

Proteome-wide Screen for RNA-dependent Proteins in Non-Synchronized HeLa cells

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Contents

1	Introduction	2
1.1	Summary of experiment	2
1.2	Goals	2
2	Materials and Methods	2
2.1	Description of the Dataset	2
2.2	T-tests	2
2.3	K-means	3
2.4	Linear Regression	3
3	Results	3
3.1	Data Clean Up	3
3.2	Normalization	3
3.2.1	Repwise Normalization	3
3.2.2	Fraction-wise Normalization - Scaling	4
3.3	Reproducibility	5
3.4	Smoothing the Curve	5
3.5	Maxima	5
3.5.1	Identification of global Maxima	5
3.5.2	Finding significant peaks	6
3.6	Clustering with K-means	7
3.7	T-test to compute p-values	7
3.8	Linear Regression	8
3.9	Comparison of models for RDP identification	8
4	Discussion, Outlook and Conclusion	8

1 Introduction

Cancer, neurological diseases and muscular atrophy - What do these diseases have in common? All of them include RNA-binding proteins. RNA-binding proteins (RBP) are proteins that bind RNA and form ribonucleoprotein particles. They are involved in a number of processes like translation, alternative splicing, RNA editing and chemical modification as well as protein modification. RBPs belong to the RNA-dependent proteins (RDP) together with RBP-binding proteins. These RBP-binding proteins bind RBPs when they assemble with RNA. Their complex interactions are crucial for cellular functions and therefore it is of utmost importance to identify RDPs.

1.1 Summary of experiment

To identify RDPs a proteome-wide screen was conducted with mass spectrometry. HeLa S3 cells in different cell cycle stages were lysed (Figure 1). The cell lysate was split into a control and an RNase group. The RNase group was treated with RNase, which causes the degradation of RNA. RBPs can no longer bind to RNA and RBP-binding proteins to RBPs. The control group did not receive any further treatment and therefore all RNA and RDPs interact. The cell lysate was transferred onto a sucrose density gradient.

After ultracentrifugation the RNA-protein complexes or proteins alone will end up in one of 25 fractions, depending on their molecular weight. A heavy RNA-protein complex will travel further and land in a higher fraction than a single protein from the RNase treatment. This would create a left shift and is expected to happen for RDPs.

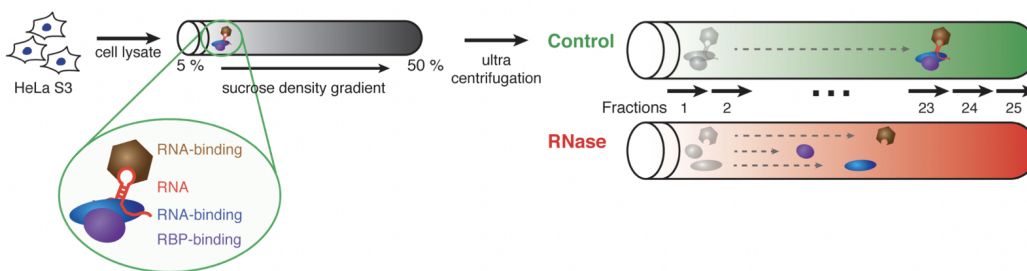


Figure 1: Generation of data

1.2 Goals

The main goal is to automatically identify RDPs on the basis of the fraction shift between control and RNase treatment.

2 Materials and Methods

2.1 Description of the Dataset

The dataset consists of 4765 rows which represent different proteins and 150 columns. Those include 3 replica for each of the 25 fractions for control and RNase treatment ($3 \times 25 \times 2 = 150$ columns). For every protein, the protein amount in a certain fraction is given in arbitrary units.

2.2 T-tests

The t-test is a statistical hypothesis test. It follows the student's t-distribution under the null hypothesis. It can be used to identify whether two sets of data are significantly different when compared to each other. A two-sampled t-test checks if the means of the two data sets are equal. The p-value computed, is the probability of obtaining a more extreme value of the test statistics if the null hypothesis is valid. To determine when the probability is very low or high a significance level alpha has to be defined. If the p-value is smaller than the significance level, the null hypothesis can be rejected and the observed

effect is significant. If the p-value is larger than the significance level this means that the effect is not sufficient to reject the null hypothesis and there can be no significant effect seen. The standard value for alpha is 0.05. Furthermore, it is important to check the false discovery rate. For this, the Benjamini-Hochberg procedure can be used. It controls for the that some small p-values happen by chance and this could lead to an incorrect rejection of the null hypothesis. It therefore reduces Type 1 errors [1].

