK         A6NIH7  35.9  19.0  36.3  38.8  36.6  38.8
## 4    AAAAAWEEPSSGNGTAR         Q9P258 103.1  46.1  81.0 110.6 108.8 110.6
## 5              AAAACLDK         Q9H3U1  43.9  18.5  36.1  43.2  39.9  43.2
## 6            AAAAGSLDR         Q9Y2U8  59.1  38.3  61.1  97.3  79.9  97.3
```

2d. Dealing with missing values

We still have peptides with missing values

```
options(width = 60)
quant_columns <- colnames(peptides_quant)[4:9]

missing_values_pre_agg <- peptides_quant  %>%
  select(one_of(quant_columns)) %>% # only select the quantification columns
  is.na() %>% # returns a matrix of booleans (TRUE, FALSE), where TRUE = values is NA
  sum() # TRUE=1, FALSE=0 so sum of booleans is the number of TRUEs
```

```
missing_values_post_agg <- peptides_quant_no_mods %>%
  select(one_of(quant_columns)) %>%
  is.na() %>%
  sum()

message(sprintf("Before aggegation, we had %i peptides with missing values,
after aggregation we have %i peptides with missing values",
                missing_values_pre_agg, missing_values_post_agg))
```

```
## Before aggegation, we had 138 peptides with missing values,
## after aggregation we have 137 peptides with missing values
```

We could impute the missing values but this is beyond the scope of this practical. For our purposes, we will remove any peptide with a missing value

```
rows_without_na <- rowSums(is.na(peptides_quant_no_mods[,3:8])) == 0

peptides_quant_no_mods_no_na <- peptides_quant_no_mods[rows_without_na,]
```

Check your knowledge

1. Why should we remove NA? Here, we will aggregate peptide-level intensities to protein-level using a simple approach of median averaging. Missing values will invalidate this approach.
2. Are there biological reasons for NA? A protein may be very low or zero abundance in a given sample.
3. What imputation stratagies exist? Again, a very open question. In simple terms, it's helpful to consider if the missing values being imputed are missing at random (MAR) or missing not at random (MNAR). This makes a big difference to how to impute the missing values. For instance, in a simple case where the values are MNAR and expected to be missing due to falling below some threshold value, it may be appropriate to impute the minimum observed value, or a value selected from a distribution around the minimum observed value. Standard procedures for imputation vary between fields and this is an active area of research. ***

2e. Normalisation: What is it and why do we do it?

Before we proceed with the aggregation to protein-level quantification, we should consider whether we need to perform any normalisation to the data. The total peptide abundance in each column should be approximately the same. However, we can see below that this is not the case.

Below, we obtain the sum of peptide abundances in each sample (column). Note these are relatively similar but M_2 is ~2-fold less than M_1 or G1_1.

```
pep_tots <- peptides_quant_no_mods_no_na %>%
  select(one_of(quant_columns)) %>%
  colSums()

# formatC(format='e') formats numeric values into scientific notation. See ?formatC
formatC(pep_tots, format='e', digits=1)
```

```
##       M_1       M_2       M_3      G1_1      G1_2      G1_3
## "1.9e+06" "9.9e+05" "1.7e+06" "1.9e+06" "1.6e+06" "1.9e+06"
```

Check your knowledge

1. Why should the total peptide abundance be approximately the same (see experimental design)? We have labelled the same amount of peptide from each sample.
2. What could cause it to be different?

- Could have inaccurately quantified peptide concentration prior to labeling.
- Pipetting error
- Differences in TMT labeling efficiency (unlikely)
- Not all peptides in the sample are quantified since the MS can only focus on the most intense MS1 ions (unlikely since the most abundance peptides will have been quantified and these make up the bulk of the sample)

3. What impact could this have on any downstream statistical analysis? If the overall peptide abundance was systematicall lower in one condition due to a technical reason, this could generate false difference between conditions.

Next, we normalise the peptide-level quantification to adjust for the differences in the total peptide abundance

```
# make a correction factor using the total peptide abundance
pep_tots_correction_factors <- pep_tots/mean(pep_tots)

peptides_quant_norm <- peptides_quant_no_mods_no_na

# the divide operation works on each cell in the dataframe by column so we need to transpose (t)
# the dataframe so columns = peptides and rows = samples before performing the divide operation.
peptides_quant_transposed <- t(peptides_quant_norm[, quant_columns])

#Perform data normalisation
peptides_quant_norm_transposed <- peptides_quant_transposed/pep_tots_correction_factors

# then re-transform back to the initial layout
peptides_quant_norm[, quant_columns] <- t(peptides_quant_norm_transposed)
```

We can check that the total are now the same:

