MHC Class II Transactivator CIITA as a Potential Therapeutic Target for Amyotrophic Lateral Sclerosis

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Claudia Leonard

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Abstract

The incidence of ALS is rising, and with onset of the disease usually occurring around 50-60 years of age, an effective treatment is urgent. Many of the proposed ALS pathogenic mechanisms, including neuroinflammation and oxidative stress, are currently being investigated. However, the effect of antigen presentation on ALS disease progression is unknown. Therefore, my goal for this project is to uncover therapeutic targets for ALS centered on: 1) the expression of MHC class II genes, encoded by the two proteins of the HLA-DR heterodimeric complex in humans and 2) regulation of the cytoskeleton, through genes such as MYBPC2 and RAB4B. The transactivator CIITA regulates the transcription of these MHC class II proteins; a process that contributes greatly to immunocompetence. Based on our recent unpublished findings about the downregulation of the HLA-DR genes in cell lines with ALS-causative mutations, we hypothesize that loss of their master regulator CIITA plays a role in ALS. Since microglia are the immune cells of the central nervous system, we will examine CIITA, HLA-DR, CD74, MYBPC2 and RAB4B expression in the microglial cell line HMC3, as well as in HeLa cells to study the basic science of these ALS-causative proteins. This study, if successful, could lay the groundwork for small molecule drug screening for CIITA agonists as a new strategy for discovering treatments for ALS.

Dedication

I would like to dedicate this body of work to my mother, father, and sister. Their endless love and support throughout my entire journey has continued to give me strength to pursue both my personal and professional goals.

Acknowledgments

First and foremost, I would like to thank Dr. Robin Reed for her amazing mentorship and guidance as my thesis director throughout the entire development of my thesis. In addition, thank you to my fellow colleagues in the Reed Lab for their support on the execution of these experiments. Thank you so much to my research advisor Dr. James Morris and thesis coordinator Gail Dourian for their counseling and encouragement throughout this process. Finally, I want to extend my utmost gratitude to the Harvard Extension School and its amazing ALM program, which gave me with the incredible opportunity to pursue my Master's degree in Biology while working full-time as a Lab Manager and Researcher at Harvard Medical School.

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Chapter I.

Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurological disease that involves the progressive loss of upper and lower motor neurons (Zarei et al., 2015). There are two types of this disease: sporadic and familial ALS. Sporadic ALS, described to have no known genetic cause, makes up about 90-95% of all diagnosed cases (Zarei et al., 2015). Familial ALS, characterized by the presence of a dominant inheritance component, comprises the remaining 5-10% of cases (Zarei et al., 2015). Common symptoms include muscle weakness, twitching/cramping and decreased coordination. As the disease worsens, patients will begin to experience muscle paralysis in not only the extremities, but also within the tongue, pharynx, diaphragm and intercostal muscles (Nichols et al., 2013). As a result, respiratory failure begins to develop, often resulting in death in those diagnosed with severe cases (Nichols et al., 2013) .

Neuroinflammation, one of the the hallmark symptoms of ALS, caused by microglial activation, the presence of reactive oxygen species, and acetylation-induced dysfunction of mitochondrial proteins have all been postulated to correlate with the severity and advancement of the disease (Obrador et al., 2020). Although there are emerging studies looking into therapies, not many drug therapies have been approved by the Food and Drug Administration, and have only been shown to moderately slow the progression of the disease, rather than completely eradicating symptoms. Therefore, more

research on the mechanism of ALS pathogenesis is necessary in order to find an effective treatment for the disease.

The major phenotypes of ALS are usually described as follows: 1) Limb-onset ALS, which includes both upper and lower motor neuron dysfunction; 2) Bulbar-onset ALS, which is mostly characterized by difficulty swallowing and speaking; 3) primary lateral sclerosis, which is usually accompanied by lower motor neuron issues, but upper motor neuron dysfunction may occur later on, as there is a slower progression with this phenotype; and 4) ALS-plus syndrome, which usually manifests as a combination of additional symptoms such as dementia, loss of sensory processing ability, and lack of autonomic regulation (Chen, 2020). Considering the various phenotypes of ALS, it is difficult for current therapies to completely reverse disease progression. However, identifying additional genes that provide an immunological perspective on ALS pathogenesis could be monumental for discovering a potential cure.

Multiple therapeutic strategies have been used for the treatment of ALS. For example, the first FDA-approved treatment, Riluzole is a glutamate antagonist that inhibits glutamate binding at its excitatory receptors, and also inactivates sodium channels (Chen, 2020). Another example, Mastinib, is a tyrosine kinase inhibitor that targets microglia and mast cell activity (Chen, 2020). Even though these types of treatments slow disease progression, reduce excess oxidative stress and lower the incidence of respiratory depression, only a few treatments had an effect on the survival rate of ALS patients (Chen, 2020). Recently, RNA-targeted therapeutics have been used to target downstream gene expression through interactions with the RNA-induced silencing complex and RNase H-mediated degradation, indicating that a focus on RNA-

binding proteins may be promising for therapeutic strategies centered around subsets of populations of ALS patients (Mejzini et al., 2019).

Epidemiology of ALS

While there are slight differences among populations, recent studies have shown an increase in number of patients being diagnosed with ALS, reporting an overall incidence between 0.6 and 3.8 per 100,000 person-years (Longinetti & Fang, 2019). Population-based investigations have observed a prevalence of 5.2 per 100,000, revealing a major increase since 2015 (Longinetti & Fang, 2019). The mean age of onset for diagnosis is approximately between 54 and 65 years of age (Longinetti & Fang, 2019). Research also suggests that for ALS, there exists a diagnostic delay that may be attributed to the inability to differentiate the ALS from other neuromuscular disorders and the lack of biomarkers to pinpoint during the different stages of the disease (Longinetti & Fang, 2019). Further epidemiological studies provide substantial evidence that the majority of ALS patients have a limited survival rate post-diagnosis, averaging within a 24-50 month window (Longinetti & Fang, 2019). Tracheostomy is often used as an invasive attempt to increase longevity of life; however, studies have shown an increase in lifespan of only up to five years, which tends to include complications from this invasive procedure and is rarely seen in patients over 60 years of age, when the disease stage is the most critical (Longinetti & Fang, 2019).

Other Proposed Causes of ALS

Currently, there are more than 25 genes that have been associated with ALS pathogenesis (Martin, Khleifat, & Al-Chalabi, 2017). The three most studied ALS-causative genes are SOD1, TARDBP, and C9orf72 (Mejzini et al., 2019). The first gene linked to ALS, SOD1, is a gene that encodes mitochondrial-based superoxide dismutase enzymes that initiate a defense mechanism against toxic molecules during cellular respiration (Mejzini et al., 2019). Point mutations in the SOD1 gene are associated with the augmentation of oxidative stress, mitochondrial dysfunction and protein aggregation. Consequently, these cellular imbalances accelerate disease progression and decrease survival time in ALS patients (Mejzini et al., 2019). Many clinical trials are underway for SOD1 inhibitors in attempts to halt the severity of ALS disease progression.

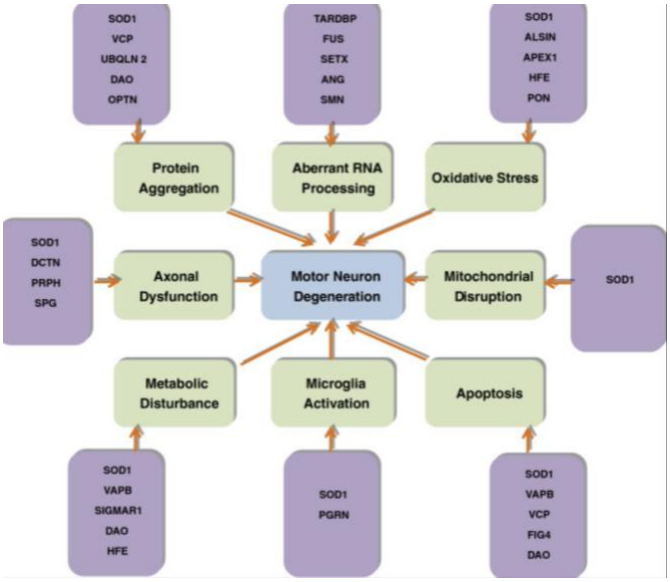

Figure 1. Genes Associated with Motor Neuron Degeneration.

Description: Gene expression in various cell type have been associated with the physiological consequences caused by ALS (Chen et al., 2013).

Another commonly studied ALS protein is TDP-43, which is a DNA/RNA binding protein that is encoded by the gene TARDBP. TDP-43 has numerous functions, including pre-mRNA splicing regulation, maintaining mRNA stability and transport, and translation (Martin, Khleifat, & Al-Chalabi, 2017). Missense mutations at the carboxy-terminal of the gene transcript in the TARDBP gene have been linked to neurodegeneration, confirming its candidacy as ALS-causative gene (Martin, Khleifat, & Al-Chalabi, 2017).

The Dual Role of Microglia in ALS

Microglia-induced neuroinflammation is a major component of the pathogenesis of multiple neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ALS. However, recent studies suggest that microglia have both toxic and protective roles in the central nervous system. Microglia protect the CNS from pathogens and debris from damaged neurons through phagocytosis (Geloso et al., 2017). Microglial activation has two phenotypes: M1 (proinflammatory) and M2 (anti-inflammatory/neuroprotective). The M1 phenotype, termed "classical activation" involves the production of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, interferon-γ, reactive oxygen species, and proteases (Geloso et al., 2017). On the other hand, the M2 phenotype, known as "alternate activation", is displayed when microglia are exposed to cytokines such as IL-3 or IL-4 (Geloso et al., 2017). A major feature of this microglial phenotype is the production of anti-inflammatory cytokines that promote reparation of the extracellular matrix. Another anti-inflammatory M2 phenotype ("acquired activation") is induced by exposure to IL-10 or TGF-β (Geloso et al., 2017). This

demonstrates that a proper balance of both M1 and M2 microglia is essential for the maintenance of the immune response and for identifying therapeutic candidates.

However, novel arguments state that proper microglial function is necessary for the survival of motor neurons. Recent studies demonstrate that microglial density increases dramatically during the early stages of recovery, in addition to the upregulation of genes associated with phagocytosis, as shown in Figure 2 (Spiller et al., 2018).

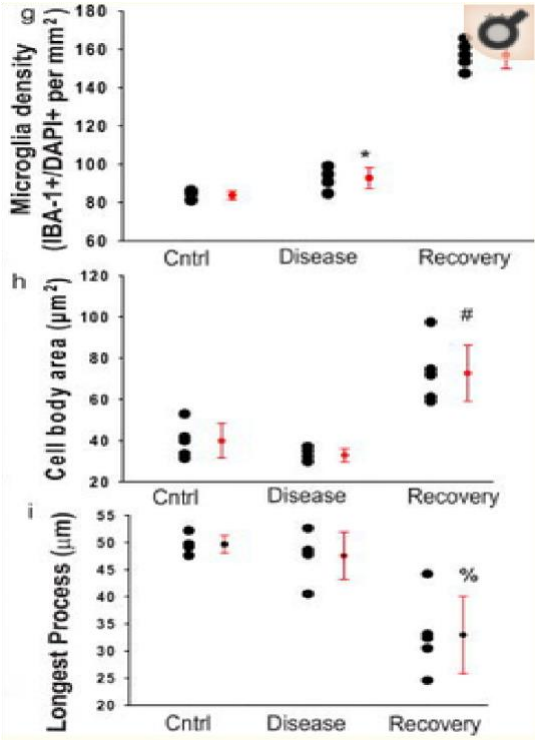

Figure 2. Microglia density in ALS patients.

Description: Comparison of microglial density and morphology changes throughout the progression of the disease and during recovery stage (Spiller et al., 2018).

Microglial density and morphology changes are also proportional to protein aggregate clearance in ALS mouse models, indicating that glymphatic function may be

correlated to extent of recovery (Spiller et al., 2018). Therefore, we hypothesize that microglia play a neuroprotective role in ALS through regulation of multiple interconnected pathways, and we will investigate whether one of these pathways is immunological.

The Glymphatic System and ALS

The glymphatic system, which is the collective name for the paired functions of microglia and the lymphatic system, is known as the main clearance pathway of the brain by way of metabolizing glucose and lipids. Factors that affect glymphatic function include arterial pulsation, respiration, sleep, and aging. The glymphatic system functions through a constant exchange of cerebrospinal fluid (CSF) and interstitial fluid (Jessen et al., 2015). Pressure gradients within the cerebrospinal fluid cause it to enter the perivascular paces of the brain; AQP4 water channels that enclose these blood vessels distributed throughout the glia facilitate this convective transport (Jessen et al., 2015). This continuous movement of the CSF proceeds to drive the interstitial fluid into the perivenous space, after which the interstitial fluid is drained out of the brain and into the lymphatic system (Jessen et al., 2015). Subsequently, the lymphatic system filters out the protein aggregates (now antigens) and allows them to bind to major histocompatibility class (MHC molecules), which ultimately present these antigens to the $CD4^+$ and $CD8^+$ T lymphocytes for initiation of the immune response (Cueni & Detmar, 2008).

