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Investigating the Link between mRNA Binding to Hts1/Tys1 tRNA Synthetases and Charcot Marie Tooth Neuropathy

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Introduction

RNA binding proteins (RBPs) are critical in regulating gene expression as well as the intermediate steps of RNA processing in the nucleus, RNA export from the nucleus, RNA localization within the cell, translation, and mRNA degradation (2,3).[1] RBPs make up a large portion of the entire cell's proteome, and as a result, many mRNA-binding proteins have yet to be identified. However, recent proteome-wide studies have revealed hundreds of novel RBPs showing new modes of binding through disordered regions, scaffolding, shape complementarity, and protein-protein interactions.[2] Previously, RNA binding domains (RBDs) were understood to consist of a limited number of known RNA motifs.[3] Current research looking at ribonucleoprotein complexes like ribosomes suggests their complex protein and RNA interactions do not exist only through conventional RBDs, but can include indiscriminate RNA binding.[4] Indiscriminate RNA binding is seen in the aggregation of intrinsically disordered regions (IDRs) of Tyr amino acids that tend to bind RNA through specific and nonspecific interactions.[5]

A particular subset of RBPs is aminoacyl tRNA synthetases (aaRS). These RBPs are known to primarily catalyze the covalent ligation of an amino acid with its corresponding tRNA in the synthesis of aminoacyl-tRNAs.[6] Recent studies have shown aaRS to regulate gene transcription, apoptosis, translation, and RNA splicing.[7] Through evolution, aaRS gained domains

[1] Levi, Arava, 2019.
[2] Hentze, 2018.
[3] Ibid.
[4] Ibid.
[5] Ibid.
[6] Sampath et al., 2004.
[7] Smirnova et al., 2011.

and insertions into their structure which created novel functions.[8] aaRS were found to have novel extracellular, cytoplasmic, nuclear, and mitochondrial functions. Among these, tyrosyl aminoacyl tRNA synthetase (TyrRS) has a domain within its tertiary structure that prevents the binding of the motif to corresponding receptors, blocking the pro-angiogenic activity that would otherwise occur.[9] Once TyrRS is secreted from the cell, the blocking effect is removed.[10] Additionally, the nuclear functions of aaRS studied revealed that certain mutations in TyrRS led to defects in the export of tRNA from the nucleus, signifying the importance of aminoacylation in nuclear export.[11] Similar domains have been studied in the corresponding histidyl aminoacyl tRNA synthetase (HisRS) domain, indicating that this domain, known to bind tRNA, could facilitate not only the correct binding of tRNA, but potentially its incorrect binding.[12] The varied functions of aaRS could potentially be driven by an evolutionary goal to maximize the diversity of protein capabilities while minimizing the number of genes in the cell's genome.[13]

A recent study used an unbiased approach to isolate and identify polyadenylated RNAs and associated proteins, finding that a significant number of the novel mRNA-binding proteins were cytosolic aaRS.[14] After reviewing the existing data on novel mRNA binding by tRNA synthetases, *in vivo* genomic experiments were performed to identify the mRNA bound by *S. cerevisiae* aaRS.[15] RNA immunoprecipitation and RNA sequencing showed that aaRS bind their own mRNA tighter than other mRNAs, and that HisRS binds its own mRNA the strongest of all tested tRNA synthetases.[16] Further examination of the structure of the bound mRNA sequence revealed

[8] Ibid.
[9] Ibid.
[10] Ibid.
[11] Ibid.
[12] Ibid.
[13] Ibid.
[14] Levi, Arava, 2019.
[15] Ibid.
[16] Ibid.

mutations showing structural similarity to the histidyl tRNA anticodon loop.[17] This confirms a new translational control mechanism through self-associative protein binding at high rates to an anticodon mimic.[18] The finding that translation decreases when its own mRNA binds to the HisRS anticodon mimic suggests a relationship exists between tRNA acylation, mRNA binding, and HisRS translation that could play a role in ribosomal association.[19]