```
norm_pep_tots <- peptides_quant_norm %>%
  select(one_of(quant_columns)) %>%
  colSums()

formatC(norm_pep_tots, format='e', digits=1)
```

```
##       M_1       M_2       M_3      G1_1      G1_2      G1_3
## "1.7e+06" "1.7e+06" "1.7e+06" "1.7e+06" "1.7e+06" "1.7e+06"
```

We plot the distribution of abundance values before and after normalisation allowing us to see the impact of normalisation. Here we create a small function to perform the plotting to save space from copying code. Note that we are plotting the log of the abundance values. This is because the abundance values extend across 4-orders of magnitude and are not Gaussian (normally) distributed. However, they are approximately log-Gaussian distributed.

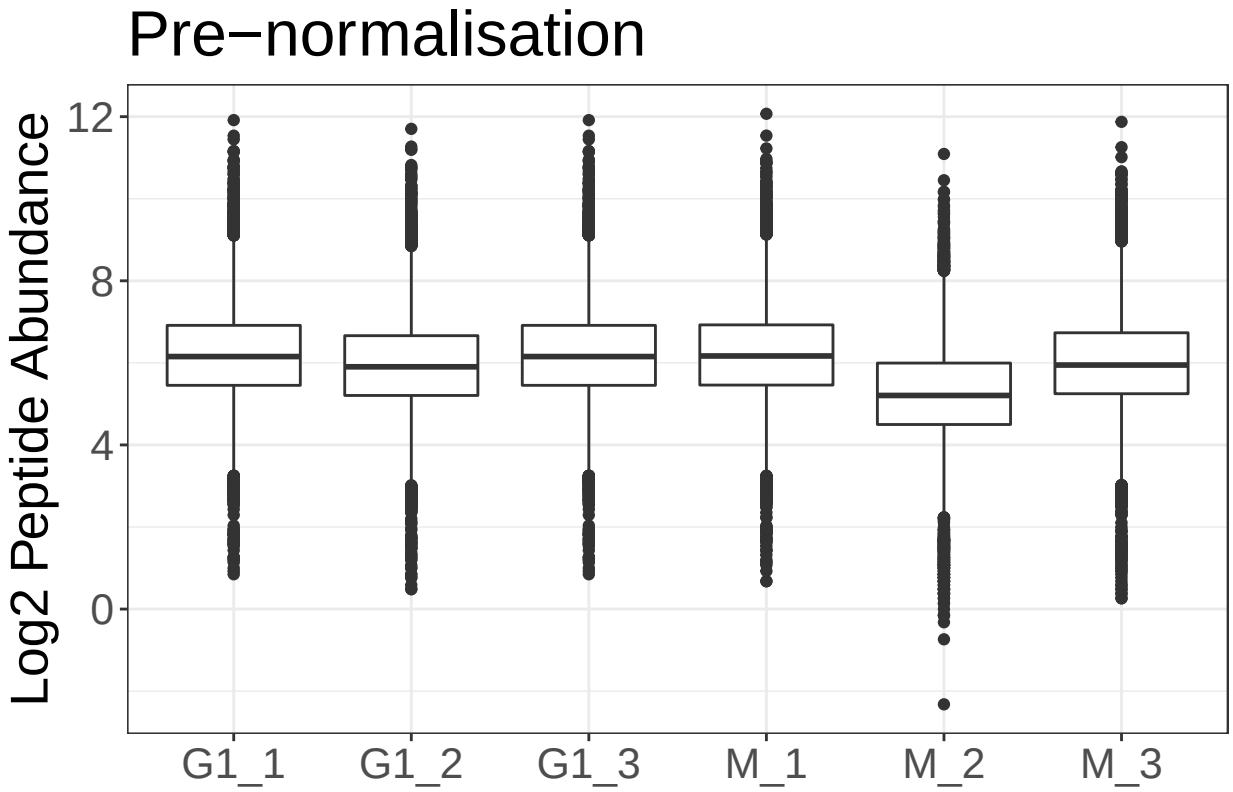
```
plotPeptideAbundance <- function(peptide_data, title=""){