2.3 K-means

K-means is a method for clustering that aims to structure n observations into k clusters. Each observation n is assigned to the cluster with the smallest Euclidean distance to the clusters centroid. After the initial cluster is assigned to each observation, new centroids are set up to have the smallest distance to all its observations. To quantify which amounts of clusters k is the best, the silhouette or elbow method can be used [2].

The silhouette method calculates the distance of every point to its own and the neighbouring cluster (smallest distance of point i to a point that is not a member of its own cluster). Every point obtains a silhouette value between -1 and 1. 1 meaning that the observation fits perfectly into its cluster and -1 meaning that it would be more appropriate to place it in a neighbouring cluster. By changing the amounts of clusters, the overall mean of the silhouette value of all points change. The higher this mean is, the more accurate the clustering works [3].

The elbow method calculates the Within-Cluster Sum of Squared Errors (WSS) for different values of k . When plotting the WSS against the amount of clusters k a declining curve is produced. By choosing the k , for which the WSS first starts to diminish, you receive the optimal amount of clusters.

2.4 Linear Regression

Linear regression is a method used to model the linear relationship between a scalar and an explanatory variable. When there is more than one scalar variable the process is called multiple linear regression. The relationship is modeled using a linear predictor function "the line of best fit". Therefore, the explanatory variable can be estimated by using only existing parameters. The parameters of the regression line are estimated using the least-square method to minimize the sum of squares of the deviations. Since the estimated value and the real value do not generally coincide, their difference is called residuals. The correlation between the estimated parameter and the residuals should be close to zero. The residuals should also have a mean value of zero and be normally distributed.

To determine the quality of a linear model the F-test is applied. The F-test is a statistical test that compares the linear model with test statistic that has an F-distribution under the null-hypothesis. The null model uses the equation $Y = b_0$ with $b_0 = \text{mean}(Y)$. While the full model uses the determined parameters $Y = b_0 + b_1X$ for a simple linear regression [4].

3 Results

3.1 Data Clean Up

Clean Up of the raw data is needed for the following analysis. First we split our dataset into two dataframes containing only the control and RNase group. Those were split further into three subtables representing the three reps. 33 proteins were deleted because they contained at least one zero row in one rep of control or RNase.

3.2 Normalization

Goal of the normalization is to make the three different reps more similar by applying statistical and mathematical functions.

3.2.1 Repwise Normalization

For simpler calculation we created subtables separately for control and RNase containing only one fraction with all three reps (for both control and RNase group, 50 subtables were created). For every

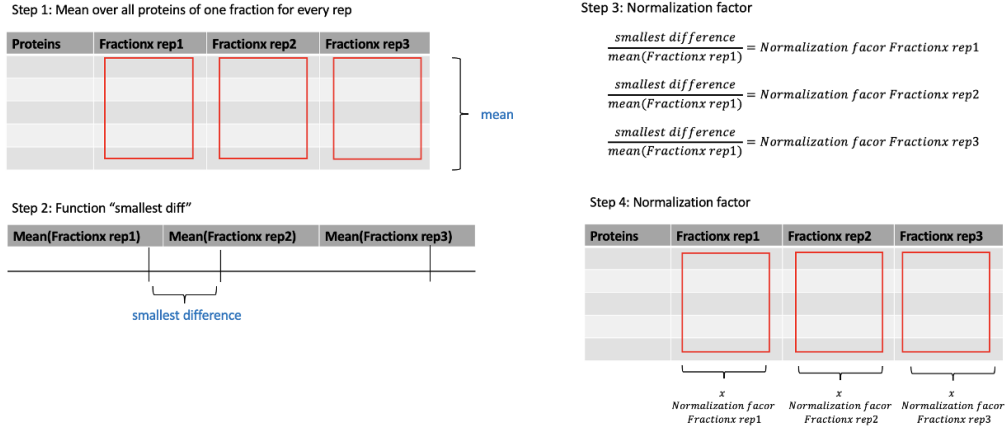


Figure 2: Steps of Normalization

subtable, the mean over all the proteins for every rep from one fraction was calculated, creating 3 mean values for each of the 50 subtables. The function *smallest diff* was created to find the two reps for every individual fraction with the smallest difference of their mean values. The mean value of these two reps over all the proteins from one fraction was determined (Figure 2). The normalization factors for every fraction for each rep were calculated by dividing the mean from the most similar two reps by the individual means of one fraction for each rep (for each of the 50 subtables containing one fraction with three reps, three normalization factors were determined). By multiplying all the values of one fraction of one rep with its corresponding, individual normalization factor, we received the normalized values for all reps and for both control (Figure 3a) and RNase (Figure 3b) group. When comparing the unnormalized values with the normalized values, by summing up all the proteins of all fractions for each rep, it is noticeable how the reps have been statistically adjusted.