One of the most important components of the immune response is the proper functioning of the lymphatic system. The lymphatic system is made up of lymphatic

vessels that drain into larger blood vessels (Cueni & Detmar, 2008). These vessels are connected to lymph nodes, which filter bacteria, foreign soluble antigens and other waste products into lymph-filled interstitial fluid that is returned back into the systemic circulation for removal from the body (Cueni & Detmar, 2008). Lymph nodes also contain T lymphocytes and B-lymphocytes, the key players of the both the innate and adaptive immune responses (Cueni & Detmar, 2008). In addition, the lymphatic vessels also transport antigen-presenting cells (APCs) to lymph nodes, which indicates that the lymphatic system plays a direct role in modulating the immune response (Cueni & Detmar, 2008).

However, recent studies have shown a link between the components of the glymphatic system and the ALS pathogenesis. Specifically, hESC-derived and iPSC-derived motor neurons displayed higher levels of degeneration when treated with CSF from ALS patients (Ng Kee Kwong et al., 2020). In addition, primary rat motor neurons exposed to cerebrospinal fluid from ALS patients experienced a lower survival rate when co-cultured with glia (Barber et al., 2011). Both studies attributed these observations to the increased toxicity of the CSF in ALS patients.

ALS-Causative DNA/RNA Binding Proteins

Among the proteins that have been postulated to cause ALS, a few of the most prominent candidates are the members of the FET family of DNA/RNA binding proteins: Fused in Sarcoma (FUS), Ewing Sacroma RNA binding protein 1 (EWSR1) and TATA-

Box Binding Protein Associated Factor 15). While these three proteins are structurally similar and have shared functions in mRNA processing, alterations in their expression have been associated with the etiology of different diseases. However, all of these genes have been shown to cause or be associated with the development of familial ALS. The FUS protein is part of a complex that regulates pre-mRNA splicing and mRNA export to the cytoplasm. Numerous severe dominant mutations in the FUS gene cause toxic phenotypes in ALS. For example, the R495X mutation, which inserts a premature stop codon in place of the amino acid arginine, promotes a mislocalization of the FUS gene and results in its accumulation within the cytoplasm and induces neurotoxicity (Nakaya & Maragkakis, 2018).

While this implies that FUS is associated with a toxic gain of function, other recent studies have shown that a loss of FUS results in neuronal death (Therrien et al., 2016). Loss of FUS expression is associated with impaired cellular proliferation, decreased mitotic rate, and downregulation of the processes involved with the organization of the cytoskeleton (Ward et al., 2014). In tandem with this evidence, the Protein Atlas indicates that FUS is highly expressed in the brain. Therefore, loss of the FUS protein supports our model of loss of immune function in ALS.

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The Mechanisms of the Immune Response: Antigen Presentation

ALS has different forms of onset and severity. Therefore, it is plausible that defects in the immune system could play a role in ALS pathogenesis. The innate immune system is the body's defense against foreign invaders, otherwise known as infectious pathogens, that disrupt homeostasis by causing disease.

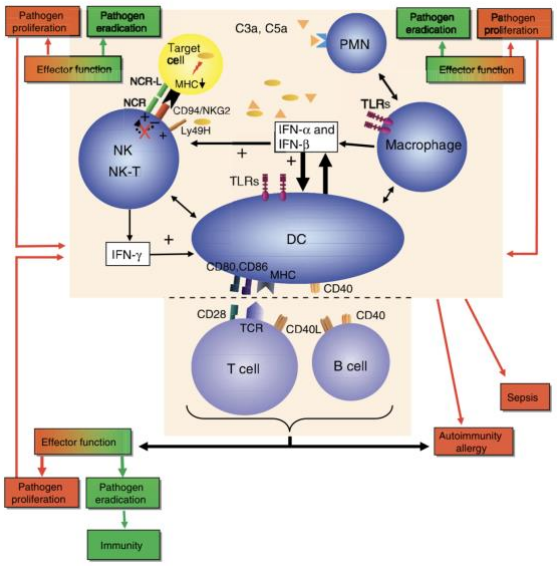

Figure 3. The interface between innate and adaptive immunity.

Description: The innate and adaptive immune systems work together to combat infection by foreign invaders. Receptor-mediated uptake and phagocytosis of pathogens by APCs jumpstarts the innate immune response. Maturation of the APCs allows for the upregulation of MHC class I and MHC class II molecules, providing the basis for increased antigen presentation to B cells and T cells. Antigen presentation leads to killing of the microbes and host immunity, due to the ability of the adaptive immune system to exhibit immunological memory through the activation of macrophages and antibody-producing B cells, respectively (Clark & Kupper, 2005).

The nonspecific, innate component of the immune response involves the activation and recruitment of immune cells such as B lymphocytes, T lymphocytes, macrophages, and dendritic cells to the site of infection (Clark & Kupper, 2005). Once these immune cells bind to the foreign antigen, these cells become antigen-presenting cells that travel through the lymph to present the antigens to major histocompatibility (MHC) molecules, which kickstarts the complementary specific adaptive immune system (Figure 3) (Clark & Kupper, 2005).

There are two classes of MHC molecules, each with their own target functions. MHC class I are molecules (encoded by the genes HLA-A, HLA-B, and HLA-C among others) bind to $CD8^+$ (cytotoxic) T lymphocytes, which directly kill their target cells by inducing them to undergo apoptosis through the secretion of granzymes (Clark & Kupper, 2005). MHC class II molecules, primarily encoded by the two genes of the heterodimeric HLA-DR complex HLA-DRA and HLA-DRB1, bind to $CD4^+$ (helper) T-lymphocytes, which have two functions: 1) activating cytotoxic T cells to kill infected cells and 2) activating B cells to secrete antibodies and macrophages to destroy the internalized microbes (Clark & Kupper, 2005).

CIITA: Master Regulator of MHC Class II Expression and Antigen Presentation

MHC class II expression is regulated by class II transactivator (CIITA), a protein that functions concurrently with the induction of IFN-γ. CIITA does not bind DNA

directly, but it regulates transcription as a transcriptional activator and general transcription factor (Devaiah & Singer, 2013). To complement its various modes of action (Figure 6), ranging from acetyltransferase activity to activation, the structure of CIITA contains multiple domains. In addition, CIITA also modulates the expression of several immune regulatory genes such as IL-4 and IL-10, indicating that this protein is essential for sufficient activation of the immune response (Devaiah & Singer, 2013).

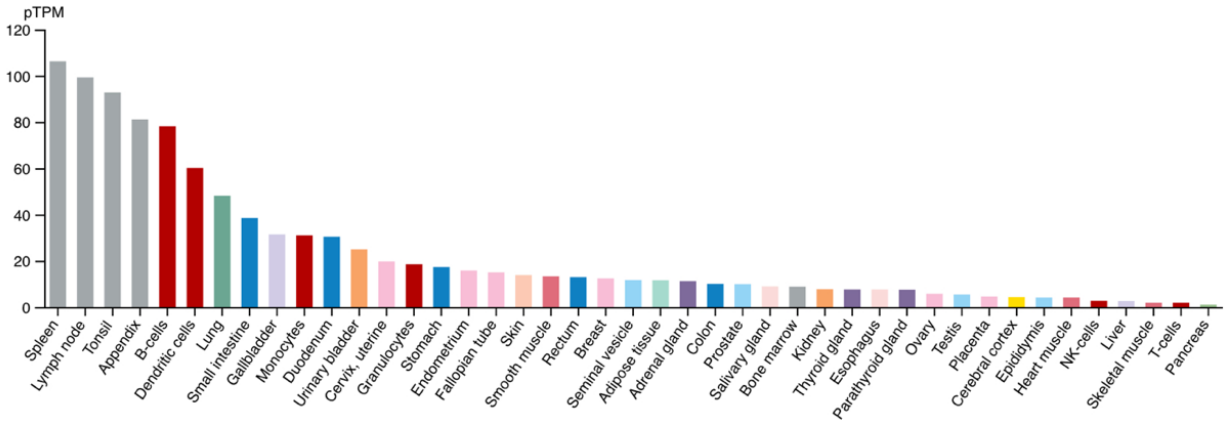

Figure 4. CIITA RNA Expression in Human Tissues.

Description: Expression of CIITA RNA in different human tissues, indicating tissue specificity. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419

CIITA is expressed mainly in secondary lymphoid organs, such as the spleen, lymph nodes, tonsils in addition to B cells and dendritic cells, confirming that it directly regulates the process of antigen presentation (Figure 4). Within the central nervous system, expression of CIITA is highest in microglia, the spinal cord, midbrain and basal

ganglia, providing further evidence that CIITA is likely to play a major role in ALS pathogenesis (Figure 5).

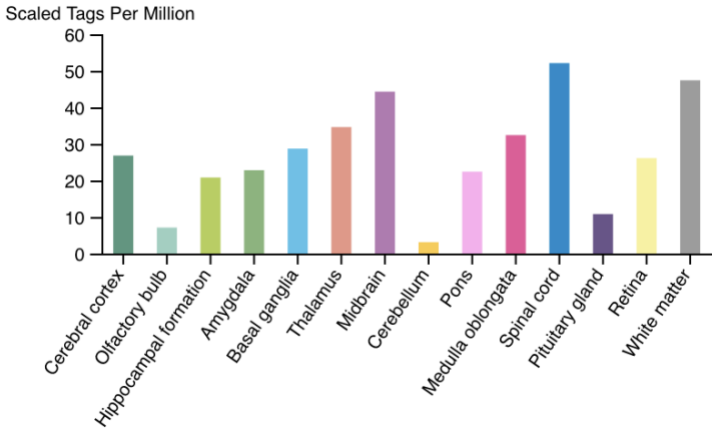

Figure 5. CIITA Expression in Various Human Brain Regions.

Description: CIITA Expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

Furthermore, aside from HLA-DR, CIITA regulates the expression of the CD74 gene, which is the invariant chain that is involved in the formation of MHC class II complexes. CD74 expression is highest in antigen-presenting cells such as microglia, dendritic cells, B cells and macrophages (Schröder, 2016). Functioning as both a chaperone and trafficking protein, CD74 interacts with HLA-DR, and both proteins are co-localized on the cell surface of antigen-presenting cells (Karakikes et al, 2012).

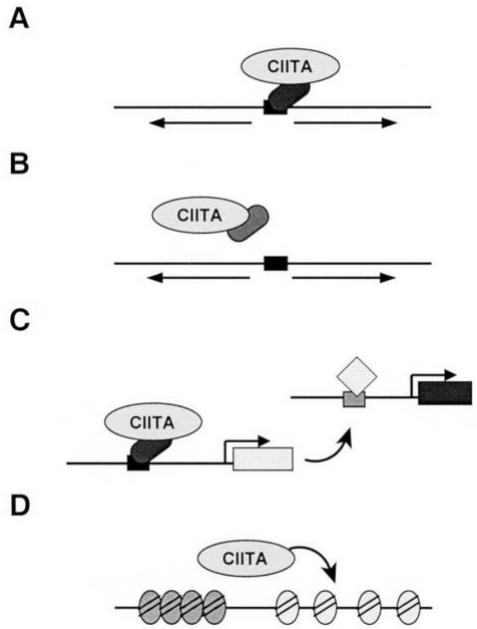

Figure 6. Models of CIITA action on downstream expression.

Description: A: CIITA interacts with DNA-bound transcription factors; B: CIITA sequesters/titrates a factor that activates or represses a specific gene; C: CIITA itself activates a transcription factor/activator; D: CIITA is directly involved in the chromatin modulation of a gene region (Nagarajan et al., 2002).

The Role of CIITA in MHC Class II Trafficking

It is widely known that CIITA regulates HLA-DRA and HLA-DRB1 expression. However, its function in regulating the cytoskeleton has not been studied. Two CIITA-regulated proteins that are directly associated with the cytoskeleton are MYBPC2 and RAB4B (Krawczyk et al. 2008) (Figure 7). MYBPC2, a myosin-binding protein, is part of the MyBP-C family that binds to myosin heavy chains (MHC), a process that regulates MHC class II trafficking and antigen presentation. The MyBP-C family has two functions: 1) stabilizing myosin filaments and 2) modulating cross-bridge formation between myosin and actin (Vascotto et al., 2007).