The novel functions of RBPs and aaRS are important to consider for their potential relationships to human disease pathology. Dominant intermediate Charcot Marie Tooth type C, also known as DI-CMTC, is in part caused by mutations in the YARS gene that encodes tyrosyl-tRNA synthetase and mutations in the HARS gene that encodes histidyl-tRNA synthetase.[20] This disease is characterized by progressive impairment and loss of motor control, distal muscle wasting, and sensory loss, among other symptoms.[21] Mutations related to DI-CMTC have been identified in the Tyr tRNA, and current research in *Drosophila* models confirms expressing the YARS mutants induces phenotypes related to the human disease and impaired translation.[22] Expression of mutations on glycyl-tRNA synthetase (GARS) resulted in DI-CMTC-like symptoms of motor performance and cellular-level reduced global protein synthesis in motor and sensory neurons.[23] The mutations seen across various aaRS confirms a shared dominant "gain of toxic function," and a potential connection to the disruption of noncanonical functions of aaRS causing the neurodegenerative symptoms of CMT.[24] Current research works to understand how these

[17] Ibid.
[18] Ibid.
[19] Ibid.
[20] Storkebaum, 2009.
[21] Ibid.
[22] Ibid.
[23] Ibid.
[24] Ibid.

disrupted noncanonical functions of aaRS may cause inhibited protein translation and result in CMT phenotypes.[25]

Materials and Methods

Over the course of this project, yeast strains expressing Hts1 and Tys1 proteins were studied by growing the yeast strains, isolating the protein through various methods, running SDS-PAGE gels, and performing western blot analysis. Eventually, after the presence of desired proteins is confirmed, this project will move towards isolating bound mRNA, performing reverse transcriptase PCR, and building a sequencing library of the resulting DNA to identify bound mRNA. This analysis would then be performed with mutant Hts1 and Tys1 to contrast changes in mRNA binding by CMT-associated mutants. Methodology is shown below:

Yeast Strains and Growth Conditions

Tys1-GFP and Hts1-GFP yeast from the yeast GFP library in a BY4741 background were used, available through Thermofisher.[26] GFP-tagged yeast were grown overnight to OD_{600} 5.0 in 10 mL (0.5mL dextrose, 9.5 mL YEP), diluted to OD_{600} 0.1, and grown to mid-logarithmic phase (OD_{600} 0.5) in 50 mL (1 mL yeast, 2.5 mL dextrose, 46.5 mL YEP) at 30°C. Yeast cultures were then spun at 4000 rpm for 2 minutes at 25°C and frozen until needed. HA-tagged genes Tys1 and Hts1 were cloned into a BG1805 plasmid and are in the yeast host strain Y258.[27] These yeast were grown first in 10 mL (0.5 mL sucrose, 9.5 mL -URA), transferred to a new 10 mL (9.475 mL -URA, .5 mL sucrose, .025 mL dextrose) grown overnight, and diluted to OD_{600} 0.1, and grown in

[25] Nihues, 2015.
[26] Huh et al., 2003.
[27] Gelperin et al., 2005.

a new 10 mL (0.5 mL 40% galactose, 9.5 mL -URA) to mid-logarithmic phase. After this, the yeast were spun down and the pellet was resuspended in a 50 mL solution of -URA and 40% galactose. An additional 50 mL culture with only sucrose and dextrose was prepared as a control to show significance of galactose expression. Proper fusion and expression of the GFP and HA tags, respectively, were in the process of being verified by western analysis.

Protein Extraction

After yeast culture was pelleted, the pellet was washed with 1 ml H_2O and transferred to a new microfuge tube. The solution was spun at 15K rpm for 30 seconds and the liquid was removed. The pellet was resuspended in 50 uL 5 M urea, boiled for two minutes at 100°C, acid washed glass beads were added, and the tubes were vortexed for five minutes. 125 uL Solution A (125 mM TrisCl pH 6.8, 2% SDS) was added, the solution was vortexed for one minute, and boiled for two minutes at 100°C. The liquid was removed from the tube through a hole made by a heated 18-gauge needle, then spun at 2K rpm for one minute. The collected protein extract was spun in a new tube at 15K rpm for one minute, and the supernatant was transferred to a new tube. Protein concentration in the extract was measured via Bradford Assay.