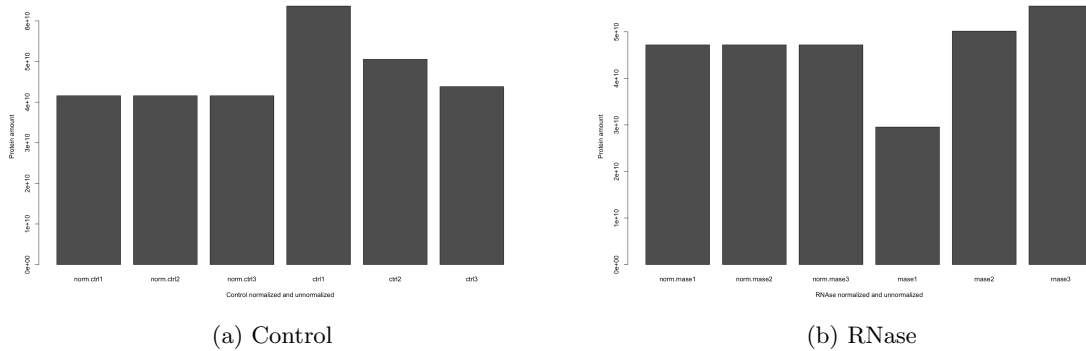


Figure 3: Barplots before and after normalization

3.2.2 Fraction-wise Normalization - Scaling

After creating the vectors with the normalized values for each fraction, we were ready to scale our data. We created a for loop for each sample which first sums up the values of each fraction and sets this sum to 100. We then calculated the proportion for each protein amount in comparison to the scaled sum of the whole row. This gave us values of protein amount between 1 and 100 and a sum of 100 for the sum of a protein over all fractions. This facilitates the comparison of our values between the reps.

3.3 Reproducibility

The three reps for control and RNase group were adjusted during normalization. Nevertheless, it is possible that they differ too much in order to be used in further analysis. Therefore we examined the reproducibility of the reps with correlation. First the normalized data was transformed so that the columns consist of proteins and rows of the different protein amounts in each fraction. We created the function *low.cor* that first calculates the Pearson correlation over all fractions of each protein of two reps. It returns only the column numbers of proteins, with reps that have a correlation coefficient beneath 0.7. By applying *low.cor* six times (rep1 rep2, rep1 rep3, rep2 rep3 for control and RNase) we could create a dataframe that contains only proteins with low correlation and includes how often a protein did not correlate well. Proteins that have a low correlation coefficient twice or thrice (thrice meaning that no rep correlates well) are deleted.

3.4 Smoothing the Curve

To smoothen the curve, we created two for loops. The first loop goes through each row so that the second for loop is applied to each protein. The second loop takes fraction i and calculates the mean of fraction i , $i-1$ and $i+1$. This mean will then replace the original value of the protein amount in fraction i . Since there is no fraction before fraction 1 and no fraction after fraction 25, the second loop is only applied to fraction 2-24 for every protein of each rep. We will still have 6 vectors containing every protein and its 25 fractions, but these values will now be more similar to its neighboring values due to the smoothing method we have applied. This step would be essential before fitting the curve to a Gaussian curve for certain proteins. In Figure 4 and 5 the result of smoothing can be seen for the protein AHNK_HUMAN. Individual fractions are corrected and the curve looks more similar to the Gaussian curve.

