Final Report: Proteome-wide Screen for RNA-dependent Proteins *non-synchronized A549 cells*

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1. Introduction

RNA-binding proteins (RBPs) constitute one of the largest families of proteins in the cell, with over 4000 RBPs identified to date (Gebauer et. al,2020). In addition to the examination of proteins that directly bind RNA, this analysis encompasses RNA-dependent proteins whose interactome relies on RNA, even without direct binding (Caudron-Herger et al., 2019). Unlike proteins with classical RNA-binding domains, these proteins can engage with RNA through intrinsically disordered domains (Corley et al., 2020). For the sake of simplicity, the term “Rdeep” will be used in this report to refer to both RNA-binding and RNA-dependent proteins. These proteins play a crucial role in controlling various aspects of RNA life, function, and efficiency,

thereby acting as essential regulators in numerous cellular processes. Rdeeps are involved in extensive regulatory networks that govern critical processes, including transcription, splicing, RNA modification, intracellular trafficking, translation, and decay (Corley et al., 2020). The significance of Rdeeps in human health is underscored by their involvement in a wide range of diseases. Mutations in genes encoding Rdeeps have been identified as the underlying cause of various disorders, leading to malfunction and tissue-specific defects. Rdeeps are particularly implicated in diseases of the nervous system and cancers, making them promising targets for therapeutic interventions. Remarkably, the prevalence of RBP mutations in diseases is substantial, with nearly one-third of Rdeeps implicated in various pathologies, encompassing over a thousand disease-related Rdeeps identified thus far. In mendelian disorders, Rdeeps outnumber other classes of proteins, including transcription factors, in terms of the prevalence of mutations (Gebauer et. al,2020). To comprehensively elucidate the involvement of Rdeeps in translational control and their roles in disease pathogenesis, further investigations are required. Understanding the mechanistic interplay between Rdeeps and RNA in cellular processes holds great promise for developing targeted therapeutic strategies to rectify RBP-related dysfunctions. Therefore, the goal of this analysis is, to identify which proteins in the given dataset are Rdeeps. The dataset to be analysed contains 3680 different proteins from synchronized mitotic A549 cells. At the end of this project, a linear regression will be developed, which will make it possible to predict RNA dependence for proteins based on their distribution of the control and RNase treated sample.

1.1. Experimental Setup

To collect the mass spectrometry data, a strict protocol was followed. The non-synchronized A549 cells were centrifuged and lysed. One sample was treated with RNAse, while the other served as the untreated control. Both samples were divided into 25 fractions using a sucrose gradient. Ultracentrifugation was performed, allowing the proteins to distribute based on their density. For statistical relevance, the protocol included three repetitions of the experiment. Therefore, triplicates are available for each protein for both Control and RNase. The fractions were then subjected to mass spectrometry analysis to determine the protein abundance, measured in arbitrary units (Caudron-Herger et al., 2019). Furthermore, it should be noted that the protein amount is represented by the y-value whilst the number of the fraction is the x-value.

2. Methods

2.1. Data cleanup

The data clean up consists of checking for missing values and if necessary deleting rows of zeros. Furthermore, the columns are reordered to facilitate the separation of the dataset into two dataframes one containing Control while the other consists of the RNase group.

2.2. Reproducibility

The reproducibility of the replicates (rep) for the Control and the RNase group is calculated separately by computing the pearson correlation between rep1 and rep2, rep1 and rep3 as well as between rep2 and rep3. There are 2 scenarios where a protein is seen as not reproducible. Firstly, if a replicate of a protein has only zeros, its correlation can not be calculated (NA) and the protein is discarded. 83 proteins are affected. Moreover, if all 3 correlations either in the Control or RNase group of one protein are below 0.9 the protein isn't reliable enough for further analysis. Thus 523 proteins are additionally deleted, resulting 3074 proteins from initially 3680 proteins are left for further analysis. Some proteins contain two replicates similar to each other (correlation < 0.9) and a third one that completely differs. Knowing that these proteins have one high and two smaller correlations, the deviating replicate was set to NA and will be ignored when uniting the replicates per protein. Consequently, important data is preserved without loosing too many proteins.