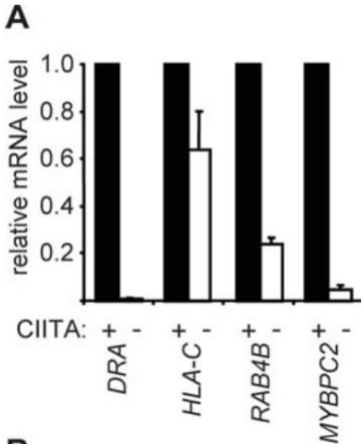

Figure 7. Regulation of DRA, HLA-C, MYBPC2 and RAB4B by CIITA.

Description: Expression of HLA-DRA, RAB4B, and MYBPC2 is rescued by the addition of CITA (black bars), indicating that these genes are directly regulated by CIITA (Krawczyk et al., 2008).

When B cells are activated by antigens, myosin and actin interactions allow for intracellular (vesicular) trafficking of MHC class II molecules for antigen presentation (Vascotto et al., 2007). Non-muscle myosin II, which is highly expressed in activated microglia, is essential to their proper migration and ability to phagocytize debris and toxic molecules, as shown in Figure 8 (Janßen et al., 2014) . The expression of MYBPC2 may be involved in activating the immune system, a response that we hypothesize is negatively impacted by the progression of ALS. MHC molecules become attached to the contractile actomyosin network through an interaction between myosin II and the cytosolic tail of the MHC class II chaperone CD74, allowing for MHC molecules to become mobile and successfully present antigens to effector T cells (Vascotto et al., 2007). It is also likely that MYBPC2 has a role in antigen presentation, whether it be direct or indirect.

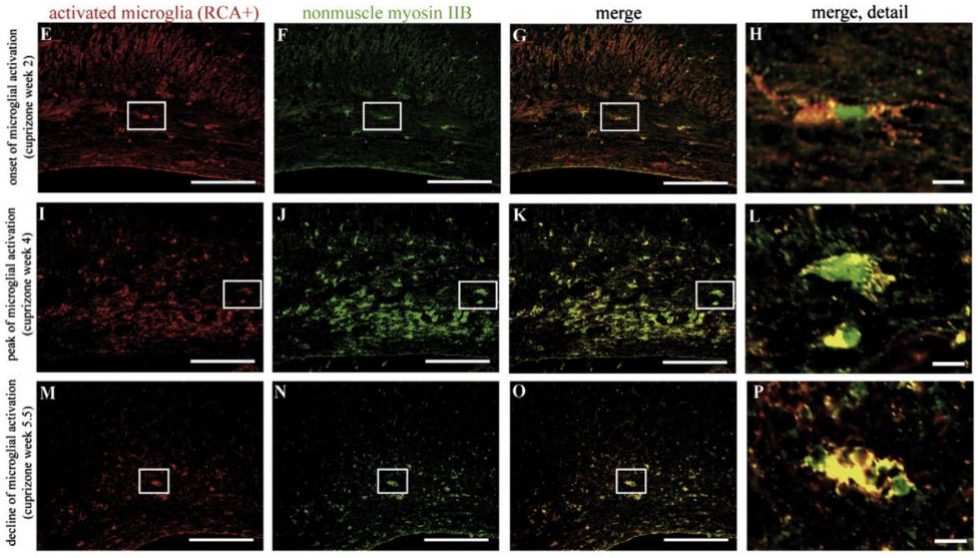

Figure 8. IF analysis of non-muscle myosin IIB expression in activated microglia.

Description: Non-muscle myosin IIB is expressed in activated microglia, indicating that the cytoskeleton is actively involved in the removal of debris and pathogens (Janßen et al., 2014).

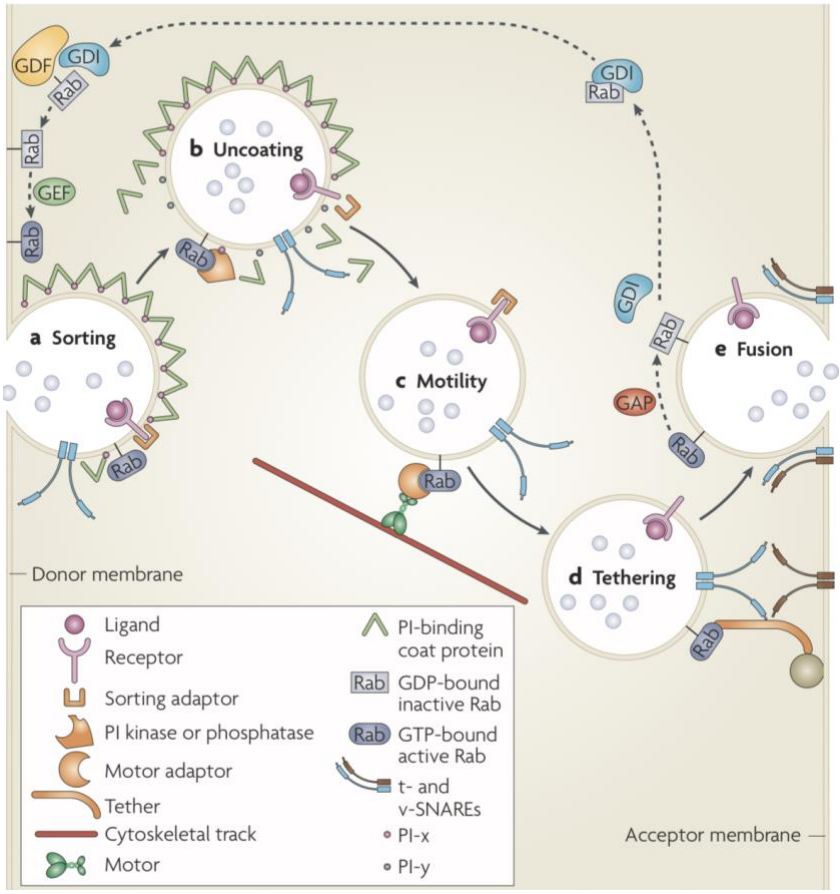

Figure 9. RAB4B functions in vesicle trafficking.

Description: a) Rab is activated by binding to GFP; sorts receptor into transport vesicle. b) Rab recruits phosphoinositide kinases; composition of vesicle is altered (uncoating). c) Rab mediates transport by recruiting motor adapters or direct motor protein binding. d) Rab mediates vesicle tethering by recruiting tethering factors that interact with molecules in the acceptor membrane. e) Membrane fusion and exocytosis; Rab is converted to its inactive form by binding to GDP after GTP hydrolysis; Rab-GDI complex is sent back to donor membrane (Stenmark, 2009).

Unlike MYBPC2, RAB4B has a direct role in antigen presentation. RAB4B encodes an isoform of the small GTPase RAB4, which regulates recycling of membranes and proteins from these compartments back to the cell surface (Figure 9) (Stenmark, 2009). Co-regulated with the MHC class II genes, RAB4B also regulates many other

antigen presentation mechanisms such as MHC-II restricted presentation of peptides derived from antigens internalized by receptor-mediated uptake in B cells, cross-presentation of endocytosed antigens by MHC-I molecules in DC and presentation of intact antigens to the antigen receptors of B cells (priming) (Stenmark, 2009).

Primary Goals, Specific Aims and Hypotheses

Our unpublished whole cell proteomics data revealed that both subunits of the HLA-DR heterodimer were significantly decreased in FUS, TAF15, and MATR3 HeLa KO lines. The most robust downregulation was observed in the FUS KO line (Figure 10). HLA-DRA was down 13-fold and HLA-DRB1 was approximately down 10-fold. Strikingly, HLA-DRA and HLA-DRB1 were the top two most downregulated genes in all HeLa KO lines, except for EWSR1. GSEA analysis of our KO lines indicated that many essential processes including antigen presentation and positive regulation of the immune response, are among the most negatively enriched processes (Figure 29 in Appendix). We conclude that these ALS-causative proteins are essential for the activation of the immune system.

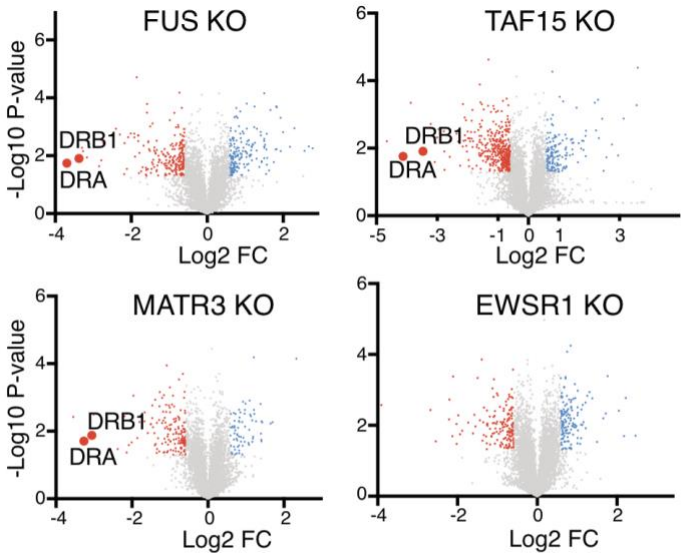

Figure 10. Volcano plot of whole cell proteomics of HeLa KO lines.

Description: HLA-DRA and HLA-DRB1 were the top downregulated genes in FUS KO, TAF15 KO and MATR3 KO lines. Downregulation of HLA-DR was not observed in the EWSR1 KO line.

Subsequently, we confirmed this result by Reverse Transcription-PCR, quantitative (Real-Time) PCR and Western blotting. With further diving into current research, we recently discovered that class II transactivator (CIITA) is the master regulator of the MHC gene expression (Devaiah & Singer, 2013). CIITA controls IFN-γ induced transcription of MHC class II genes and promotes constitutive expression of MHC class I genes (Figure 11) (Devaiah & Singer, 2013). This demonstrates that CIITA activation is required for the efficiency of the immune response, and that loss of CIITA may be involved in ALS.

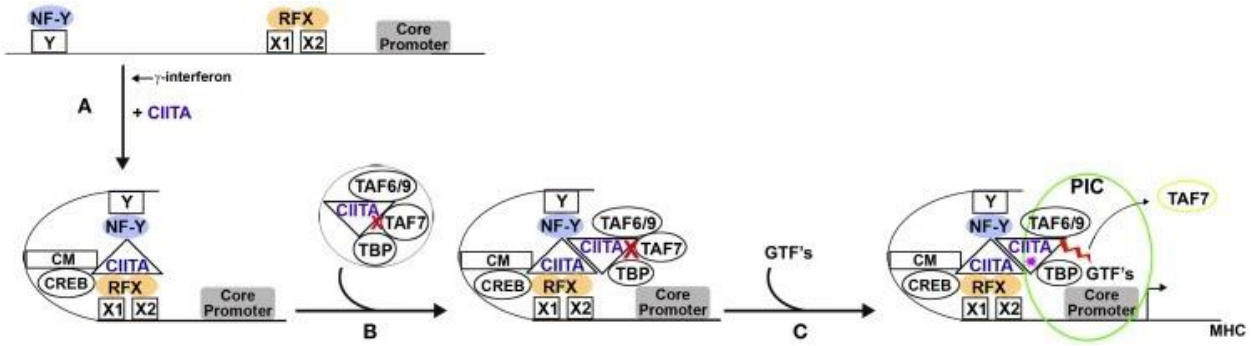

Figure 11. Proposed Model for the Roles of CIITA in MHC Transcription.

Description: CIITA expression is induced by IFN-γ and activates MHC gene expression by recruiting histone-modifying proteins within an enhanceosome (Devaiah & Singer, 2013).

In examining the literature, we learned that CIITA regulates the expression of the HLA-DR heterodimer, which led to our further investigation into whether CIITA was also downregulated in our KO lines. Excitingly, this turned out to be the case. These results provide evidence that these ALS-causative proteins all converge on a common pathway that involves CIITA and antigen presentation genes.

The primary goal of this project is to identify CIITA as a therapeutic target that may be used for ALS treatments. To achieve this goal, our aims are as follows:

Specific Aim #1: To confirm that loss of CIITA directly decreases HLA-DR and CD74 expression.

Specific Aim #2: To demonstrate that ALS-causative gene FUS controls CIITA, HLA-DR and CD74 expression.

Specific Aim #3: Our third approach is to rescue HLA-DR and CD74 expression by adding-back CIITA to our HeLa KO and HMC3 lines.

Specific Aim #4: To determine whether loss of FUS and CIITA affects MHC molecule trafficking in ALS. To do this, we will analyze the expression of two other CIITA-regulated genes mentioned above, MYBPC2 and RAB4B, in FUS KDs and CIITA KDs.

We hypothesize that the loss of FUS and CIITA will not only downregulate HLA-DR and CD74 expression, but will also decrease the expression of the MYBPC2 and RAB4B. We also hypothesize that adding-back CIITA will restore the expression of HLA-DR and CD74 in our ALS model systems.

Chapter II.