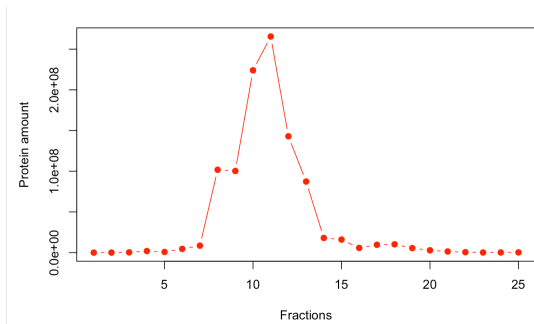


Figure 4: AHNK_HUMAN before Smoothing

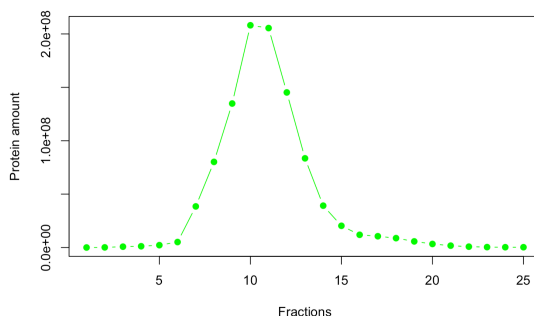


Figure 5: AHNK_HUMAN after Smoothing

3.5 Maxima

The relevance of the maxima for the definition of a Protein as one that binds to RNA lies in the premise, that the peaks of protein seen in a certain fraction within our control group will then have a peak shift once the same mass spectrometry has been performed in the presence of RNase. Thus, it is essential that we are able to find the maxima of each and every single protein, so that we can later on compare these values.

3.5.1 Identification of global Maxima

With our now smoothened data, we can now create a function that defines our maxima and apply it, with the goal of creating a new dataframe consisting of just the fractions where the global maxima for control and RNase can be found.

We defined a function that takes a protein from our normalized dataframes and goes through each fraction, comparing the protein amount in it to a m -number of values before and m -number of values after the to be determined fraction (m being an adjustable parameter, which allows for heightened robustness against outliers and fluctuation).

```

find_peaks <- function(x, m=50) { #In this case m was set to m=50, since for our
  #first run we mainly want to observe the global maxima of each protein
  peaks_pos <- sapply(1:length(x), FUN = function(i) { #In "peaks_pos" all values
    #are saved of positions where the function has found a peak in, while iterating
    #over all fractions of all proteins
    if (x[i] == 0) return (numeric(0)) #If any value found is equal to 0, then said
    #value will be returned as an empty one
    lborder <- max(c(1, i-m)) #This part of the function makes sure, we stay within
    #our borders. While the function is iterating through the values it, it will do
    #so and either return the value of the position it finds itself at or, in the
    #most extreme of cases, return the value "1"
    rborder <- min(c(i+m, length(x))) #Similarly to the lborder, rborder makes sure
    #we stay within our borders, but on the other extreme (length(x) = 25)
    if(all(x[lborder:rborder] <= x[i])) return(i) else return(numeric(0)) #This part
    #of the function will return the position of a maxima, in the case that when
    #comparing its neighboring values of lborder and rborder, the maxima "i" is great
    #or equal; if not, it will return an empty value and continue with the next
    #position
  } )
  return(unlist(peaks_pos)) #Finally, we return the unlisted list of "peaks_pos"
}

```

3.5.2 Finding significant peaks

In the following step, we created a second part of the function, that should take the values out of the *peaks_pos* list (or peaks positions list), and see if the protein amount in said peaks was higher than the defined threshold. If the values in *peaks_pos* are higher than the threshold, they are put into the peaks list. Our intent is to just use the global maxima for the evaluation of the shifts, so by the end, we only had one peak per protein, whenever the function was applied on a dataframe. When comparing the global maxima of the same protein in the control experiment and the RNase experiment, we can appreciate a variety of shift types. These can be assigned to one of four groups: There are left shifts (Figure 6A), where the RNase peak is seen further left of the control peak. Similarly, the right shift (Figure 6B), portrays the RNase peak to the right of the control peak. No shift (Figure 6C) involves no difference in fractions between the peaks of control and RNase and precipitated (Figure 6D) means both peaks are found in the last few fractions.