2.3. Normalization methods and Reduction

Since each normalisation method has advantages and disadvantages, we apply three different methods to the dataframe. The **mean-value method (mvm)** is the first data normalisation we use. The mean protein amount of each protein is substracted from the protein amount in each fraction.The values that are zero or smaller than the mean become negative through this subtraction. We set these negative values to zero to simplify further analysis. Afterwards, the sum of the proteinamount in each row is scaled to 100. Furthermore we used the **z-transformation**, which transforms our data into a standard normal distribution with a mean of 0 and a standard deviation of 1 by using the formula: $Z = (X - \mu) / \sigma$. To avoid negative values and not lose to much information as we did with the mean-value method, the smallest value per protein is added to each fraction of the corresponding protein, meaning the smallest value is now 0. In case of the z-transformation we first normalized than scaled to 100. Afterwards we reduced by calculating the mean between the replicates with a higher correlation than 0.9 and scaled again to 100. The last used normalization method is **Min-Max scaling (mms)**, which is a very simple scaling-method where the normalized value x' is calculated

from the original value x as follows: $x' = \frac{x - \min(x)}{\max(x) - \min(x)}$. This means that the highest value is automaticly set to one and the lowest values to zero. With this method it is very easy to calculate the global peaks, but on the other hand, the protein amount, so the area beneath the graph cannot be normalized.

2.4. Gaussian fit

Goal of the gaussian fit is to fit a gaussian function to the data points, in our case the protein amount in each fraction of one protein. Therefore, we establish a list in which the parameters, which describe the distribution are saved. To fit the parameters to our data we used the optim() function implemented in R.

2.6. Data description via Parameters

To identify whether a Protein is RNA dependent or not each protein is tested on 4 parameters. The goal of those 4 parameters is the identification of significant differences between the Control and RNase sample. In the following sections the parameters will be elaborated.

2.6.1. Parameter 1: Significant change of protein amount under global peak

The first parameter identifies a significant change of the protein amount under the global peak for each protein. The global maximum represents the fraction of the sucrose gradient containing the highest protein amount. It is determined by using the *which.max()* function for each protein. If the Protein amount of the global peak fraction is either in the Control or RNase sample 1.7 times higher than the other sample, we defined it as a significant change.

2.6.2. Parameter 2: Significant change of protein amount under local peaks

The next parameter recognizes a significant change in the total protein amount under the local peaks between the Control and RNase sample for each protein. New local peaks can occur if after RNase treatment the protein either dissociates or gains new interaction partners. To detect a significant change the local peaks have to be identified. To be defined as a local peak four criteria have to be fulfilled. First, the y-value of the local peak fraction has to be higher than the y-value of its neighbor fractions. Afterwards we checked if the sd of the local peak's y-value and its neighbors is higher than the sd of the y-values which contain less than 8 % of the total protein amount (*sd.threshold*). The aim is to sort out small fluctuation between the y-values. The third criteria selects the relevant local peaks by sorting those out which have a smaller protein amount than 3 % of the total protein amount. Most of the already mentioned

criteria fit to the global peaks as well and thus global maxima have to be removed. The total protein amount under the local peaks for each protein in the Control and RNase sample is of interest. If it differs more than 7.5 from each other a significant change of the protein amount under the local peak is present.

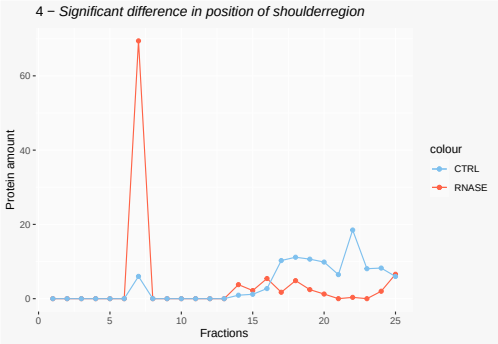
2.6.3. Parameter 3: Significant fraction-shift of global peak

The third parameter focuses on the x-axis depicting the fractions. If the fraction of the global peak in either the Control or RNase sample differs more than two fractions in the positive or negative x-direction compared to the other sample, the protein is defined as RNA-dependent. It lost or gained an interaction partner due to the RNase treatment.