Materials and Methods

Cell Lines

HeLa Cells

HeLa cells were purchased from the ATCC. HeLa cells were grown in DMEM containing 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% Heat-Inactivated FBS, and 1% Penicillin/Streptomycin. Cells were passaged every two to three days, with medium changes in between passages. Knockout lines were generated using CRISPR gene editing at the CRISPR Core at Harvard Medical School. The following KO lines were made: FUS KO, EWSR1 KO, TAF15 KO, and MATR3 KO.

HMC3 Microglial Cell Line

HMC3 cells were grown in EMEM containing 1% FBS. Cells were passaged every to to three days, with medium changes in between passages.

siRNA knockdown in HMC3 cell line (FUS and CIITA)

HMC3 cells were transfected with the CIITA and FUS siRNAs, along with scrambled siRNA as a negative control. All siRNAs were purchased from Dharmacon. Transfection was performed in a 6-well tissue culture plate using the Lipofectamine RNAiMAX Kit from Thermo Fisher Scientific. In a separate tube for each, the siRNAs were diluted with ddH2O according to desired concentration (20 uM). In labeled tubes, 250 uL of Opti-MEM was added to each of the labeled tubes. 1 uL of diluted CIITA and FUS siRNA was then added to designated tubes to create mixture 1. In another separate tube, 22.5 uL of Lipofectamine RNAiMAX was added to 750 uL of Opti-MEM to create mixture 2. After subsequent mixing and a wait time of 5 minutes, 245 uL of mixture 2 was added into each of the two tubes containing the siRNA and Opti-MEM (mixture 1). After 15 min of incubation at room temperature, old media was aspirated from the cells and 1 mL of new media was added to each well. Then after a total wait time of 20 min, 500 uL of this final mixture was added into each of the wells (drop-wise). The 6-well plates were then shaken back and forth to make ensure sufficient mixing of all reagents and incubated for a total of 48 hours. 24 hours after siRNA transfection, IFN-γ (diluted 1:3000 with EMEM) was added to the cells for a subsequent 24 hour incubation until harvesting.

Add-Back of CIITA into HeLa KO lines and HMC3 KD cells

HeLa KO lines and HMC3 KD cells were transfected with a myc tagged CIITA plasmid and a myc-PK plasmid was transfected as a negative control. Both plasmids were purchased from Addgene. Transfection was performed in a 6-well tissue culture plate using the Lipofectamine 3000 Kit from Thermo Fisher Scientific. In a separate tube for each, the plasmids were diluted with ddH_2O according to desired concentrations (10 ng ng/uL for HeLa and 90 ng/uL for HMC3s). In labeled tubes, 125 uL of Opti-MEM and 5 uL of P3000 was added to each of the labeled tubes. Specific concentrations of diluted myc-CIITA and myc-PK were then added to designated tubes to create mixture 1. In another separate tube, 11.25 uL of Lipofectamine 3000 (3.75uL per well +1) was added to 375 uL of Opti-MEM (125 uL per well+1) to create mixture 2. After subsequent mixing, 125 uL of mixture 2 was added into each of the two tubes containing the plasmid, P3000 and Opti-MEM (mixture 1). This final mixture was incubated at room temperature in the cell culture hood for 15 min. After 10 min, old media was aspirated from the cells 2 mL of new media was added to each well (1 mL initially for transfected wells). Then after a total wait time of 15 min, 250 uL of this final mixture was added into each of the wells (dropwise). The 6-well plates were then shaken back and forth (to ensure sufficient mixing of all reagents) and placed into the incubator for 48 hours until harvesting.

RNA Extraction, DNase, and cDNA Synthesis

RNA Extraction

Harvested cells were resuspended in 1 mL of Trizol reagent. Subsequently, 200 uL of chloroform was added to each sample for an incubation time of 3 minutes at room temperature. Samples were centrifugated for 15 minutes at 12,000 x g, at 4^{o}C . 500 uL of the upper colorless aqueous phase was transferred to a new Eppendorf tube. 500 uL of isopropanol and 3 uL of glycogen was added to each sample. Inversion of each tube 5 times occurred after this step to ensure maximum precipitation. Samples were then incubated at room temperature for 10 minutes. Samples were centrifugated for 10 minutes at 12,000 x g at 4^{o}C. After centrifugation, the supernatant was discarded. Remaining pellets of RNA were washed with 500 uL of 75% ethanol. Samples were vortexed briefly for 10-15 seconds and centrifugated for 5 minutes at 7,500 x g at 4^{o}C. Supernatant was discarded, and RNA pellets were left to air dry for 2-3 minutes. RNA pellets were dissolved in 20 uL of before the RNA concentration was measured on a spectrometer. RNA was stored at -20 ^{o}C until DNase treatment.

DNase Treatment and cDNA Synthesis

Extracted RNA received. DNA treatment using the following reagents: 1) DNase Buffer, 2) DNase, 3) ddH_2O, and 4) Stop Solution from the DNase kit from Promega^{TM}. After reagents 1-3 were added, RNA incubated at 37 ^{o}C for 30 minutes. Subsequently, 1

uL of Stop Solution was added to all samples. RNA was then incubated at 65 ^{o}C for 15 minutes.

The Ultrascript 2.0 cDNA Synthesis Kit from PCR Biosystems^{TM} was used to obtain complementary cDNA from extracted RNA. Samples were incubated at 50-55 ^{o}C for 30 minutes. As a final step, the complementary cDNA was incubated at 95 ^{o}C for 10 minutes.

Reverse Transcription PCR and Quantitative Real-Time PCR

10X PCR Buffer, forward and reverse primer-pairs, dNTPs, ddH20 and Taq Polymerase were added to cDNA in order to catalyze the RT-PCR reaction. Agarose gel electrophoresis was used to visualize our results under UV light. Quantitation of results was achieved using the Fiji (Image J) Software.

For quantitative PCR, Power SYBR Green Mix, forward and reverse primer-pairs, and ddH_2O were added to the cDNA before placement into the qPCR machine. Melting curves and amplification plots were analyzed using the QuantStudio^{TM} 7 Flex Real-Time PCR System Software. Fold change in expression was calculated for each experimental sample with respect to the corresponding wild-type (control) sample.

Protein Extraction and Western Blotting

Western blotting was used to confirm the efficiency of the CRISPR KO editing in all HeLa KO lines.

Protein Extraction

In order to extract the protein from cell pellets for each condition, 100 uL of PGB-DTT was be added to each sample. Samples were then incubated at 95^{o}C for 5 minutes on a heat block.

Western Blotting

10 uL of each sample was loaded onto a Bolt^{TM} Bis-Tris Plus 4-12% polyacrylamide gel. The gel was run in 250 mL 1X MOPS (3-(N-Morpholino)propanesulfonic acid) Buffer at 175 V for 30-40 min. The gel was then transferred onto a nitrocellulose membrane by running in 1X transfer buffer (10X transfer buffer, methanol, and ddH_2O) at 0.15A for 40 min at 4^{o}C. Primary (for each target gene) and secondary antibodies (Rabbit/Mouse) was prepared using 5 mL of Intercept Blocking Buffer and 10 uL of Tween 20. Membranes were blotted for either 1 hour or overnight at 4^{o}C on a shaker. Gene expression at the protein level was analyzed after scanning on the Odyssey CLx Imaging System. Ratios for comparison between the wild-type and experimental samples were calculated using the ImageStudio^{TM} software.

Statistical Analysis

Three biological and technical replicates will be obtained in order to perform a two-tailed student's t-test in order to determine the statistical significance of our results. Statistical significance will be determined based on the standard p-value threshold of 0.05.

Chapter III.

Results

HLA Class I and Class II Expression in HeLa KO Lines

HLA-DRA, HLA-DRB1, and CIITA were all downregulated in our FUS, TAF15 and MATR3 HeLa KO lines. In marked contrast, these three genes were all upregulated in the EWSR1 KO line (Figure 12). MHC class I gene expression was also analyzed in the HeLa KOs. There was no significant difference in the expression of MHC class I genes, demonstrating that our ALS-causative genes of interest specifically target MHC class II expression.

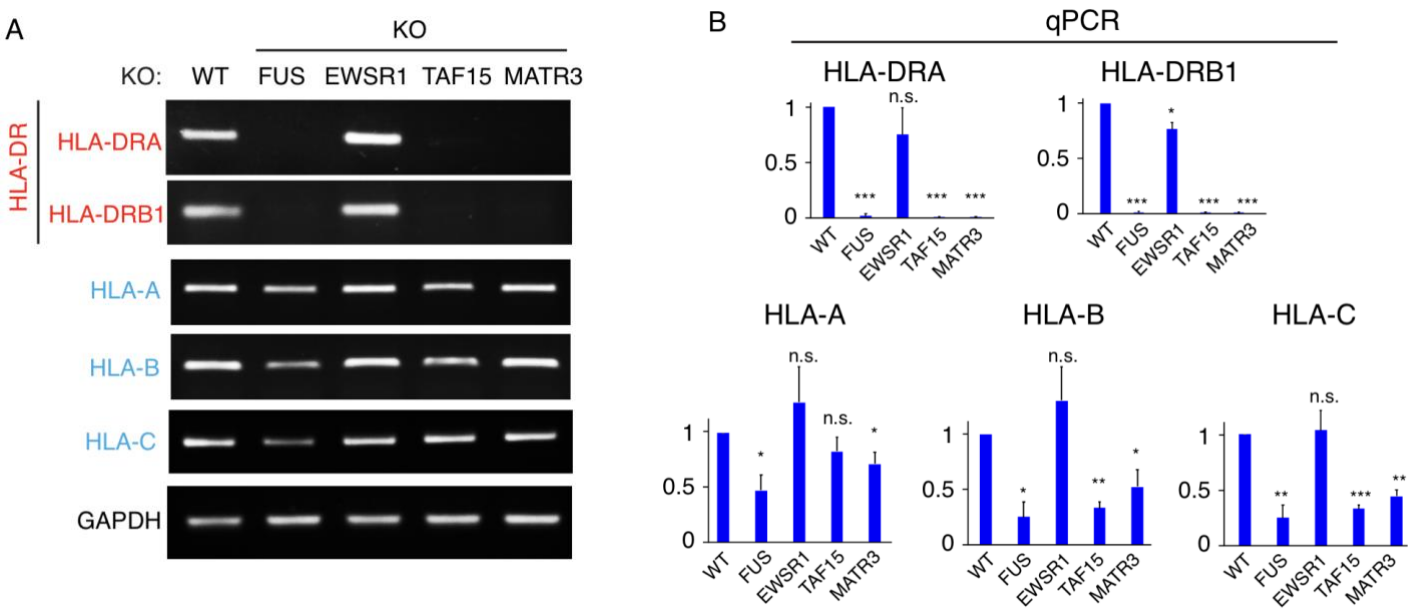

Figure 12. MHC Class I and MHC Class II Expression in HeLa KO lines.

Description: In HeLa KO lines, aside from EWSR1, major downregulation of HLA-DR was observed both RT-PCR and qCR analysis. No significant downregulation in MHC class I gene expression was observed.

In addition to HLA-DR, we also looked at CD74 expression in our HeLa KO lines. Excitingly, we also found that the CD74 was significantly downregulated in all KO lines except for EWSR1 (Figure 13). We conclude that CD74 was also regulated by our ALS-causative genes of interest.

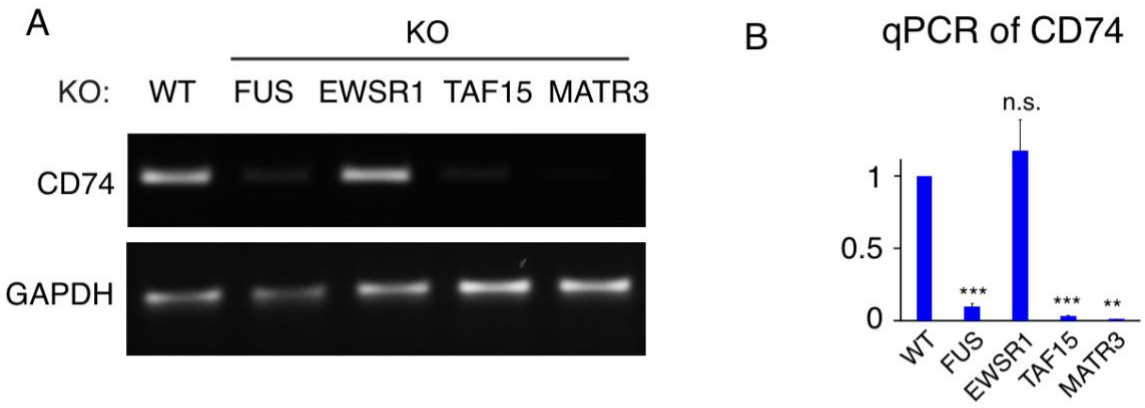

Figure 13. CD74 Expression in HeLa KOs.