  p <- peptide_data %>%
    pivot_longer(cols=-c(Sequence, master_protein), # reshape data into 'long' format for ggplot
                 names_to='sample', values_to='abundance') %>% # set names and values columns
    ggplot() +
    aes(sample, log(abundance, 2)) +
```

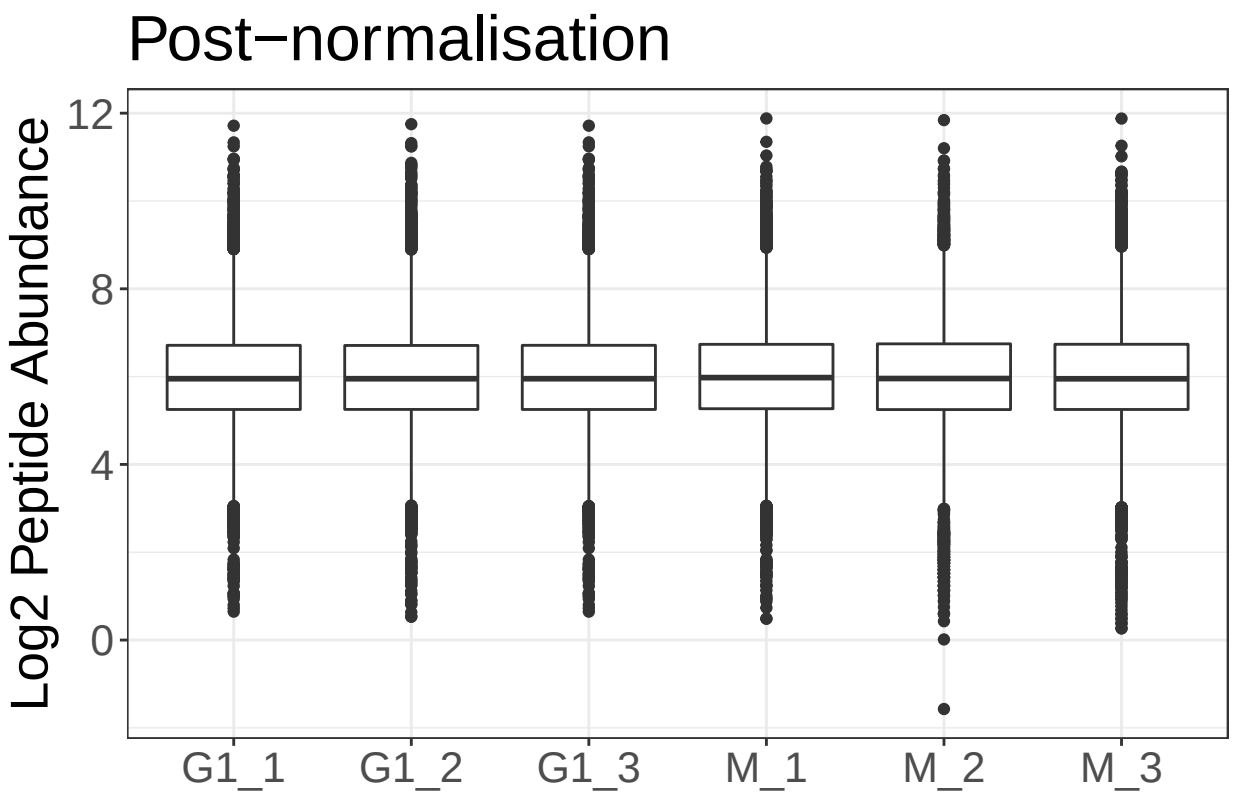
```
    geom_boxplot() +
    xlab("") + ylab("Log2 Peptide Abundance") +
    theme_bw() +
    theme(text=element_text(size=20)) +
    ggtitle(title)

  print(p)
}


plotPeptideAbundance(peptides_quant_no_mods_no_na, "Pre-normalisation")
```




```
plotPeptideAbundance(peptides_quant_norm, "Post-normalisation")
```



Now we have a single quantification value for each peptide, we can aggregate across the different peptides for the same protein to obtain the protein-level quantification. Here we are using the median.

Check your knowledge

1. Why do you think we are using the median to aggregate across the different peptides? The median has an advantage over the mean in that it is less sensitive to outliers.
2. What other mathematical operations could we use and what are the relative merits? The mean or sum (equivalent in this case) have some merit in that they weight the final protein-level abundances more towards the higher abundance peptides, which arguably will have more accurate quantification estimataes. There are a miriad of more sophisticated functions we could use to aggregate but we won't go into these now
3. What information do we lose by aggregating peptides into peptides? By simplifying multiple peptide level abundances to a single protein abundance, we lose all information about the variance between different peptides for the same protein.

```
protein_quant <- peptides_quant_norm  %>%
  select(-Sequence) %>% # exclude the Sequence column
  group_by(master_protein) %>% # group by the master_protein column
  summarise_all(funs(median(.))) # aggregate the quantification values using the median function
```

```
## Warning: `funs()` is deprecated as of dplyr 0.8.0.
## Please use a list of either functions or lambdas:
##
##   # Simple named list:
##   list(mean = mean, median = median)
##
```

```
##   # Auto named with `tibble::lst()`:
##   tibble::lst(mean, median)
##
##   # Using lambdas
##   list(~ mean(., trim = .2), ~ median(., na.rm = TRUE))
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_warnings()` to see where this warning was generated.
```