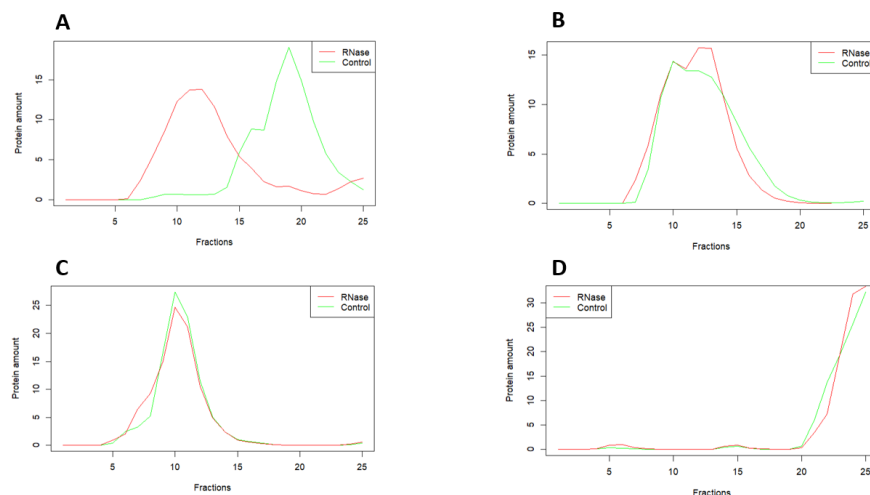


Figure 6: Types of shifts

3.6 Clustering with K-means

K-means was used to identify proteins with similar characteristics in their shifts between control and RNase. We tested different cluster amounts ranging from two to six. To determine which amount of clusters is the best, we used the silhouette and elbow method (Figure 7). We then used the depicted amount of clusters to develop the k-means clustering (Figure 8). For our proteins, the highest mean silhouette value can be seen for $k=5$ with a mean of 0.526. This indicates that 5 centroids, placed at different points throughout the graph, allow the proteins to be assigned optimally to their closest cluster. In the elbow plot it is difficult to decide on a perfect cluster amount. Since we always need to have the biological question in mind, we decided to choose 5 centroids for clustering. That way RDPs are in cluster number 4 (indicated with round dots showing left-shifting proteins). By isolating all proteins in cluster 4 RDPs were discovered by k-means.

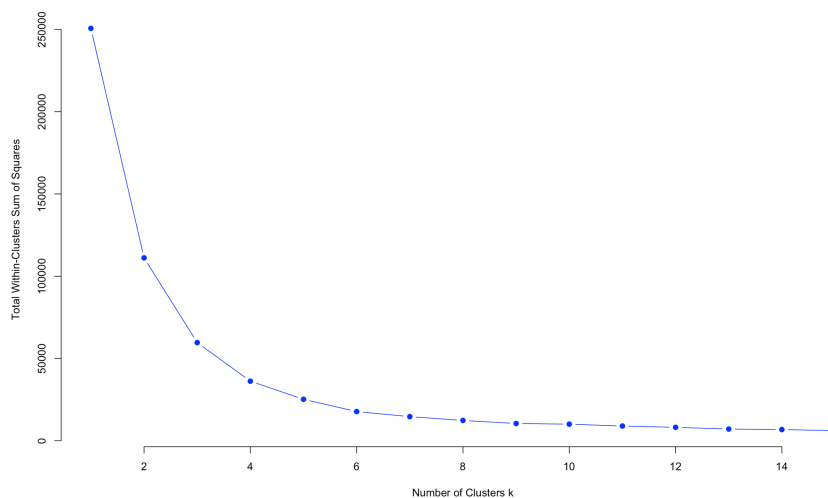


Figure 7: K-means elbow plot

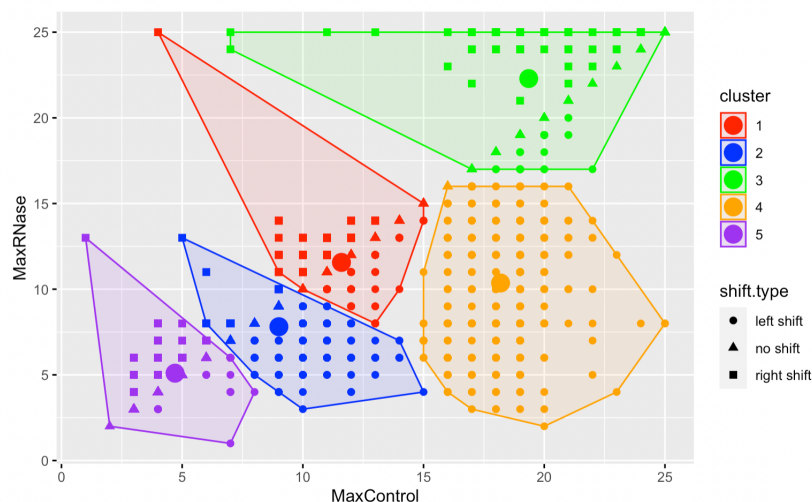


Figure 8: K-means clustering

3.7 T-test to compute p-values

Before coding the actual function to compute our t-test, we firstly created two empty vectors. One to put the p-values in and one for the significant proteins. Afterwards, we created a for loop that firstly

creates a vector for control and RNase respectively and that contains the peaks of each protein. As we encountered some problems with essentially constant values, we used an if function to set the p-value for proteins, which either have a duplicate in the control or RNase group, to 1. We set this value to 1 as this shows that there is absolutely no difference between the values, which in turn indicates that there cannot be a significant shift. This did sort out 78 proteins containing constant values and therefore we were then able to compute the two-sided t-test for all other proteins that differ in the amount of the six replicates. The computed p-value was then adjusted using the method of Benjamini, Hochberg and Yekutieli to decrease the false discovery rate. This left us with an amount of 1921 proteins that did show a significant shift.