Description: RT-PCR and qPCR analysis of CD74 expression in HeLa KO lines. Downregulation of CD74 is observed in FUS KO, TAF15 KO and MATR3 KO, but not EWSR1 KO line.

HLA-DR and CD74 Expression after siRNA knockdown in HMC3 Lines

In order to determine whether CIITA regulates these genes, we first performed an siRNA knockdown of CIITA in HMC3 cells with subsequent induction by IFN-γ 48 hours after transfection.. The expression levels of HLA-DRA, HLA-DRB1 and CD74 were analyzed using qPCR. Levels for all three genes were significantly downregulated in the CIITA KD compared to the control (scrambled) siRNA, confirming that HLA-DRA, HLA-DRB1 and CD74 are all directly regulated by CIITA (Figure 14).

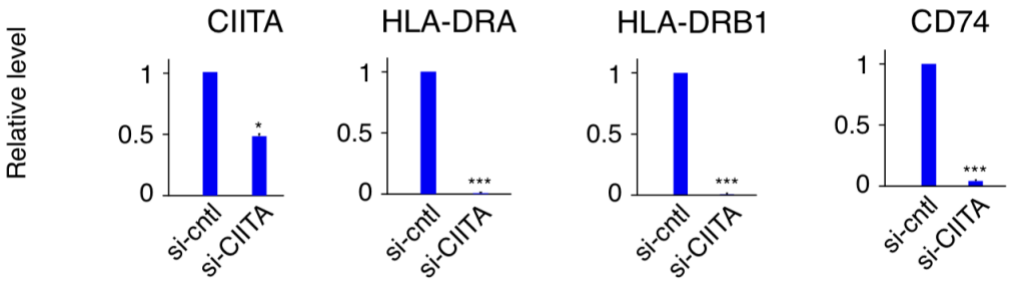

Figure 14. HLA-DR and CD74 Expression in CIITA KD HMC3s.

Description: qPCR analysis of HLA-DR and CD74 expression in CIITA KD HMC3s. All three genes are significantly downregulated in CIITA KD HMC3s compared to scrambled KD HMC3s.

After observing the regulation of our initial target genes by CIITA, we then performed an siRNA knockdown of the FUS gene with activation by IFN-γ after 48 hours of incubation after transfection. Here, we observed whether loss of FUS would directly affect the expression of CIITA, HLA-DRA, HLA-DRB1 and CD74.

Using analysis by qPCR, we found that the expression levels of all of these genes were significantly downregulated in the FUS KD cells, compared to the cells that contained the control (scrambled) siRNA (Figure 15). We conclude that FUS regulates expression of CIITA, HLA-DR, and CD74.

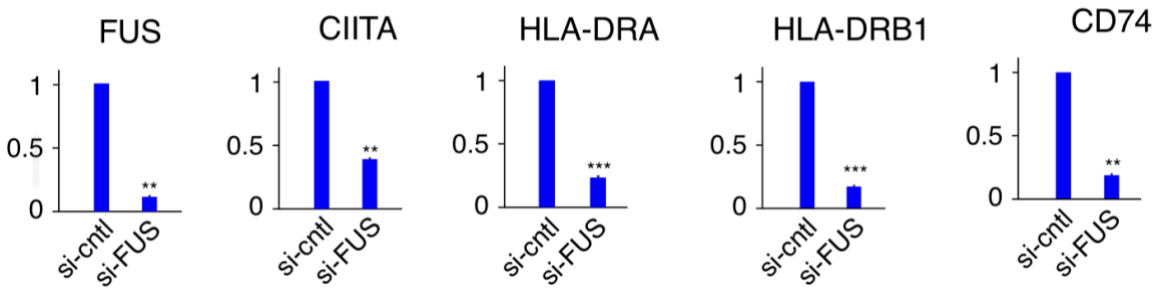

Figure 15. HLA-DR and CD74 Expression in FUS KD HMC3s.

Description: qPCR analysis of CIITA, HLA-DR and CD74 expression in FUS KD HMC3s. All four genes are significantly downregulated in FUS KD HMC3s compared to scrambled KD HMC3s.

Add-Back of CIITA into HeLa FUS KO

With the knowledge that CIITA regulates these downstream genes, we hypothesized that adding-back CIITA would restore their expression. Initially, we performed the plasmid transfection in our HeLa FUS KO line and assayed for CIITA, HLA-DR and CD74, using comparisons to two controls: HeLa WT and HeLa FUS KO transfected with the myc-PK plasmid.

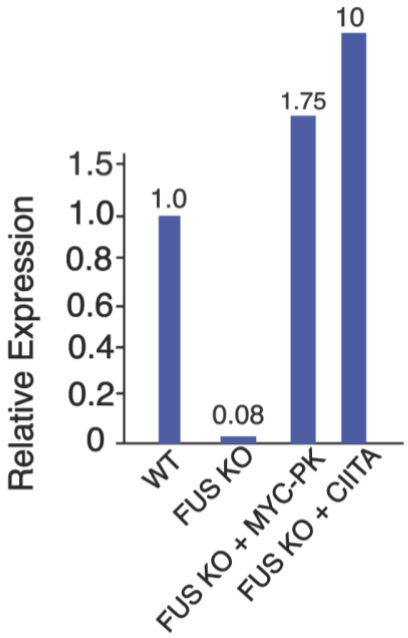

Figure 16. CIITA Expression after CIITA add-back in HeLa FUS KO.

Description: qPCR analysis of CIITA expression in FUS KO line before and after add-back of CIITA. CIITA expression was significantly rescued indicating we had a successful and efficient transfection of the CIITA plasmid.

Analysis of CIITA expression demonstrated a 6-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 16). This indicated that the CIITA plasmid did enter the cells and had a high efficiency. In addition, we observed that the expression of CIITA in FUS KO was 13-fold down compared to WT, demonstrating that loss of CIITA is observed in our ALS model (Figure 16).

Looking at HLA-DRA expression, our results indicated a 6.25-fold difference between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 17). In addition, we observed that the expression of CIITA in FUS KO was approximately 17-fold down compared to WT, demonstrating that loss of HLA-DRA is also observed in our ALS model (Figure 17).

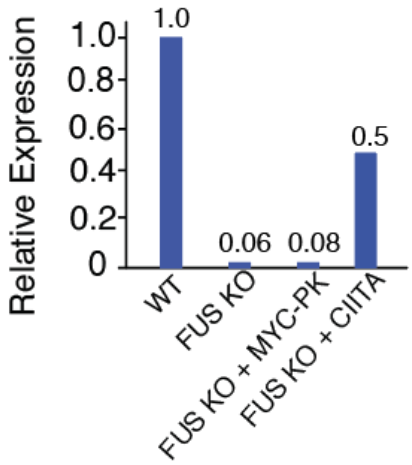

Figure 17. HLA-DRA Expression after CIITA add-back in HeLa FUS KO.

Description: qPCR analysis of HLA-DRA expression in FUS KO line before and after add-back of CIITA. Transfection of CIITA successfully rescued HLA-DRA expression.

Analysis of DRB1 expression demonstrated a 10-fold difference between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 18). In addition, we observed that the expression of DRB1 in FUS KO was 100-fold down compared to WT, demonstrating that loss of HLA-DRB1 is observed in our ALS model (Figure 18). Therefore, our results excitingly confirmed that CIITA directly regulates both components of the HLA-DR complex, and that CIITA expression is required for the the expression of these genes that regulate antigen presentation. These observations also demonstrate that the loss of FUS causes a dramatic decrease in the expression of these

target genes, indicating that FUS expression may be necessary for the process of antigen presentation to occur, through its regulation of these downstream genes.

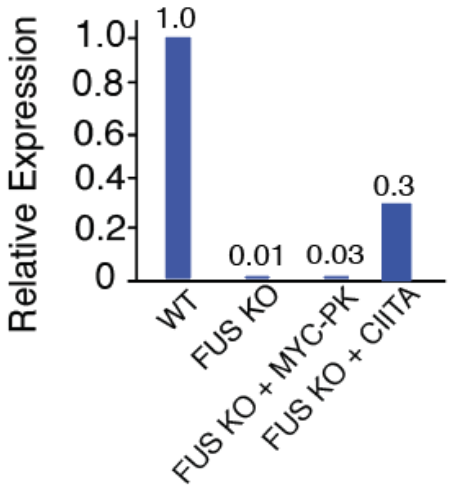

Figure 18. HLA-DRB1 Expression after CIITA add-back in HeLa FUS KO.

Description: qPCR analysis of HLA-DRB1 expression in FUS KO line before and after add-back of CIITA. Transfection of CIITA successfully rescued HLA-DRB1 expression.

Subsequently, analysis of CD74 expression demonstrated a 4.4-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 19). These results confirmed that CIITA directly regulates CD74, allowing us to establish CD74 as an integral component of our proposed pathway. Conclusively, given that all of these target genes were rescued, it is clear that CIITA is indeed a master regulator of the many components of antigen presentation. In addition, we observed that the expression of CD74 in FUS KO was 8-fold down compared to WT, demonstrating that loss of CD74 is regulated by both CIITA and FUS (Figure 19).

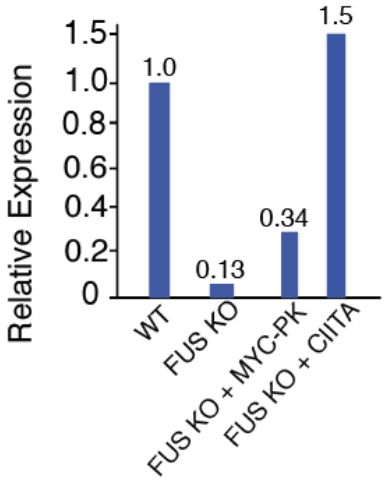

Figure 19. CD74 Expression after CIITA add-back in HeLa FUS KO.

Description: qPCR analysis of CD74 expression in FUS KO line before and after add-back of CIITA. Transfection of CIITA successfully rescued CD74 expression. Add-Back of CIITA into CIITA KD HMC3s.

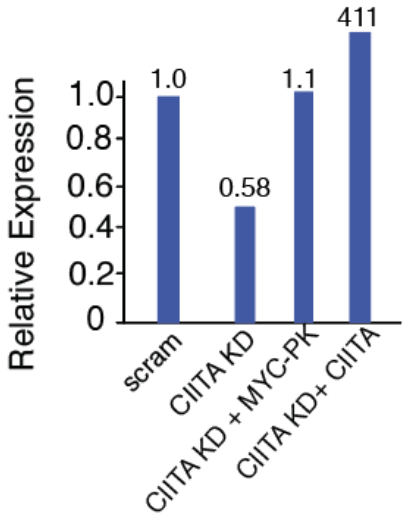

Figure 20. CIITA Expression after CIITA add-back in CIITA KD HMC3s.

Description: qPCR analysis of CIITA expression in CIITA KD HMC3s before and after add-back of CIITA. CIITA expression was significantly rescued indicating we had a successful and efficient transfection of the CIITA plasmid.

Analysis of CIITA expression demonstrated a 400-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 20). This indicated that the CIITA plasmid entered the HMC3s at a higher efficiency than in HeLa cells. In addition, we observed that the expression of CIITA in CIITA KD was approximately 2-fold down compared to our scrambled siRNA, demonstrating that knockdown of CIITA did successfully work in our HMC3s before the rescue (Figure 20).

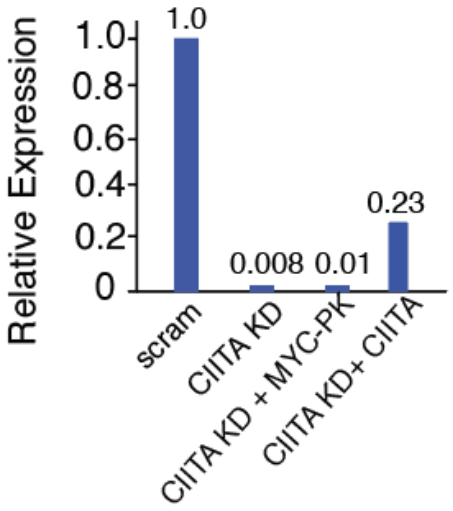

Figure 21. HLA-DRA Expression after CIITA add-back in CIITA KD HMC3s.

Description: qPCR analysis of HLA-DRA expression in CIITA KD HMC3s before and after add-back of CIITA. HLA-DRA expression was significantly rescued indicating we had a successful and efficient transfection of the CIITA plasmid.

Analysis of HLA-DRA expression demonstrated a 29-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 21). This indicated that the CIITA plasmid efficiently rescued DRA expression in HMC3s. In addition, we observed that the expression of HLA-DRA in

CIITA KD was approximately 125-fold down compared to our scrambled siRNA, demonstrating that the knockdown of CIITA significantly decreased the expression of this downstream gene before the rescue was completed (Figure 21).