```
head(protein_quant)
```

```
## # A tibble: 6 x 7
##   master_protein   M_1   M_2   M_3  G1_1  G1_2  G1_3
##   <chr>          <dbl> <dbl> <dbl> <dbl> <dbl> <dbl>
## 1 A0AVT1          75.2  66.5  70.2  76.7  79.0  76.7
## 2 A0MZ66          52.4  53.0  52.1  45.8  49.2  45.8
## 3 A1L390          51.4  51.5  54.3  49.4  57.7  49.4
## 4 A1X283          43.3  42.5  49.4  45.5  45.3  45.5
## 5 A2RTX5          35.0  36.2  33.0  32.4  38.0  32.4
## 6 A3KN83          13.5  15.3  14.9  20.6  18.6  20.6
```

---

3. Performing a statistical test to identify changes in protein abundance

We now want to identify the proteins with significant changes in abundance between M and G1 phases. For the purposes of this practical, we consider hypothesis-based statistics. Note there are other schools of statistics and they might have different tests.

Formally, we state our null hypothesis:

(H0) The change in abundance for a protein is zero

We want to test this against the alternative hypothesis:

(H1) The change is greater than zero.

For each protein, we conduct an independent statistical test. The important points to consider when choosing the appropriate statistical test are:

- We have independent samples from two populations and wish to test whether the difference in the mean of the two populations is non-zero.
- The samples are not paired, e.g M-phase replicate 1 and G1-phase replicate 1 are not related samples.
- We want to identify changes in both directions.
- The protein abundances are not Gaussian (normal) distributed. However, they are approximately log-Gaussian distributed.
- The cell cycle stage is not expected to have a large impact on the biological variability.

This is a very simple experimental design and we recommend that you use a student's t-test for this practical. However, there are other statistical tests which would also be suitable. For example, if one was concerned about whether the protein abundances are truly approximately Guassian, a non-parametric test such as the Mann-Whitney U test or Wilcoxon rank-sum tests may be used depending on the exact experimental design. Alternatively, if the experimental design was more complicated and we wished to take into account confounding factors, or investigate interactions between factors, we could use ANOVA or Linear Regression. Choosing the correct test must be based on the assumptions of your experimental design, choosing a test so that you see the results you want is academic fraud.

Check your knowledge

1. Should the t-test be one-tailed or two-tailed? Two-tailed. We are not specifying beforehand that we are only interested in changes in one direction.
2. Should you perform a paired t-test? No. The samples are not paired.
3. A two-sample equal variance t-test or a two-sample unequal variance t-test? Since the cell cycle stage is not expected to impact biological variability, we can reasonably assume equal variance. Ideally, we would test whether this assumption is reasonable.
4. Do you need to perform any transformation on the data before conducting the t-test? The proteins abundances are expected to be be log-Gaussian distributed. Therefore we need to log-transform the values before applying the test.
5. What are the assumptions of the t-test and do these assumptions hold for these data?

a) Continuous dependent variable - Yes
b) Gaussian distributed dependent variable - Yes (when logged)
c) Independent and identically distributed random variables (i.i.d) - Yes. Replicates are independent samples. As noted above, we don't expect condition to affect variance so they are identically distributed.

6. How low does the p-value have to be before you reject H0 (what's your value for alpha)? 0.05 is commonplace in biological experiment, though there's nothing special abou this values. You can use whatever value you feel is justified.
7. What effect size do you think would be biologically relevant?
8. Would a 2-fold increase/decrease in abundance be relevant?
9. What about a 0.5-fold or a 0.1-fold increase/decrease? The above are open questions to stimulate discussion. There are no correct answers here as it very much depends on the experiment and protein in question.

We perform a log-transform on the data before conducting the t-test. We use `log2()` since it's intuitive to have a single increase by a point in the `log2()` scale indicate a doubling and a two point increase indicate a 4-fold increase.

```
protein_quant_log <- protein_quant # create a new copy of the protein quant data

# log2-transform the quantification values
protein_quant_log[,quant_columns] <- log2(protein_quant_log[,quant_columns])
```

In order to perform the t-test on each row, we create a function which performs a t-test on a vector of six values (where 1-3 = group 1, 4-6 = group 2) and returns the p-value, the difference between the group means, and the 95% confidence interval for the difference.

```
runsingleTTest <- function(quant_values){

  test_results <- t.test(x = quant_values[4:6],
                         y= quant_values[1:3],
                         paired = FALSE,
                         alternative = "two.sided",
                         conf.int = TRUE,
                         var.equal = TRUE)

  p.value <- test_results$p.value # extract the p-value

  # extract difference between the groups
  difference <- as.numeric(test_results$estimate[1] - test_results$estimate[2])
```

```
  ci <- as.numeric(test_results$conf.int) # extract the 95% CI

  return(c(p.value, difference, ci))
}
```

We now `apply` this function over the rows and reformat the output