2.6.4. Parameter 4: Significant difference in position of shoulderregions

At last it is observed if shoulderregions occur or disappear after the RNase treatment. A shoulderregion contains more than 2 consecutive fractions with a sd less than the *sd.threshold*. On the contrary to the local peak identification the fractions with small fluctuations are sorted. Of interest are those fractions belonging to shoulderregions which either occur in the Control or RNase sample but not in both.Often parts of the shoulderregions are overlapping, resulting in shoulderregions of interest with less than 3 consecutive fractions. So, a shoulderregion of interest is only significant if it has three or more consecutive fractions.

```
## Warning: Paket 'ggplot2' wurde unter R Version 4.2.3 erstellt
```



2.6.5. Boundaries and Precipitated proteins

The local peakfinder can't find local maxima at the boundaries (fraction 1 and fraction 25) because they have only one neighbor fraction. A local peak at the boundary has to fulfill following requirements. Firstly, it has to have a higher y-value than the two succeeding fractions. Secondly, the protein amount of those three fractions has to be bigger than 10. Smaller protein amounts are not relevant enough. Also precipitated proteins are not described by the parameters and have to be identified separately. They have a global peak in fraction 25 and their total protein amount of 100 has to be split between fraction 23, 24 and 25.

2.7. K-means clustering

Kmeans is used to group a set of data points of a d - dimensional space into a certain number k of clusters. Each cluster has a center, the so called centroid. At the beginning of the clustering process k number of clusters are set randomly. Then, the following steps have to be repeated again and again (n-times), until no further change can be obeserved: 1. The points are assigned to the cluster to whose centroid they are closest to. The distance is commonly the euclidean distance. 2. Thus, the centers of the clusters change and the centroids move. In our case we grouped our proteins in a two-dimensional space (fraction of control-peak and fraction of rnase) into $k = 4$ clusters. We performed kmeans to have an alternative Method for identification of RNA-dependent Proteins, besides our Parameters.

2.8. Regression analysis

The linear regression models the mathematical relationship between a dependent variable and a independent variable. The linear equation is represented as y = mx + n. To analyse the model two values have to be taken into account. At first if the *p-value* is above 5 %, the model has to be discarded. Furthermore, the R-squared value describes the model's accuracy representing the proportion of the variance in the dependent variable that can be explained by the

independent variables. Tow regression models are generated, both working with the pearson correlation between the Control and RNase protein amounts as the independent variable. The two models differ in their dependent variable. One uses the result of our four parameters, while the other uses the global shift amount in fractions. They are trained with 80 % of our dataset, so a prediction can be made for the remaining 20 %.

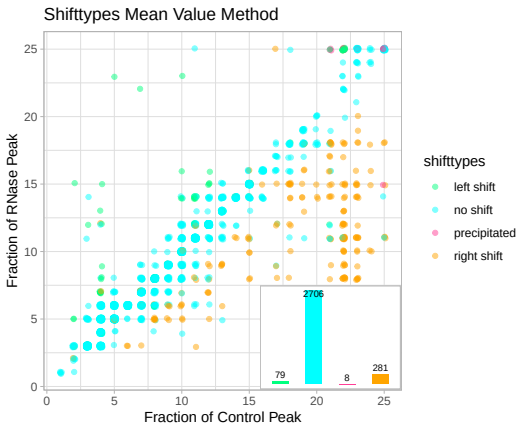
3. Results

3.1 Gaussian fit

On the grounds of our lists, containing the parameters for the gaussian distribution for every protein, it is possible to plot the gaussian curves. The control and RNase curves can be plotted separately or together in one plot for better comparison. Because, we use the optim() function and to not implement further parameters ourselves, the distribution does not show local peaks or shoulder regions. Therefore,the gaussian fit is only used for visualisation not for further analysis. **Graph ???**

3.2. RNA-dependent Proteins

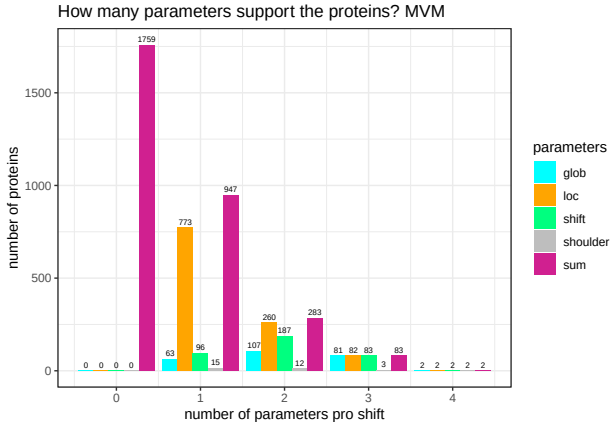
Using just parameter 1 (global peaks), we were able to characterize the shift-type, so whether the peaks show no shift, a right shift, a left shift, or are precipitated. The following graph visualizes our results for MVM:



Using only parameter 1 we were able to identify 368 RNA-dependent Proteins with MVM, 349 with z-transformation and 320 with MMS.