Bradford Assay

Six standards and the two protein samples for the assay were prepared as follows:

	BSA (uL)	H_2O (uL)	Bradford Reagent (uL)
1	0	800	200
2	2	798	200
3	4	796	200
4	6	794	200
5	8	792	200
6	10	790	200
Hts1/Tys1 Sample 1	2	798	200
Hts1/Tys1 Sample 2	5	795	200

BSA or protein sample, H_2O, and the Bradford reagent were added to microfuge tubes and vortexed, then incubated for five minutes. The spectrophotometer was zeroed with a blank sample, and the absorbance of each sample was measured. A graph of the standard was created and used to determine sample protein concentrations (Figure 2).

RNA Pull-Down

Buffer Preparation[28]

Buffer B (1 liter): 20 mM Tris pH 7.5, 140 mM NaCl, 0.1% NP-40, 0.1% SDS, 0.5 mM EDTA, 1 mM DTT, 1 protease inhibitor tablet (per 10 mL)

Wash Buffer: 20 mM Tris 7.5, 1 M NaCl, 0.5% NP-40, 0.1% SDS, 0.5 mM EDTA, 0.5 mM DTT

Strains expressing GFP-tagged HisRS and TyrRS were grown in YEP to logarithmic phase in 50 mL. Cells were lysed using 1mL Buffer B, then alternately vortexed for two minutes and placed on ice for two minutes, three times in the cold room. Lysates were drained from the microfuge tubes into new tubes using the heated needle method. These tubes were centrifuged at 1000 rpm for one minute, placed in new tubes, and centrifuged again at 2000 rpm for two minutes. Each lysate was placed into new labeled tubes and kept at -80°C until needed. After thawing on ice, 500 uL of Buffer B was added to lysates and spun at 4°C for two minutes at 2500 rpm. The supernatant was removed, and two more washes and spins with 500 uL of Buffer B were performed. 50 uL of lysates at this stage were set aside for immunoblot analysis. 25 uL of GFP beads were added to tubes with the remainder of lysates and rotated for two hours at 4°C. After

[28] Levi, Arava, 2019.

two hours, the tubes were centrifuged at 2500 rpm for two minutes at 4°C. 50 uL of supernatants were set aside at this stage for immunoblot analysis. The remainder of the supernatant was discarded, and beads were resuspended in 500 uL cold wash buffer. This solution was rotated for five minutes and centrifuged for two minutes at 4°C, with the cycle repeated three times total. SDS loading buffer was added to the beads and boiled at 95°C to remove any protein bound to beads. 80 uL of 2X SDS loading buffer was added to each lysate in preparation for an SDS-PAGE gel. Samples were heated for five minutes at 95°C, spun down for two minutes at 2500 rpm, and supernatant was collected. Samples of each Hts1 and Tys1 lysate and supernatant, as well as a GFP-tagged control, were run on the SDS-PAGE gel.

SDS-PAGE

SDS-PAGE gels, 4-15% mini-protean TGX gels from BioRad, were run using a 1X SDS Running Buffer. 7 uL of Precision Plus Prestained Protein Ladder from BioRad, 20 uL of products, and a control of varying amounts were run on each gel. Gels were run at 180 V for 45 minutes.