```
TTest_results <- protein_quant_log

# apply the runSingleTTest function over each row ("MARGIN=1") for the quantification columns
# We need to transform (t) the values returned by apply to get the correct layout
TTest_results[c("p.value", "difference", "CI_diff_low", "CI_diff_high")] <- t(
  apply(TTest_results[, quant_columns], MARGIN = 1, runsingleTTest))
```

3a. Accounting for multiple hypothesis test

Check your knowledge

1. For your chosen level of alpha, how many proteins would you expect to have a significant change in abundance by chance (false positives; Type I errors) ?

If alpha = 0.05: 0.05 * nrow(TTest_results) = 144.35

The problem of multiple comparisons is the following. Imagine, I have two groups and I make 100 tests between these two groups. Let us assume I make these tests at the 5% level (alpha = 0.05) and then assume all my 100 test support the null hypothesis. In expectation ("on average"), the number of incorrect rejections would be 5.

Since we have conducted multiple tests, we get many false positives if we use the uncorrected p-values. Here are the two most popular options to deal with the multiple testing. Note that multiple testing is still an active research area.

1. Control the Family-Wise Error Rate (FWER) so that the probability of getting even a single false positive equals our initial alpha.
2. Control the False Discovery Rate (FDR) so that the percentage of false positives among the null hypotheses rejected is no greater than a chosen value.

The first approach is underpowered, since we are trying to avoid identifying even a single false positive across all the tests conducted. This stringently avoids false positives (type I errors), but leads to many false negatives (type II errors). The second approach is powerful, since we allow a certain percentage of our rejected null hypothesis to be incorrect. Thus, the number of type I errors increases, but the number of type II errors also decreases. The approach to take depends on the application.

Here, the downstream analysis focus on groups of proteins with shared functionality, rather than specific proteins which have altered abundance. Therefore, we can accept a low percentage of false positives. We calculate the FDR using the Benjamini-Hochberg method via the `p.adjust` function and reject the null hypothesis where the FDR < 0.01 (1%). This means approximately 1% of the rejected null hypothesis are false positives but we do not know which ones these are.

```
TTest_results$FDR <- p.adjust(TTest_results$p.value, method = "BH")

FDR_threshold <- 0.01
TTest_results$sig <- TTest_results$FDR < FDR_threshold
```

We can summarise how many proteins have a significant change in abundance using the `table` function

```r
table(TTest_results$sig)
```

```
##
## FALSE  TRUE
##  2731   156
```

Check your knowledge

1. Can you add another command in the cell below to work out how many of the proteins have a significant increase in abundance and how many decrease?

```r
table(ifelse(TTest_results$sig, 'Sig.', 'Not sig.'),
      ifelse(TTest_results$difference>0, 'Increase', 'Decrease'))
```

```
##
##            Decrease Increase
##   Not sig.     1217     1514
##   Sig.           76       80
```

We visualise the t-test results in a so-called "Volcano" plot (see below). In the second plot we add the 95% confidence intervals for the change in abundance. Many of the significant changes are small. In fact, just 16/156 of the significant changes in abundance are greater than 2-fold (Remember that we have log base 2 transformed the abundance data so a change in abundance of 1 on the log scale is a 2-fold change).

```r
cat("Tally of changes > 2-fold for proteins where null hypothesis rejected")
```

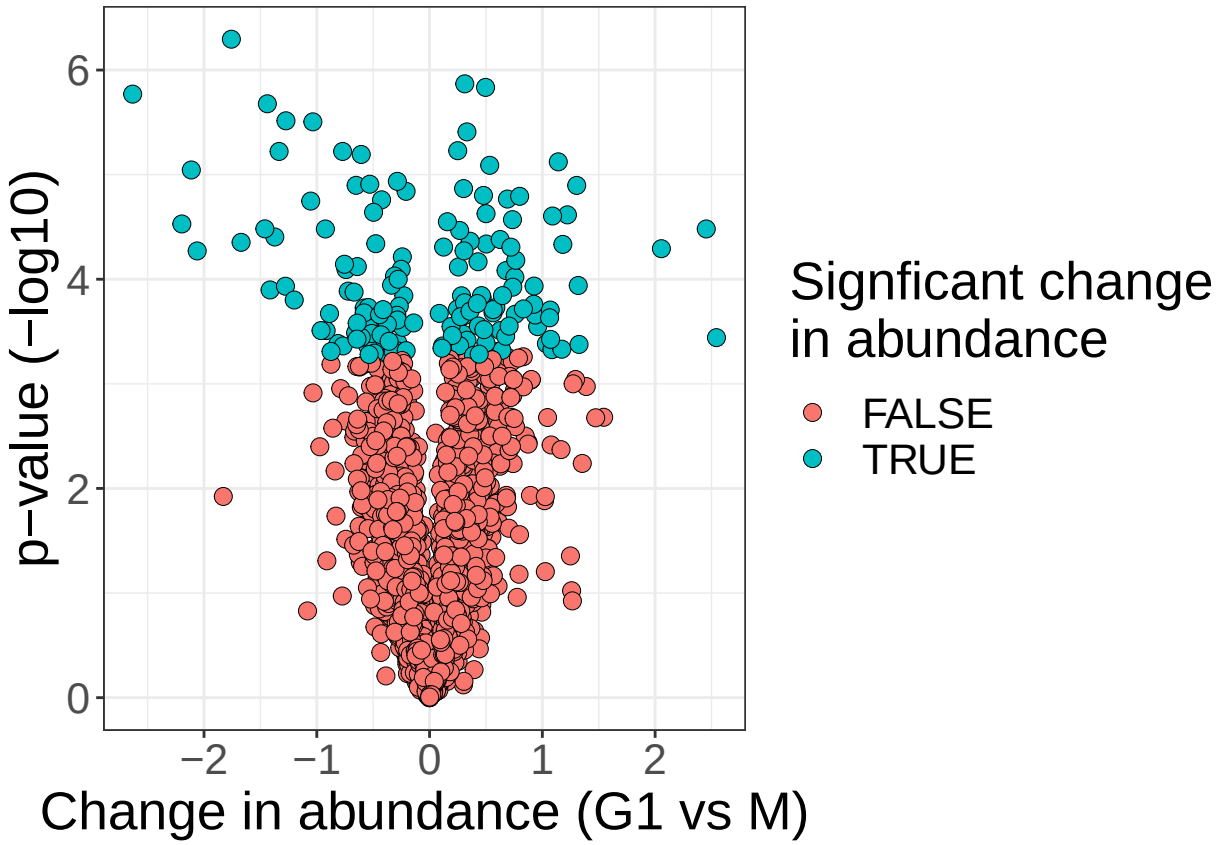
```
## Tally of changes > 2-fold for proteins where null hypothesis rejected
```