Using our parameters, we were able to identify 464 RNA-dependent proteins with MVM, 468 with z-transformation and 396 with MMS. The following plot shows the significance of the

different parameters and how they they contributed to the characterization for MVM. All proteins that have two positive parameters and all proteins that have solely a global shift are classified as RNA-dependent.



3.3. K-means clustering

We want to cluster the Proteins depending on their global control peaks and their global RNase Peaks. To find out how many proteins would be optimal in theory we used the elbow method that showed us, that two clusters would be optimal. But looking at the biological background, two clusters would not help us to identify RNA-dependent proteins, but rather group them into heavy and light proteins. To gain useful results from kmeans, we forced it to create four clusters. Kmeans clustered the proteins as followed:

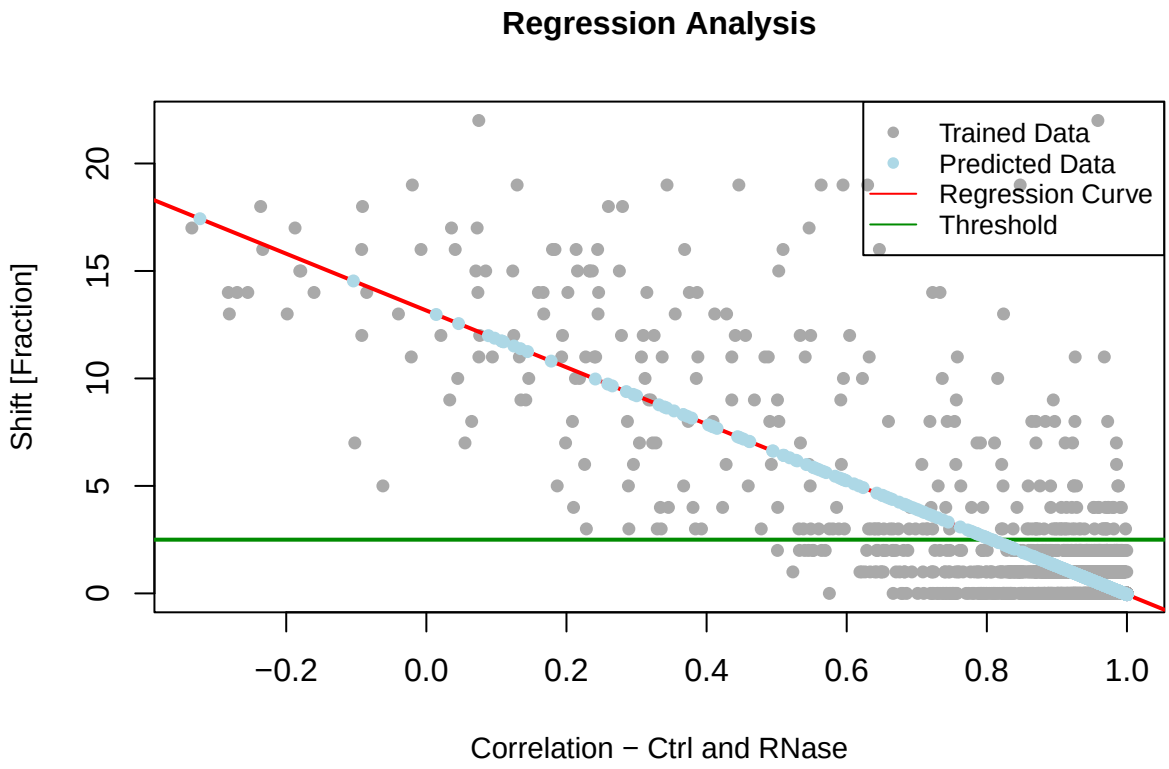
We chose the cluster at the bottom right for every normalization method, to be RNA-dependent (for MVM this would be cluster two). The other clusters can't be identified as RNA-dependent or not. There are no visible differences between the RNA-dependent proteins clusters of the different normalization methods. Using kmeans we were able to identify 160 RNA-associated Proteins for MVM, 155 for z-transformation and 159 for Min-Max-Scaling.