Western Blot

After transferring the gel to the nitrocellulose (or PVD) membrane, the membrane was blocked with TBST/5% milk solution for one hour at room temperature on the shaker. The solution was poured off, and the membrane was incubated with TBST and 10 mL of a 1:5000 dilution of the primary anti-GFP antibody from BioLegend or the anti-HA antibody from Thermofisher for one hour on the shaker. The membrane was rinsed twice with TBST, then washed three times with TBST for five minutes each wash while shaking. Then, the membrane was incubated with 15 mL of a 1:2000 dilution of the secondary antibody, a goat anti-mouse HRP from Thermofisher, and

TBST/5% milk solution for one hour at room temperature on the shaker. The second round of washes was then performed, rinsing two times with the TBST and washing once with TBST for 20 minutes shaking at room temperature. The membrane was then blotted dry, placed on Saran wrap, and incubated for five minutes with 3 mL of 50:50 SuperSignal West Dura Extended Duration Substrate, a luminol-based enhanced chemiluminescence (ECL) horseradish peroxidase (HRP) substrate for western blot analysis. The solution was drained off, then the membrane was dipped in TBST and imaged on an Azure gel imaging system.

Results

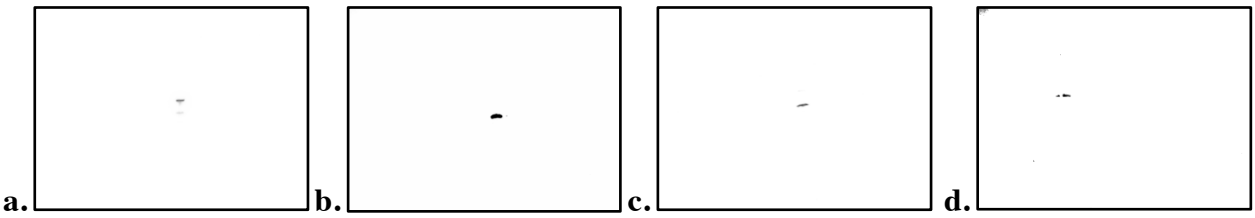

Figure 1. a. The 12/04/19 western blot of the Hts1 and Tys1 RNA pull-down products, lysates, and positive GFP control. The band shown represents the GFP control. **b.** The 01/27/20 western blot of the Hts1 and Tys1 protein extracts from the Muriel Brengues technique and positive GFP control. The band shown represents the GFP control. **c.** The 01/29/20 western blot of the Hts1 and Tys1 protein extracts from the Muriel Brengues technique and positive GFP control prepared 01/27/20, run with a higher concentration of protein. The band shown represents the GFP control. **d.** The 01/27/20 western blot of the Hts1 and Tys1 protein extracts from the Muriel Brengues technique and positive HA control grown in -GAL yeast cells. The band shown represents the HA control.

In each repetition of the methodology, after lysing and extracting the protein from the yeast cells, samples were run on SDS-PAGE gels and transferred to a membrane for a western blot. Imaging by the Azure gel imaging system for each membrane showed no protein on the membrane, with the exception of a pure GFP and HA positive control. The 12/04/19 blot using the samples from the RNA pull-down and protein lysis showed only evidence of the positive GFP control (Figure 1. a).