Subsequently, analysis of DRB1 expression demonstrated a 14-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 22). We also observed that the expression of HLA-DRB1 in CIITA KD was 14-fold down compared to scrambled siRNA, demonstrating that loss of HLA-DRB1 was successfully executed by our CIITA knockdown (Figure 22). Since these results are in our microglial cell line, this confirms that HLA-DR expression is regulated by CIITA in other cell types.

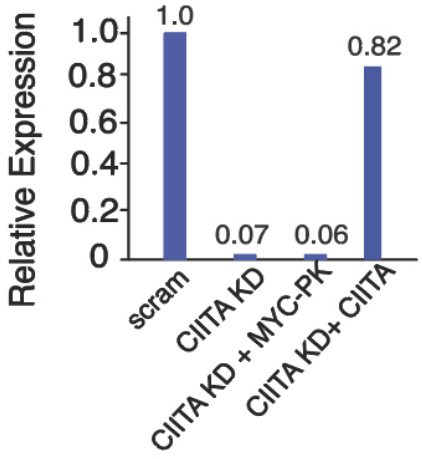

Figure 22. HLA-DRB1 Expression after CIITA add-back in CIITA KD HMC3s.

Description: qPCR analysis of HLA-DRB1 expression in CIITA KD HMC3s before and after add-back of CIITA. HLA-DRB1 expression was significantly rescued indicating we had a successful and efficient transfection of the CIITA plasmid.

Analysis of CD74 expression demonstrated a 3.5-fold difference between between the cells transfected with the myc-PK control and the cells transfected with CIITA (Figure 23). We also observed that the expression of CD74 in CIITA KD was 10-fold down compared to scrambled siRNA, demonstrating that loss of CD74 was also successfully executed by our CIITA knockdown (Figure 23). Since these results are in our microglial cell line, this confirms that CD74 is regulated by CIITA in other cell types that are directly involved in ALS pathogenesis.

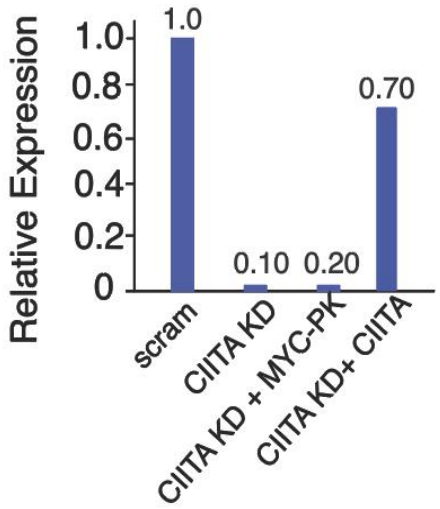

Figure 23. CD74 Expression after CIITA add-back in CIITA KD HMC3s.

Description: qPCR analysis of CD74 expression in CIITA KD HMC3s before and after add-back of CIITA. CD74 expression was significantly rescued indicating we had a successful and efficient transfection of the CIITA plasmid.

Expression of RAB4B and MYBPC2 in CIITA KD

To examine if loss of MHC class II trafficking and if the cytoskeleton is a part of our pathway, we first wanted to determine if RAB4B and MYBPC2 are regulated by CIITA. RAB4B expression was significantly downregulated 20-fold in CIITA KD, indicating that it is likely a downstream target of CIITA, similar to HLA-DRA, HLA-DRB1 and CD74 (Figure 24). MYBPC2 expression was significantly downregulated 12.5-fold in CIITA KD, indicating that it is also likely a downstream target of CIITA, in a manner similar to RAB4B (Figure 25). Its downregulation is slightly less significant than that of RAB4B, meaning that RAB4B may be more tightly regulated by CIITA.

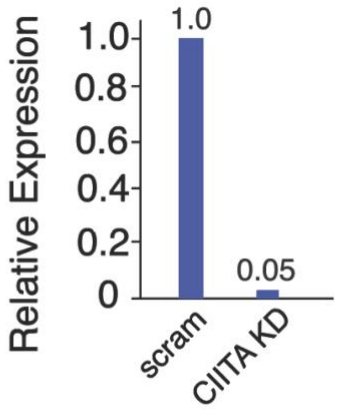

Figure 24. RAB4B Expression in CIITA KD HMC3s.

Description: qPCR analysis of RAB4B in CIITA KD HMC3s. RAB4B is downregulated in CIITA KD HMC3s, giving evidence that RAB4B is a downstream target of CIITA.

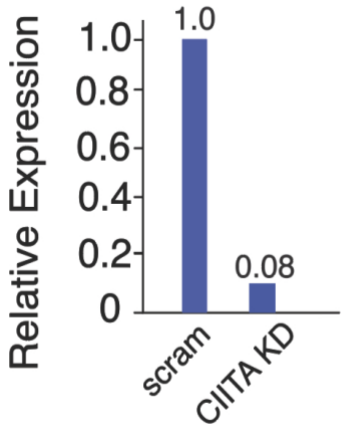

Figure 25. MYBPC2 Expression in CIITA KD HMC3s.

Description: qPCR analysis of RAB4B in CIITA KD HMC3s. RAB4B is downregulated in CIITA KD HMC3s, giving evidence that RAB4B is a downstream target of CIITA.

Expression of RAB4B and MYBPC2 in FUS KD

To further link the loss of cytoskeleton regulation to ALS, we also examined RAB4B and MYBPC2 expression in FUS KD HMC3s. RAB4B expression was significantly downregulated by 10-fold in FUS KD, indicating that it is likely also a downstream target of FUS, giving further evidence of that FUS regulates CIITA and its downstream genes (Figure 26). Meanwhile, MYBPC2 was downregulated 5-fold in FUS KD HMC3s, indicating that both of these proteins fit into our proposed pathway, since their expression is regulated by both FUS and CIITA (Figure 27).

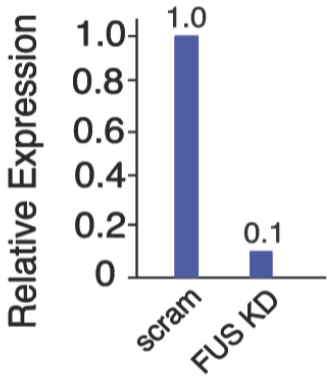

Figure 26. RAB4B Expression in FUS KD HMC3s.

Description: qPCR analysis of RAB4B in FUS KD HMC3s. RAB4B is downregulated in FUS KD HMC3s, giving evidence that RAB4B is a downstream target of both FUS and CIITA.

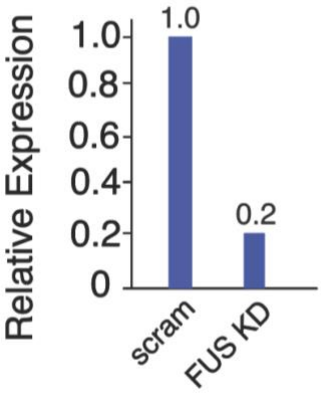

Figure 27. MYBPC2 Expression in FUS KD HMC3s.

Description: qPCR analysis of MYBPC2 in FUS KD HMC3s. MYBPC2 is downregulated in FUS KD HMC3s, giving evidence that MYBPC2 is a downstream target of both FUS and CIITA, similar to RAB4B.

Chapter IV.

Discussion

Our proposed pathway hypothesizes that our ALS-causative genes regulate CIITA, and that in turn, CIITA regulates the antigen presentation and cytoskeletal genes. However, it may be questioned whether the ALS genes regulates CIITA or if this part of the pathway is reversed, with CIITA actually regulating the ALS genes. We proved this point in our KDs by analyzing the expression of each ALS gene in the CIITA KD as well as the expression of CIITA in our KDs of the ALS genes. Our results indicate that the expression of the ALS genes were unchanged in CIITA KD HMC3s, but CIITA was dramatically decreased in our FUS KD HMC3s. Therefore, we validated that the regulation of CIITA by these ALS-causative genes is at the forefront of this mechanism.

Our results indicated that RAB4B was much more significantly downregulated than MYBPC2 in CIITA KD and particularly in FUS KD. This may be due to the fact RAB4B is more directly tied to antigen presentation so the effect of the decrease in CIITA expression is likely to be larger than that of MYBPC2 (CIITA has only been shown to modulate its expression), which could explain the inconsistencies in MYBPC2 expression in our KD HMC3s (Krawczyk et al., 2008). In our HeLa cells, we also discovered in MYBPC2 was not able to be expressed in HeLa (likely due to a lack of IFN-γ), but was able to be expressed in HMC3s that were induced by IFN-γ. These results demonstrate that IFN-γ is necessary for the expression of certain target genes that are regulated by CIITA, since IFN-γ does function in conjunction with CIITA activation (Nagarajan et al., 2002). We also postulate that this effect may be both gene-specific and

cell-type specific, because RAB4B is expressed in HeLa cells without supplementation by IFN-γ.

We suspect that MYBPC2 may have different results in KDs of other ALS-causative genes such as TAF15 and EWSR1. This potential result could be attributed to the fact that FUS and TAF15 only share a small subset of common genes. Even though both proteins are required for transcription, FUS and TAF15 may be involved in different pathways in ALS and other diseases (Kapeli et al., 2016). In contrast to our other target genes, MYBPC2 may actually be downregulated in EWSR1 KD, since studies have shown that loss of EWSR1 has been shown to decrease mitochondrial activity in skeletal muscle, the region in which MYBPC2 is highly expressed (Park et al., 2015). While myosin has been shown to affect the rate of mitochondrial fission/fusion in skeletal muscle, it is possible that the decreased expression of this myosin binding protein are associated with the lack of mitochondrial activity (Altanbyek et al., 2016).

While this study involved the knockdown of the FUS gene, we have not yet looked at factors that are upstream of FUS and affect its expression. One possible method of determining this part of our pathway from a pharmacological perspective would be to use the small molecule drug rolipram, a phosphodiesterase-4 inhibitor that ameliorates the effects of the knockdown of the FUS gene and inhibits anti-inflammatory functions (Ward et al., 2014). Research has shown that rolipram has the capacity to restore cell count initially lost by knockdown in the FUS gene. Therefore, it would be interesting to observe whether administering rolipram to FUS knockdown cells or in vivo ALS models would restore CIITA, HLA-DR, and cytoskeletal gene expression, similar to the effect of

adding-back CIITA, and whether rolipram could be further established as a treatment for ALS.

Research Limitations

Transfection (Add-Back)

Lipofectamine 3000, the transfection reagent used to transfect our HeLa FUS KO, allows for efficient plasmid entry into cells, especially when used in conjunction with the reagent P3000. While Lipofectamine 3000 is known for having a very high transfection efficiency, this comes with a higher level of toxicity to the cells over the 48 hour incubation period (Wang et al., 2018).

siRNA knockdown

Similar to Lipfectamine 3000, Lipofectamine RNAiMAX is also incubated for a period of 48 hours (Wang et al., 2018). In addition, siRNA knockdown efficiency is variable between genes and requires rounds of optimization, as it may be determined by many factors such as incubation time of the siRNA before harvesting, concentration the siRNA (frequency of addition), and appropriate timing for addition of IFN-γ (Kim & Eberwine, 2010). Therefore, optimal conditions may are difficult to obtain at first, as they depend on confluency at the time of transfection.

Polymerase Chain Reaction (RT-PCR and qPCR)

High sensitivity of RT-PCR and qPCR could lead to a high chance of contamination. Results can be misleading even with little amounts of contaminated DNA. Bands seen for PCR can include non-specific binding, as primers designed for PCR can anneal non-specifically to DNA sequences that are similar to the target sequence (Garibyan & Avashia, 2013). In addition, incorrect nucleotides could be inserted into the DNA sequence by DNA polymerase, which may cause other bands to appear in the gel electrophoresis results (Garibyan & Avashia, 2013).

Western Blotting

Western blotting only indicates whether a protein is present or not, not how much protein is present. This technique also only gives an estimation of a molecular weight. Antibodies used for Westerns may exhibit non-specific binding, which may lead to inaccurate results (the wrong band size may appear on the nitrocellulose membrane). Weak signals due to the concentration of the primary antibody (or amount of washing) also may lead to inconclusive results (Mahmood & Yang, 2012).

Future Directions

Based on our results, we can conclude that CIITA is a therapeutic target for ALS. Future research could include looking at whether the expression of these downstream genes can be rescued by CIITA in FUS KD HMC3s. While we have confirmed that

CIITA directly regulates MHC class II expression, it would be interesting to observe whether MHC class I expression (HLA-A, HLA-B, and HLA-C) is affected by adding-back CIITA, to validate that the rescue of expression is specific to MHC class II. Inserting point mutations in the CIITA gene into in vitro or in vivo ALS models and assaying for these downstream genes, would give more evidence that alterations in CIITA function and expression plays a major role in ALS pathogenesis. In addition, since the receptor that CIITA is a member of the NOD-like receptor (NLR) sub-family, upcoming studies could look at restoring this expression from a pharmacological approach through the development of an NLR agonist, and then proceed to observe if that could also rescue CIITA, HLA-DR, CD74, RAB4B and MYBPC2 expression. During the immune response, antigen presentation leads to the activation of T cell activation. As a follow-up to these pharmacological studies, the level of T-cell activation after the administration of potential CIITA agonists would useful in determining whether CIITA can rescue the immune response in ALS patients to the same extent as it did in our in vitro ALS models.