3.4. Regression analysis

The two already described regression models show both a p-value below 0.05 and therefore they can be used for further analysis. The highest R-squared values are detected for the z-transformed data: the model based on the parameters shows a R-squared value of 0.43, while in the model using the global shift amount the R-squared value is 0.66. The model with the higher R-squared value is visualized in the plot below. Each protein is depicted as a dot. As expected the majority of the trained data, marked in grey shows a high correlation and a shift under 2 fractions. On the other hand those with a small correlation have a shift upwards of 2

	mvm	zt	mms
True Positives	422	417	356
False Positives	39	47	37
True Negatives	695	687	697
False Negatives	1905	1910	1971

fractions. As already decided for the parameter **Significant fraction-shift of global peak**: a protein is classified as RNA-dependent protein if it has a shift higher than two fractions. The threshold is marked as a green line in the plot.



3.5. Comparison with Database

We compared our results with the RDeep Database and a table containing non-RNA-associated Proteins from another paper (Quelle?). The following table shows how well the Method via our parameters worked. The table on the left shows the number of true positives, false positives, true negatives and false negatives. The table on the right shows the false negative rate (FNR), the false positive rate (FPR) and the precision.

The following tables do the same for our kmeans results:

	mvm	zt	mms
FNR	0.8187	0.8208	0.8470
FPR	0.0531	0.0640	0.0504
Precision	0.9154	0.8987	0.9059

	mvm	zt	mms
True Positives	152	145	151
False Positives	7	9	7
True Negatives	727	725	727
False Negatives	2175	2182	2176

The following tables do the same for our results that only depended on out global shift:

There are thirteen proteins, that are not present neither in the RDeep data set, nor the table containing non-RNA-associated Proteins from the paper. The following tables show the names of these proteins and whether they are considered as RNA-dependent by our analysis or not. On the left side are the results using our parameters, on the right side the ones obtained by kmeans:

4. Discussion

The goal was to identify RNA-dependent proteins using the data provided by mass-spectrometry. -> t-test

Regarding the regression analysis, the R-squared values differ notably. This can be explained with the following information: for the model based on the results of the parameters, we used zeros for non-Rdeep proteins and ones for proteins we classified as Rdeep, resulting in a low range and therefore a worse linear relationship. The second model has a higher range because we used the shift amount in fractions as the dependent variable, thus has a better linear relationship, shown by the higher R-squared value. To find the Rdeep proteins, we used several methods to find out which variant of our analysis was the best. Overall, we had a very high false negative rate, independent of the methods we used. The lowest was 81.9%. This could be, because our criteria were to strict (e.g. **w**). But it has to be taken into account that the method used in the experiment is not able to detect all RNA-dependent proteins. The RDeep Database uses the results of many different papers that used all sorts of different experiments to identify RNA-dependent proteins. So it is impossible for our results to have a low false-

	mvm	zt	mms
FNR	0.9347	0.9377	0.9351
FPR	0.0095	0.0123	0.0095
Precision	0.9560	0.9416	0.9557

	mvm	zt	mms
True Positives	339	315	336
False Positives	26	31	31
True Negatives	708	703	703
False Negatives	1988	2012	1991

	mvm	zt	mms
FNR	0.8543	0.8646	0.8556
FPR	0.0354	0.0422	0.0422
Precision	0.9288	0.9104	0.9155

mvm	shift?	zt	shift?	mms	shift?
PBIR3_HUMAN	0	PBIR3_HUMAN	0	PBIR3_HUMAN	0
MPP6_HUMAN	0	MPP6_HUMAN	0	MPP6_HUMAN	0
F207A_HUMAN	1	F207A_HUMAN	1	F207A_HUMAN	1
CCD58_HUMAN	0	CCD58_HUMAN	0	CCD58_HUMAN	0
BZW2_HUMAN	0	BZW2_HUMAN	0	BZW2_HUMAN	0
UBIM_HUMAN	1	UBIM_HUMAN	1	UBIM_HUMAN	1
ACOC_HUMAN	0	ACOC_HUMAN	0	ACOC_HUMAN	0
PHB_HUMAN	0	PHB_HUMAN	0	PHB_HUMAN	0
UTRO_HUMAN	0	UTRO_HUMAN	0	UTRO_HUMAN	0
RT36_HUMAN	1	RT36_HUMAN	1	RT36_HUMAN	1
DIEXF_HUMAN	0	DIEXF_HUMAN	0	DIEXF_HUMAN	0
BZW1_HUMAN	0	BZW1_HUMAN	1	BZW1_HUMAN	0
WDR92_HUMAN	0	WDR92_HUMAN	0	WDR92_HUMAN	0
13	3	13	4	13	3