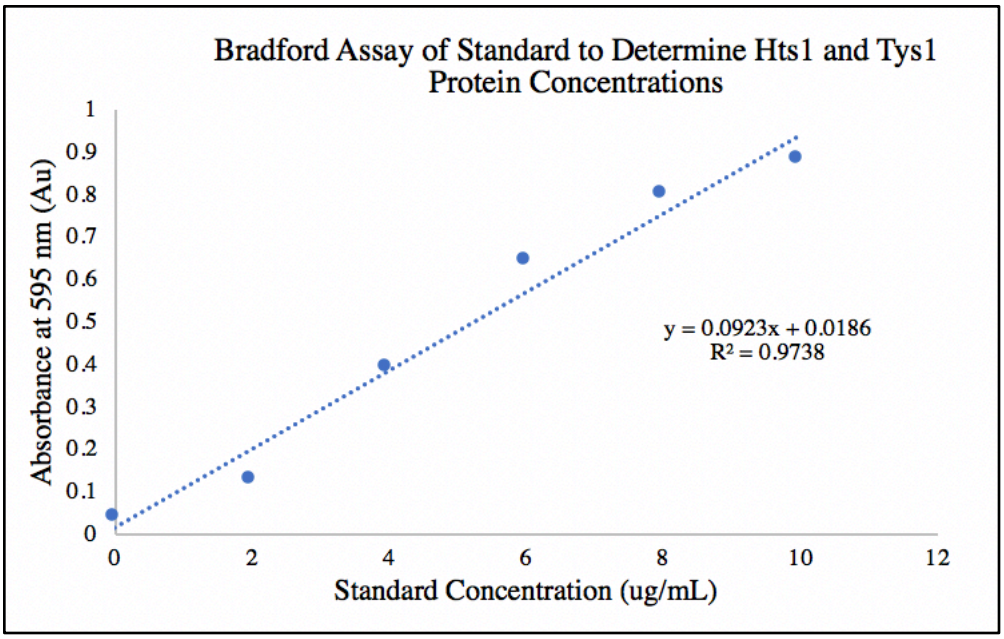

Figure 2. Bradford Assay of the standard for determining Hts1/Tys1 protein concentrations.

Due to this result, a Bradford Assay was performed to confirm the presence of protein in the samples. The assay of the protein extracts confirmed 2-3 ug/uL in each Hts1 and Tys1 protein extract. Based on the equation $y = 0.0923x + 0.0186$, a 1 uL sample of Tys1 protein extract contains 2.77 ug protein (Figure 2). A 1 uL sample of Hts1 protein extract contains 2.93 ug protein (Figure 2).

Because no RNA was seen in the RNA pull-down samples, future Western blots contained only samples from the protein extraction. The 01/27/20 western blot shows only the positive GFP control, and to address this, the 01/29/20 western blot increased the protein concentration in each sample four-fold, but still only the positive GFP control was seen (Figure 1. b, c). Considering the potential low expression in the current yeast cells, new -GAL yeast cells were grown to test for increased expression of the Hts1 and Tys1 proteins. Both a nitrocellulose and PVD membrane were used to test for the possibility of sample loss during membrane washing, but this modification saw no change in results, and even the positive GFP and HA controls were not seen. Using a new

HA antibody with the -GAL cells, the western blot still resulted in only a positive HA control on the image (Figure 1. d).

Discussion and Future Directions

The lack of results seen on the western blots could be explained by many reasons. Despite the Bradford Assay showing a concentration of 2-3 ug/uL in the protein extract samples, over the course of the multiple western blots performed, protein concentration could have varied, and too little protein could have been present in some prepared gel samples. Additionally, there is the possibility that the entirety of protein in the sample could have not been tagged with the GFP or HA tags, and the Bradford Assay showed protein that would not have appeared on the blots. A positive control of pure GFP and HA was used in respective gels to determine if there was an issue with the antibody working properly, but when the positive controls were shown in the images (Figure 1), it was concluded there was no issue with the antibody. The possibility of the proteins being present in minimal amounts was addressed by loading samples with higher concentrations of proteins, but still no protein showed on the membrane. Considering the lack of results could have been caused by the membrane used, a PVD membrane was used instead, but likewise showed no results, indicating the issue was not due to the membrane. After testing each of these potential sources of error, the conclusion was reached that the expression level of these specific proteins could simply be too low in these yeast strains.

Moving forward, future research could introduce new methodology to assess these proteins' functions from a more direct route. One potential source could be using a TAP-tagged plasmid in *E. coli* paired with the genes of interest. After transforming the *E. coli* into yeast strains, an expression test could be performed by growing yeast in galactose to turn on expression of the

genes, extracting the proteins, and performing a western blot. Under this method, the yeast will have TAP-tagged plasmids with the genes of interest, so the protein extracts will contain the Hts1 and Tys1 proteins. Alongside this method, Protein-A bead protein pull-down, under gentle conditions, could be used to extract the RNA with phenol chloroform to separate the protein from the mRNA. RT-PCR could be performed to make a DNA copy from the mRNA, and then perform PCR to look for specific genes present that bind to the mRNA. Once protein expression is confirmed, this procedure can be repeated with CMT causing mutants of Hts1 and Tys1 to identify any changes in mRNA binding caused by the disease associated mutants.

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