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Appendix.

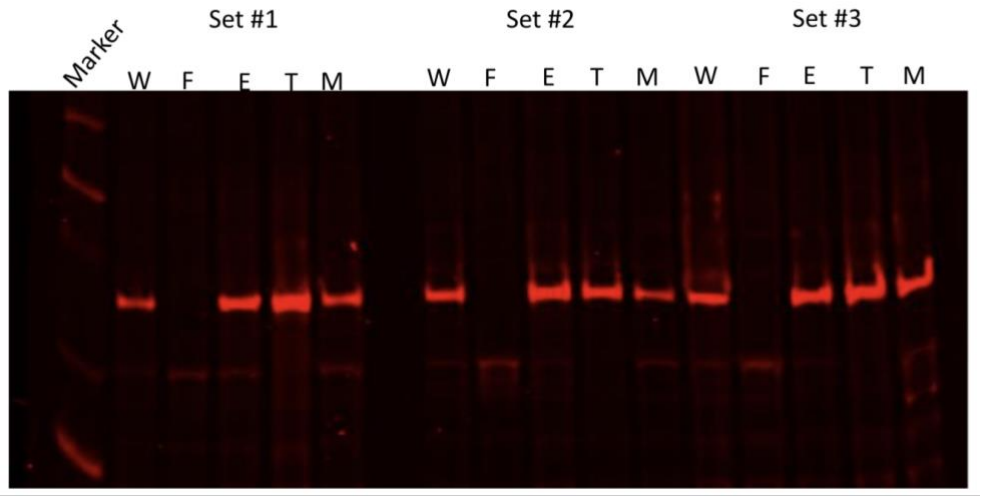

Figure 28. FUS KO in HeLa KO lines.

Description: Analysis of FUS protein expression by Western blot. FUS protein was expressed in other KO lines, but not in the FUS KO line. This result confirms that the CRISPR knockout of the FUS gene in our HeLa cell line was successful.

Downregulated pathways shared in FUS, TAF15 and MATR3 KOs

Gene set name	NES in KO lines		
	FUS	TAF15	MATR3
INNATE_IMMUNE_RESPONSE	-3.0	-2.4	-2.5
RESPONSE_TO_INTERFERON_GAMMA	-2.6	-2.3	-2.1
ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN	-2.6	-2.3	-2.0
ANTIGEN_PROCESSING_AND_PRESENTATION	-2.5	-2.4	-2.0
POSITIVE_REGULATION_OF_IMMUNE_RESPONSE	-2.4	-2.1	-2.1
ANTIGEN_RECEPTOR_MEDIATED_SIGNALING_PATHWAY	-2.2	-2.6	-2.1

Figure 29. GSEA analysis of our KO lines.

Description: Top GO terms in GSEA analysis of our KO lines indicate that genes that regulate the innate immune response and antigen presentation are highly downregulated in our in vitro models.

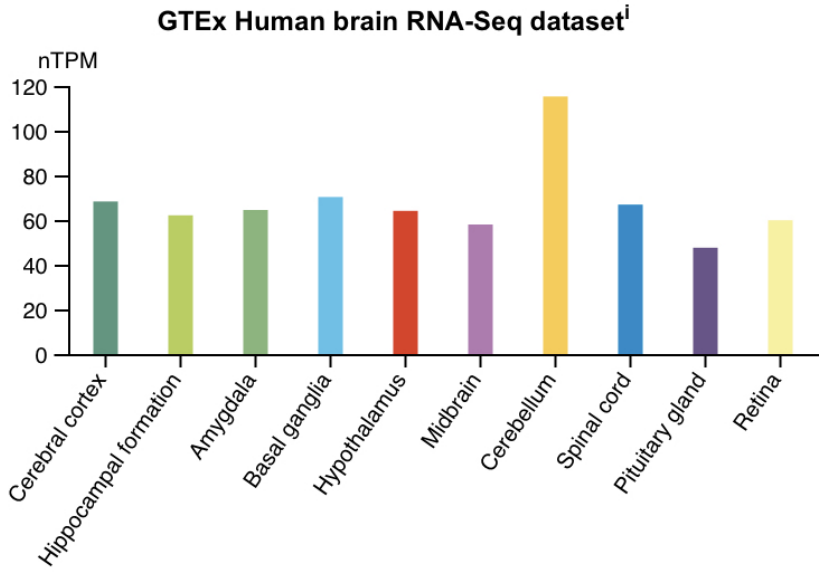

Figure 30. FUS Expression in Brain/CNS Tissues.

Description: FUS expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

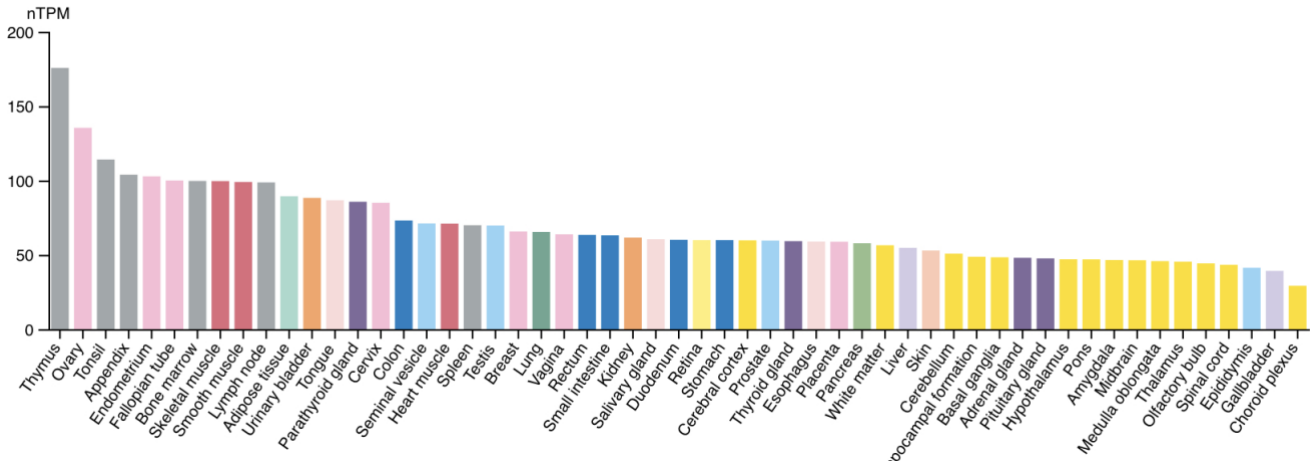

Figure 31. FUS Expression in Various Human Tissues.

Description: Expression of FUS RNA in different human tissues, indicating tissue specificity. This data indicates that FUS is likely to play a role in multiple physiological pathways. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

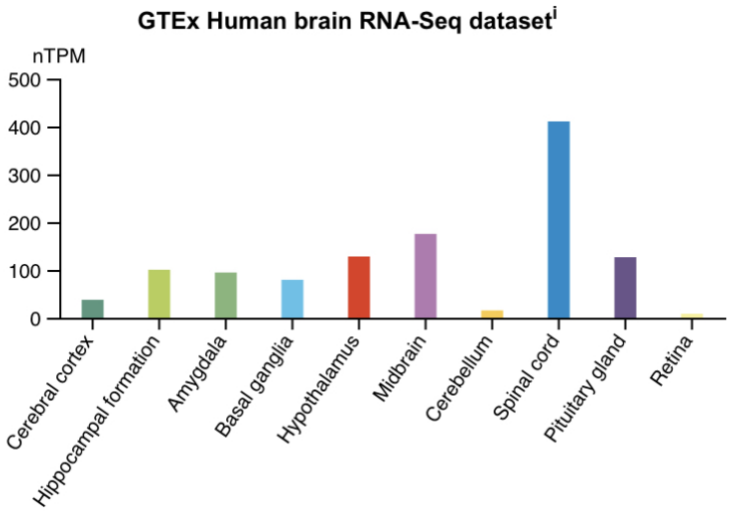

Figure 32. HLA-DRA Expression in Brain/CNS Tissues.

Description: HLA-DRA expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

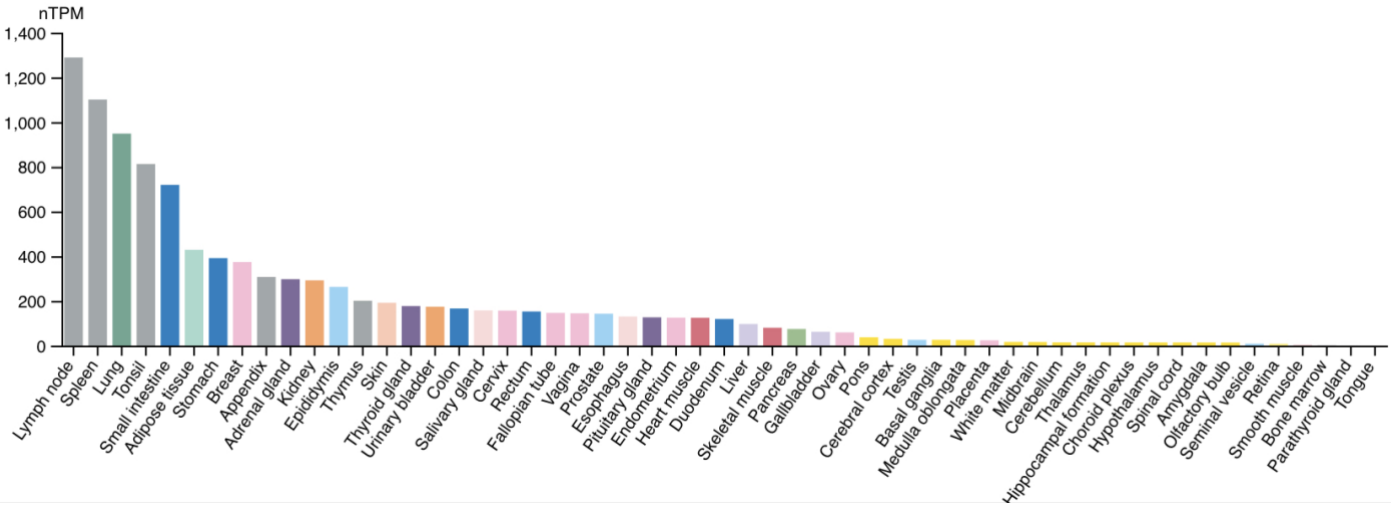

Figure 33. HLA-DRA Expression in Various Human Tissues.

Description: Expression of HLA-DRA RNA in different human tissues, indicating tissue specificity. HLA-DRA expression is highest in the secondary lymphoid organs. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419

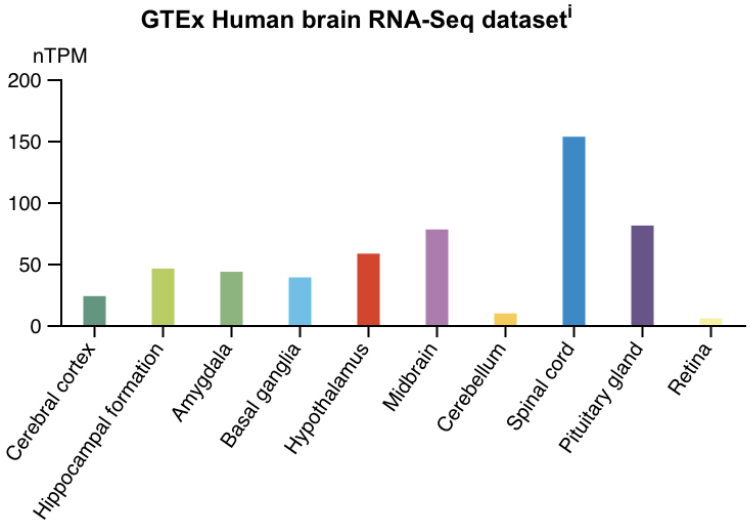

Figure 34. HLA-DRB1 Expression in Various Brain/CNS Tissues.