mvm	shift?	zt	shift?	mms	shift?
PBIR3_HUMAN	0	PBIR3_HUMAN	0	PBIR3_HUMAN	0
MPP6_HUMAN	0	MPP6_HUMAN	0	MPP6_HUMAN	0
F207A_HUMAN	0	F207A_HUMAN	0	F207A_HUMAN	0
CCD58_HUMAN	0	CCD58_HUMAN	0	CCD58_HUMAN	0
BZW2_HUMAN	0	BZW2_HUMAN	0	BZW2_HUMAN	0
UBIM_HUMAN	0	UBIM_HUMAN	0	UBIM_HUMAN	0
ACOC_HUMAN	0	ACOC_HUMAN	0	ACOC_HUMAN	0
PHB_HUMAN	0	PHB_HUMAN	0	PHB_HUMAN	0
UTRO_HUMAN	0	UTRO_HUMAN	0	UTRO_HUMAN	0
RT36_HUMAN	1	RT36_HUMAN	1	RT36_HUMAN	1
DIEXF_HUMAN	0	DIEXF_HUMAN	0	DIEXF_HUMAN	0
BZW1_HUMAN	0	BZW1_HUMAN	0	BZW1_HUMAN	0
WDR92_HUMAN	0	WDR92_HUMAN	0	WDR92_HUMAN	0
13	1	13	1	13	1

negative rate. (**unterschiedliche Zellstadien?**) More important for us would be to look at the false-positive rate and precision of our results: Through all methods, normalization via mean-values method was the best. It led to the lowest false-negative rates, the lowest false-positive rates and the highest precision. z-transformation seams to have been the worst of the three methods. So, mean-value method is the normalization method that should be used when analyzing this sort of data. (**Why?**) The following applies to our MVM-results, but is similar to our z-transformation results and MMS results. If we look at the results of kmeans, our parameters and global shift alone, we observe that kmeans is the most precise, but also leaves out a lot more RNA-dependent proteins that were detected by other methods.This is because, we only chose one cluster, that probably contained right shift proteins, leaving out all precipitated and left shifting proteins. To increase the number of proteins found this way, we would have to create more clusters and choose them accordingly. Looking at the false negative rate of the results of only the global shift, it is clear that the global shift already detects more RNA-dependent proteins than kmeans, but also has a higher false positive rate. Still, the low false-positive rate of our global shift results is quite low, and validates our decision to qualify proteins that solely have a global shift, but no other positive parameter, as RNA-dependent. If we look at the results of our four parameters, we see the same pattern. We found way more RNA-dependent proteins, but the false positive rate increased as well. In summary: If we want to be sure that the proteins we identify as RNA dependent, really are RNA dependent, we should choose kmeans. But we have to embrace the possibility that we miss a lot of proteins. If we want to detect many proteins, we should choose our parameters, but take into account that our precision will go down by about 4-5% depending on the normalization method. Looking at the global shift as sole parameter, would be a comprise between precision and quantity. But even with kmeans clustering, our false positive rate is at about 1%, so the results should be verified by another method. Using our methods, we were able to detect 13 new proteins that are not part of the RDeep Database, of which 1-4 are Rdeep, depending on the normalization method used. These proteins could be analysed further with additional experiments, and be added to RDeep.

5.Outlook

With our analysis we verified numerous Rdeeps and identified 1 to 4 new ones. Nevertheless, for medical applications their cellular function and potential mutations causing the proteins malfunction and leading to diseases have to be investigated.

6. Literature

Gebauer et al., RNA-binding proteins in human genetic disease, 2020, Nature Reviews Genetics

Caudron-Herger et al., R-DeeP: Proteome-wide and Quantitative Identification of RNA-Dependent Proteins by Density Gradient Ultracentrifugation, 2019, Molecular Cell

Corley et al., How RNA-Binding Proteins Interact with RNA: Molecules and Mechanisms, 2020, Molecular Cell

7. Appendix