3.8 Linear Regression

Our idea for model 1 was to use the correlation between the mean values of control and RNase to predict the amount of fractions for a shift for each protein. We used 90% of all proteins to create the linear regression. The p-value of the F statistic is $2.2 \cdot 10^{-16}$, meaning that the null hypothesis could be rejected. For b_0 we received 8.27 and for b_1 -8.14. When looking at these numbers it is clear that the model does not work properly. It does not predict a shift of more than one fraction, but, as our data shows, there are a number of proteins that have a shift of 2 or more fractions. This is why we rejected this model.

Therefore, we created a second model which predicts whether there is a shift or not. A matrix containing the difference between the maxima fractions of control and RNase was converted to contain 1 when there is a shift and 0 when there is no shift. Model 2 also uses the correlation between control and RNase. The p-value of the F statistic is again smaller than $2.2 \cdot 10^{-16}$, $b_0 = 1.09$ and $b_1 = -0.727$. Then we tested the model with the other 10% of our proteins. A dataframe was created containing the real shift (0 or 1) and the predicted shift. Since the predicted shift is a number between 0 and 1, we set a threshold of 0.2. If the predicted shift is 0.2 or higher we estimate a shift and assign the number 1. If the predicted shift is smaller than 0.2 we estimate that this protein will not shift and assign the number 0. The false positive rate is 10,8% and the false negative rate is 41,5%. Out of 466 proteins, 387 proteins were assigned correctly which means that model 2 predicted 83% of proteins correctly.

3.9 Comparison of models for RDP identification

The two methods we used for RDP identification were by shift and k-means. Identification by shift worked by firstly calculating the difference between the fraction with maximum protein amount of control and RNase. RDPs are characterized as left-shifted proteins and therefore have a negative difference between the maximum fractions. All left-shifted proteins were stored in a matrix *RDP.matrix* with their name and row position.

In addition a t-test was conducted on all proteins. As a result, the vector *sigproteins* contains 1921 proteins which shift significantly. Since it includes also right-shifted proteins, we created the vector *sig.RDP* that is made up of proteins that are in both *RDP.matrix* and *sigproteins* that way it is ensured that only proteins with a significant left-shift are chosen. The number of identified proteins is 401.

Another method of identifying RDPs is by using k-means clustering. Figure 8 shows that left-shifted proteins are concentrated in cluster number 4. We isolated the proteins of cluster 4 in the vector *clus4.RDP*. 551 proteins were found as RDPs.

To verify our results we compared our analysis with information from RDeeP by Maiwen Caudron-Herger containing identified RDPs. For the shift model, the false-positive rate is 6.1% and false-negative rate is 90%. For the k-means model the false-positive rate is 8.0% and the false-negative rate is 85%.

4 Discussion, Outlook and Conclusion

To conclude the report on our project we would like to discuss our results and the methods used to achieve them. The normalization of our data set as well as the data clean-up were satisfactory and in line with our expectations from the project proposal.

As a next step we were planning on conducting a Gaussian fitting for each protein, in order to obtain smoother values. However, due to time constraints, we did not end up using this method. For a higher

accuracy of results and improved noise reduction this could be added in the future.

While determining the maxima of each protein we were only considering global maxima for our final results. This could explain our high false negative rate, as local maxima potentially allow to detect more shifts in control compared to RNase. For better accuracy of the data, in the future, local maxima should be considered as well as global maxima in order to be able to identify more protein shifts and thus more RDPs. An additional improvement would be to conduct a multiple linear regression model containing more variables to expand our research and to use more methods to cluster our data (e.g PCA) in order to have an even more precise identification of left-shifting proteins.

A possible outlook into the future could be that automated RDP or RBP identification becomes a reliable and quick-to-perform method. It is important for research to know about this characteristic of proteins in order to discover all their functions. Cancer, neurological diseases and muscular atrophy have already been identified to involve RBP, but there are likely to be more diseases. Identifying their origin is a major hurdle and yet indispensable.

In conclusion, we are satisfied with our results and proud of the work we have done. However, with more time on our hands, the automated RDP detection could be improved to be more precise and efficient in the future.

References

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