```r
print(table(abs(TTest_results[TTest_results$sig == T, ]$difference > 1)))
```

```
##
##   0   1
## 140  16
```

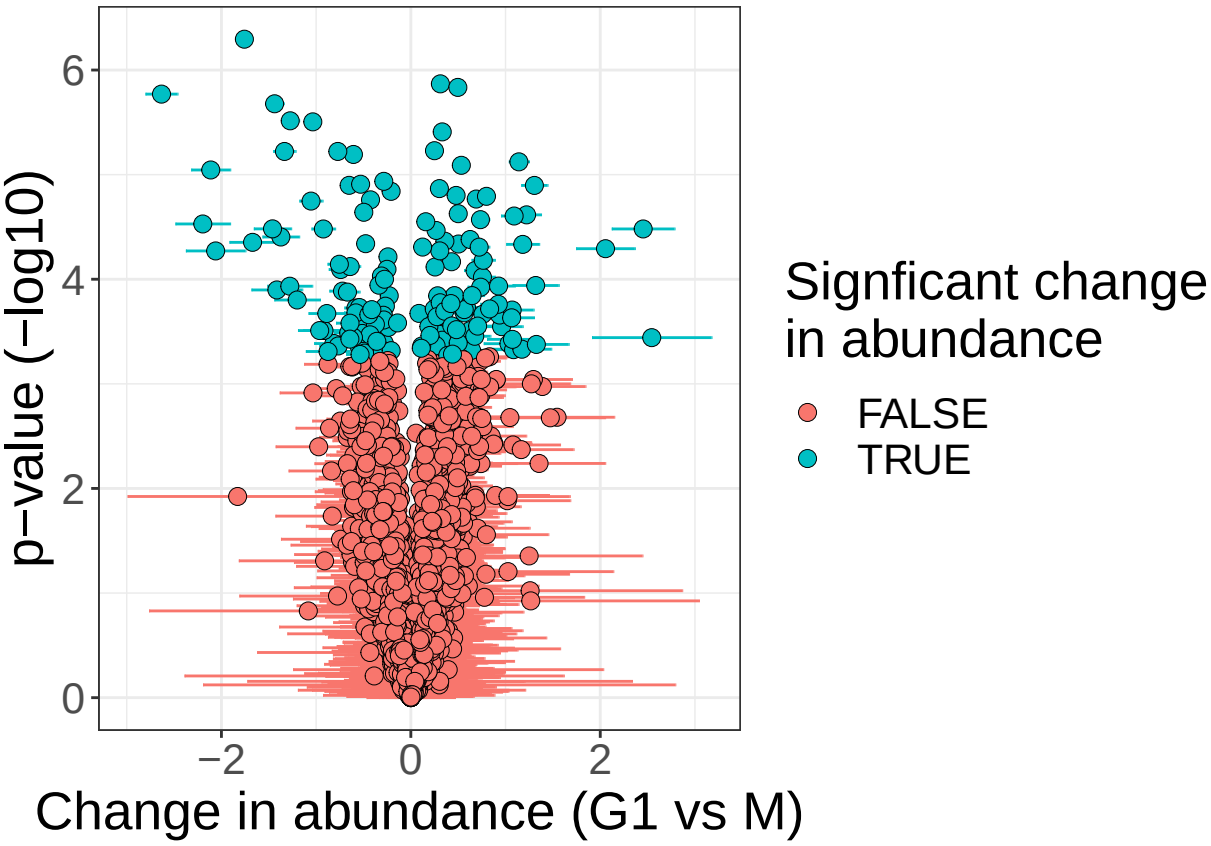
```r
p <- ggplot(TTest_results, aes(x = difference, y = -log(p.value, 10), fill=sig)) +
  xlab("Change in abundance (G1 vs M)") + ylab("p-value (-log10)") +
  scale_fill_discrete(name="Signficant change\nin abundance") +
  theme_bw() +
  theme(text=element_text(size = 20))

print(p + geom_point(pch = 21, stroke = 0.25, size = 3))
```




```
p2 <- p + geom_errorbarh(aes(xmin=CI_diff_low, xmax=CI_diff_high, colour=sig)) +
  geom_point(pch=21, stroke=0.25, size=3) +
  scale_color_discrete(guide=F)

print(p2)
```



4. Functional analysis of proteins with differential abundance

4a. What are the proteins

Now that we've identified the proteins that have a different abundance in G1-phase vs. M-phase, the next step is to investigate the functions for these proteins. First, let's add the protein names and descriptions (from another data file).