Description: HLA-DRB1 expression in different regions of the central nervous system. Similar to HLA-DRA, HLA-DRB1 expression is highest in the spinal cord, confirming that both components of the HLA-DR complex may play a huge role in neurological function. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

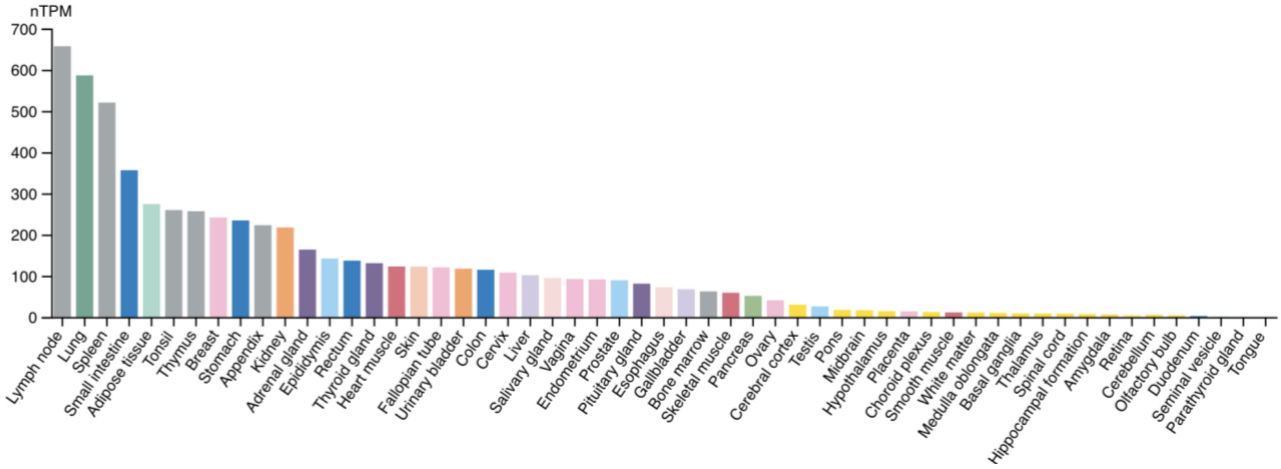

Figure 35. HLA-DRB1 Expression in Various Human Tissues.

Description: Expression of HLA-DRB1 RNA in different human tissues, indicating tissue specificity. DRB1 expression is highest in the secondary lymphoid organs. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419

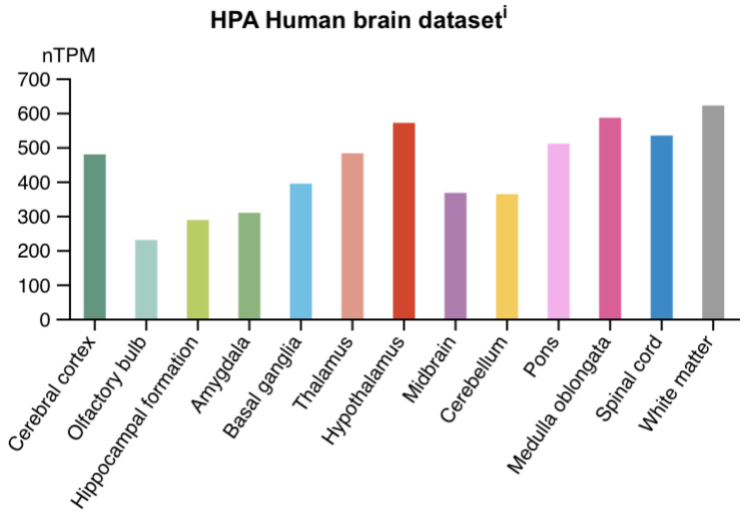

Figure 36. CD74 Expression in Brain/CNS Tissues.

Description: CD74 expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

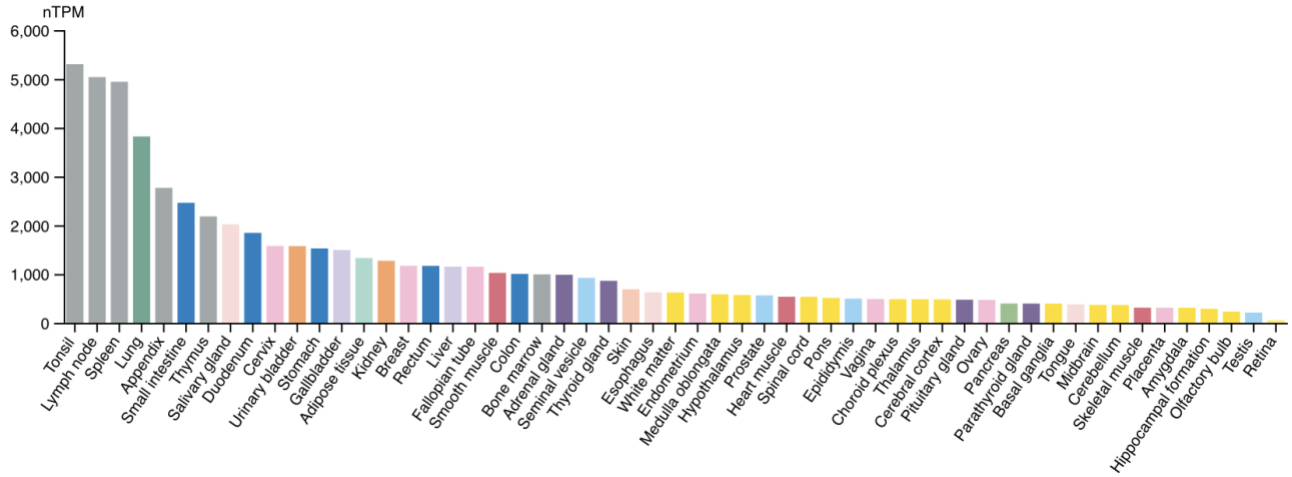

Figure 37. CD74 Expression in Various Human Tissues.

Description: Expression of CD74 RNA in different human tissues, indicating tissue specificity. CD74 expression is highest in the secondary lymphoid organs. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

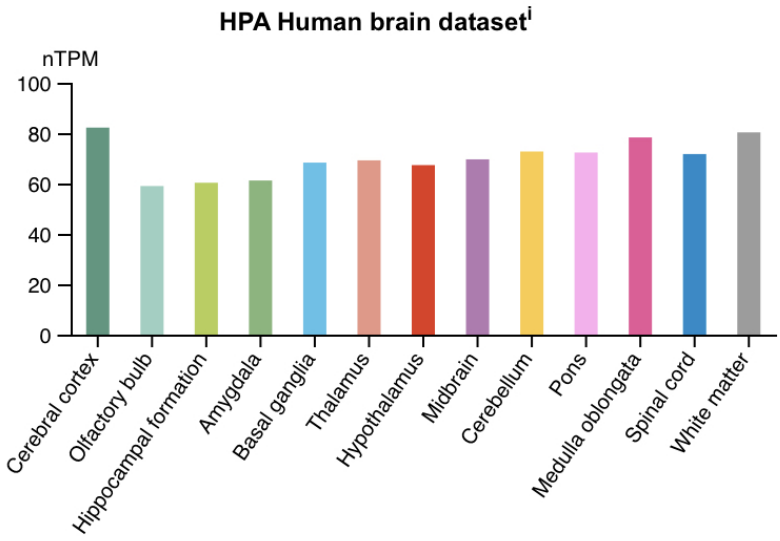

Figure 38. RAB4B Expression in Brain/CNS Tissues.

Description: RAB4B Expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.12 60419.

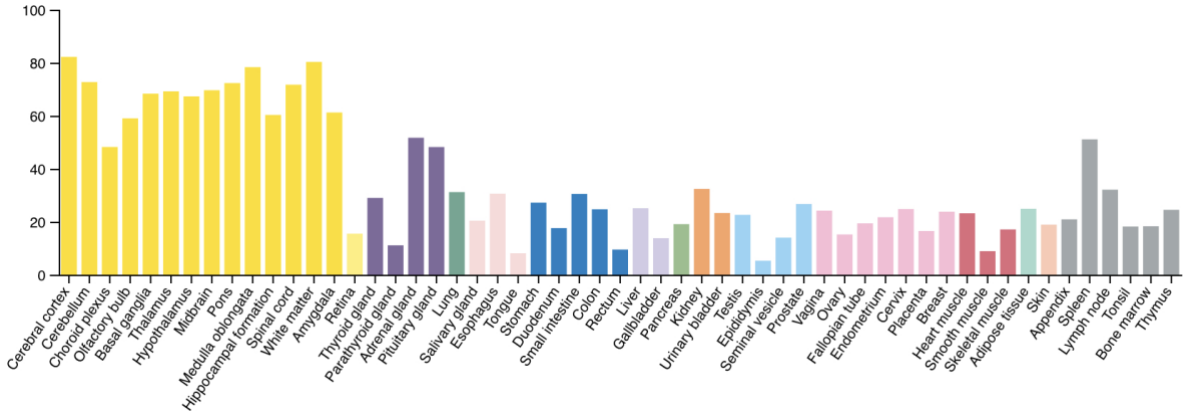

Figure 39. RAB4B Expression in Various Human Tissues.

Description: Expression of RAB4B RNA in different human tissues, indicating tissue specificity. RAB4B Expression is highest in the CNS and lymphoid organs, indicating that the cytoskeleton likely contributes to brain homeostasis and immune function. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

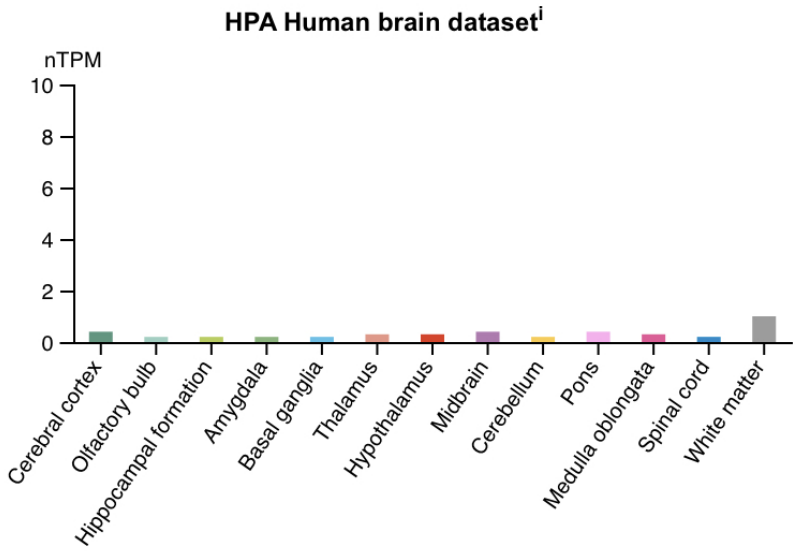

Figure 40. MYBPC2 Expression in Brain Tissues.

Description: MYBPC2 Expression in different regions of the central nervous system. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.

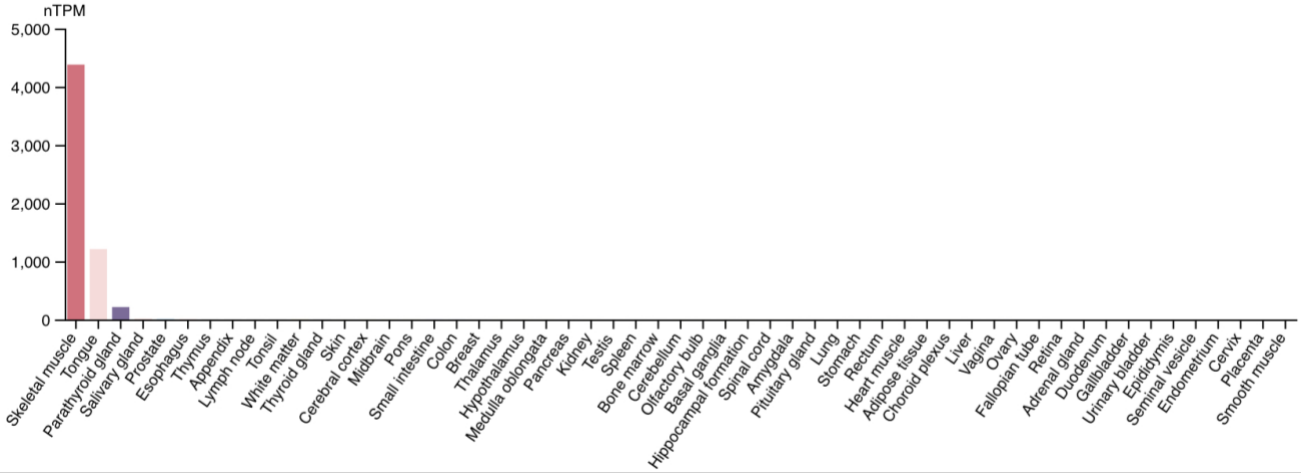

Figure 41. MYBPC2 Expression in Various Human Tissues.

Description: Expression of MYBPC2 RNA in different human tissues, indicating tissue specificity. MYBPC2 is almost exclusively expressed in skeletal muscle. Source: Uhlén M et al., Tissue-based map of the human proteome. Science (2015). PubMed: 25613900 DOI: 10.1126/science.1260419.