```
human_proteins_ids_df <- read.csv(
  "data/human_protein_ids.tsv", sep="\t", header=F,
    colClasses=c("character", "character", "character", "NULL", "NULL"),
  col.names = c("UniprotID", "Name", "Description", NA, NA))

TTest_results_annotated <- merge(human_proteins_ids_df, TTest_results, by.x="UniprotID", by.y="master_p


# we'll write the results out to file so they can be further interogated outside this notebooks
write.table(TTest_results_annotated, './data/TTest_results_annotated.tsv',
            row.names=FALSE, sep='\t')
```

We can inspect the Descriptions for the proteins which have a significant decrease in abundance in G1 vs M

```
TTest_results_annotated %>%
  filter(sig==T) %>% # significant changes
  filter(difference<0) %>% # decrease in abundance
  arrange(p.value) %>% # sort by CI
  select(Name, Description, p.value, FDR) %>% # select columns of interest
  head(20)
```

```
##           Name
## 1  CENPE_HUMAN
## 2   SGO2_HUMAN
## 3  AURKA_HUMAN
## 4   PRC1_HUMAN
## 5  BOREA_HUMAN
## 6  AKTS1_HUMAN
## 7   KITH_HUMAN
## 8  KIF11_HUMAN
## 9   ANLN_HUMAN
## 10  RT31_HUMAN
## 11 HACD2_HUMAN
## 12   VIR_HUMAN
## 13  ETFB_HUMAN
## 14  RT29_HUMAN
## 15 KIF22_HUMAN
## 16  PREB_HUMAN
## 17 KIFC1_HUMAN
## 18  TPX2_HUMAN
## 19 COCA1_HUMAN
## 20  PLK1_HUMAN
##                                          Description
## 1                       Centromere-associated protein E
## 2                                          Shugoshin 2
## 3                                     Aurora kinase A
## 4                       Protein regulator of cytokinesis 1
## 5                                           Borealin
## 6                           Proline-rich AKT1 substrate 1
## 7                             Thymidine kinase, cytosolic
## 8                              Kinesin-like protein KIF11
## 9                                           Anillin
## 10              28S ribosomal protein S31, mitochondrial
## 11 Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 2
## 12                              Protein virilizer homolog
## 13          Electron transfer flavoprotein subunit beta
## 14              28S ribosomal protein S29, mitochondrial
## 15                             Kinesin-like protein KIF22
## 16         Prolactin regulatory element-binding protein
## 17                             Kinesin-like protein KIFC1
## 18                            Targeting protein for Xklp2
## 19                            Collagen alpha-1(XII) chain
## 20                  Serine/threonine-protein kinase PLK1
##         p.value         FDR
## 1  5.071636e-07 0.001213524
## 2  1.699184e-06 0.001213524
## 3  2.101705e-06 0.001213524
## 4  3.057858e-06 0.001290723
## 5  3.129568e-06 0.001290723
## 6  6.001327e-06 0.001543256
## 7  6.003272e-06 0.001543256
## 8  6.414643e-06 0.001543256
## 9  9.024699e-06 0.001736954
## 10 1.158814e-05 0.001926388
## 11 1.235089e-05 0.001926388
```

```
## 12 1.265304e-05 0.001926388
## 13 1.444128e-05 0.001985333
## 14 1.741950e-05 0.001987746
## 15 1.790142e-05 0.001987746
## 16 2.290124e-05 0.002392901
## 17 2.961931e-05 0.002591241
## 18 3.299144e-05 0.002652798
## 19 3.307957e-05 0.002652798
## 20 3.946135e-05 0.002998024
```

Can you see what functions some of these proteins might have based on their descriptions?

As with most such analyses there are likely be some proteins you have heard of, but many more which you haven't. After all, there are ~20,000 protein-coding genes in the human genome! It's also hard looking at the proteins which have changed to understand what shared functional pathways they may have in common. Many of the protein's functions may not be obvious just from their description. Likewise, the sub-cellular localisation may not be obvious, and it could be that most of the proteins with altered abundance have the same localisation which would be an interesting observation. Even if you were a fountain of knowledge on human proteins, it's difficult by eye to know if an observation is unexpected. For example, if you see 4 kinases which are all more abundant in G1, is this a relevant observation? To answer this, you would need to know how many kinases *could* have changed abundance in your experiment, from which you can estimate how many kinases you would expect to see *by chance*, given the number of proteins with an altered abundance. In short, just looking at the protein descriptions and using your prior knowledge only gets you so far.

4b. Identifying over-representation of particular protein functions/localisations

Thankfully, the Gene Ontology (GO) consortium have defined a set of descriptions for genes and their protein products, split into 3 categories, Molecular Functions, Biological Processes and Cellular Localisations. These terms are hierarchical. For example as shown here, `RNA binding` is a child term of `Nucleic acid binding` and a parent term of e.g `RNA cap binding`.

To understand the biological relevance of the protein abundance changes between M and G1 phase, we can look for GO terms which are over-represented in the proteins with a significant change. A GO over-representation analysis involves asking whether a GO term is more frequent in a selected set of proteins (the `foreground`) relative to all the proteins which could have been selected (the `background`). As with any over-representation analysis, it's important to consider what the `foreground` and `background` should be.

In this case, we could either perform:

1. One test where the foreground is all proteins with a significant change in abundance, or
2. Two tests, one where the foreground is all proteins with a significant increase in abundance, and the other is proteins with a significant decrease in abundance.

Check your knowledge

1. What do you the most suitable background would be for the GO term over-representation analysis? All proteins in the `TTest_results` object. These are the proteins that *could* have been found to have significantly increased/decreased abundance. Other human proteins were not tested so should not form part of the background.
2. Do you think it makes more sense to perform one or two tests for the GO term over-representation analysis? Definitely two. We usually expect different biological processes to be switched 'on' and 'off' when comparing conditions

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To simplify this practical, we use GORILLA but note there are many better tools available and one should always be warying of confounding factors when performing functional enrichment analyses. For example, significant fold changes are usually easier to detect for more abundant proteins.

First we save our lists of proteins which have increased or decreased abundance in G1 vs M, and the background set of proteins.

```
background_proteins <- TTest_results_annotated$UniprotID

TTest_results_sig_relevant <- TTest_results_annotated %>%
  filter(sig==T) %>% # retains sig changes only
  select(UniprotID, difference) # select required columns

table(TTest_results_sig_relevant$difference>0)
```

```
##
## FALSE  TRUE
##    76    80
```

```
# identify proteins with increase in abundance
up_proteins <- TTest_results_sig_relevant %>%
  filter(difference>0) %>% # positive change in abundance
  select(UniprotID)

dw_proteins <- TTest_results_sig_relevant %>%
  filter(difference<0) %>%
  select(UniprotID)

write(unlist(up_proteins), "data/foreground_up.tsv")
write(unlist(dw_proteins), "data/foreground_dw.tsv")
write(background_proteins, "data/background.tsv")
```

Then go to the web-link:

http://cbl-gorilla.cs.technion.ac.il/

To use GORILLA:

- Step 1. Select `Homo sapiens` for the organism
- Step 2: Select `Two unranked lists of genes (target and background lists)`
- Step 3: Below the `Target Set` box, click browse and upload your foreground data, e.g "foreground_dw.tsv". Below the `Background set box`, click browse and upload your background data.
- Step 4: Select "All" ontologies

Leave the advanced parameters as they are and click `Search Enriched GO terms`.

The GORILLA output is split into three sections for `Processes`, `Function` and `Component`. For each section there is graph depicting the over-represented terms and their relationship to one another. Below this there is a table detailing the GO terms which are over-represented. Each GO terms is associated with a p-value for the over-representation and an estimation of the False Discovery Rate (FDR). For this practical, only consider any GO term over-representation where the FDR < 0.01 (1E-2)

Check your knowledge

1. What GO terms are over-represented in the proteins with a change in abundance between M and G1 phase? Too many to include here!
2. Why might there only be over-represented GO terms in the proteins with decreased abundance in G1 phase? The change in protein abundance from M to G1 is predominantly one of microtubule & spindle proteins no longer being required and therefore degraded. It doesn't appear that any functional group of proteins are required in G1 that aren't equally abundant in M. Increases in abundance probably reflect multiple very small changes in protein requirements in G1 vs M.

3. Why do you think there are so many related GO terms identified? GO terms are hierarchical. So the same proteins will contribute to many different terms being signficiantly over-represented.
4. From inspecting the over-represented GO terms, can you say whether the Nocodazole has had the desired effect? It appears so. The most over-represented terms (by p-value) include 'cell cycle process (BP)', 'sister chromatid segregation (BP)', 'microtubule binding (MF)' & 'spindle (CC)'.
5. What other analyses could you perform to better understand the function of these proteins? You could consider the following (suggested reference databases in parentheses)

- The biochemical pathways they are known to operate in (KEGG, etc)
- Their interacting partners (IntAct, String.db, etc)
- The co-regulation of their mRNAs (Expression Atlas)
- Protein subcellular localisation in experimental datasets
- Previously published proteomics studies of these cell cycle stages

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You've now completed the practical - Well done!

If you want to continue, you can investigate the affect of changing the thresholds used above for selecting the proteins with altered abundance. Does this change your biological interpretation of the results? Alternatively, you can have a look for R packages, software, online tools or publically available data which could help you to perform the analyses you've suggested in the final question above. Finally, if you're interested in a) further exploring how to select more 'biologically relevant' changes in protein abundance, or b) a demonstration of the pitfalls of thresholding on the point estimate of the difference between means, see the notebook entitled "Thresholding_on_point_estimate".
