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**Nonsense Mutation in AIMP2 Reduces Protein Expression and Causes a Severe
Neurodevelopmental Disorder**

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Abbreviations

AIMP- Aminoacyl tRNA synthetase-interacting multifunctional protein

ARS- Aminoacyl-tRNA Synthetases

ATCC- American Type Culture Collection

BSA- Bovine Serum Albumin

FBS- Fetal Bovine Serum

LARS- Leucyl-tRNA Synthetase

MSC- Multi-tRNA Synthetase Complex

PBS- Phosphate-Buffered Saline

PCR- Polymerase Chain Reaction

SDS- Sodium Dodecyl Sulfate

TBST- Tris Buffered Saline with Tween-20

Tris-HCl- Tris (hydroxymethyl) aminomethane hydrochloride

VARs- Valyl-tRNA Synthetase

Abstract

In mammalian cells, a multi-tRNA synthetase complex containing eight aminoacyl-tRNA synthetases, which catalyze nine different reactions, is thought to make protein synthesis more efficient by keeping components of the translational machinery in close proximity. Aminoacyl tRNA synthetase-interacting multifunctional protein 2 (AIMP2) is one of three non-catalytic components of this complex and is essential for its formation and stability. A homozygous nonsense variant (Y35Ter) of AIMP2 appears in the genotypes of patients with severe neurodevelopmental phenotypes. To determine the effect this variant has on expression at the mRNA and protein level, as well as progression of cells through the cell cycle, patient fibroblasts were studied. Fluorescent microscopy and western blotting, along with puromycin-labeling, cell synchronization, and mRNA analysis, were used to determine the cellular impact of this mutation. Through this study, it was determined that this mutation in AIMP2 causes decreased AIMP2 protein levels and altered mRNA expression. Along with that, the puromycin assay showed a lower rate of protein synthesis in patient fibroblasts when compared to ATCC control cells. There was also a slight decrease in the amount of leucyl-tRNA synthetase (LARS) in patient cells. This protein is a member of the multi-tRNA synthetase complex and the observed decrease could indicate that the protein level of other members of the complex are lower in patient cells. While the cell cycle study did not provide any significant results, this could be due to the fact that fibroblasts were used and not neuronal cells. This study showed that a homozygous nonsense variant (Y35Ter) of AIMP2 impacts expression at the mRNA and protein level while also decreasing protein synthesis. Further research into the specific mechanism behind this mutation is needed in order to possibly develop a treatment option for patients presenting with the resulting symptoms.

Introduction

Within the central dogma of cellular biology, there are many crucial molecules that allow for the replication, transcription, and translation of DNA and genes within the body. One family of molecules that are essential for the translation of messenger RNA to proteins are aminoacyl-tRNA synthetases (ARSs). These enzymes ligate amino acids to the correct tRNA allowing for the proper assembly of proteins¹. The specificity of these enzymes for a particular amino acid is critical to maintaining the fidelity of the genetic code, and thus maintenance of cellular homeostasis. Within mammalian cells, eight different ARS, which catalyze nine different reactions, are found in the multi-tRNA synthetase complex (MSC)². It is believed that this complex keeps ARS and other translational components in close proximity, in turn making protein synthesis more efficient. For this complex to assemble, three proteins called aminoacyl tRNA synthetase-interacting multifunctional proteins (AIMP 1-3), are involved with protein-protein interactions and is mediated by heat-shock protein 90¹.

AIMP2, also known as p38, is one of the non-catalytic components of the MSC and is essential for its formation and stability. The function of AMIP2 is indispensable, as evidenced by embryonic lethality observed in AMP2 knockout mice. In fibroblasts cultured from these mice prior to death, western blot analysis demonstrated that the protein levels of AIMP1, AIMP2, and various ARS that are contained within the MSC was dramatically decreased¹. AIMP2 knockout did not alter messenger RNA levels of the AIMPs or ARS that showed decreased protein levels, indicating that decreased levels of MSC components did not result from altered transcription¹.

Mutations in various components of the MSC have been shown to cause various neurodevelopmental disorders²⁻⁴. For example, the glutamyl-prolyl-tRNA synthetase (EPRS) is one member of the MSC that, when mutated, causes a loss of protein function, resulting in a

severe neurodevelopmental phenotype that presents with microcephaly, epilepsy and cognitive impairment⁴. Other ARS mutations also cause neurodevelopmental disorders, including valyl-tRNA synthetase (VARS) mutations. When the effect of VARS knockout was explored in mutant zebrafish, the resulting phenotype included epileptic activity which was also seen in patients with the mutation⁵. While determining a causal relationship between the mutant genotype and the resulting phenotype is important, it is also important to determine if there is a way to fix the neurological symptoms. In the case of the VARS mutation in zebrafish, rescuing the gene by injecting wildtype mRNA seemed to result in a normal phenotype for the early stages of growth⁵. While the zebrafish model isn't a perfect representation of this disease, these results indicate that there may be a way to mitigate the detrimental neurological effects these types of mutations have.

A nonsense variant of AIMP2 is associated with severe neurodevelopmental phenotypes similar to those that result from various ARS mutations³. This phenotype is characterized by microcephaly, seizures, spastic quadriparesis, and cognitive impairment³. This association was determined using whole-exome sequencing in patients who have the symptoms. The genotypes identified from the sequencing determined three mutations that cause the phenotype: a homozygous nonsense variant, a homozygous splice-site mutation, and compound heterozygous nonsense and splice-site mutations³. The similarity of the mutant AIMP2 phenotype with the phenotype of other mutant ARS, may indicate that the mutations cause disease through a similar molecular pathway. In this work, the effect a of homozygous nonsense variant (Y35Ter) on expression at the mRNA and protein level, as well as progression of cells through the cell cycle will be investigated to learn more about how the neurodevelopmental phenotype arises.

Materials and Methods

Cell Culture

Fibroblasts from patients were collected by collaborators and control cells were obtained from the American Type Culture Collection (ATCC). All cells were maintained in an incubator kept at 37 °C and 5% CO₂ and grown in high glucose Dulbecco's Modified Eagle Medium media supplemented with 5% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. When the cells were between 75% and 90% confluent, they were split by removing the media and washing with Dulbecco's phosphate-buffered saline. Then, 0.25% trypsin was added to the cells, incubated for 30 seconds, and then removed. The plate and flask were placed in the incubator for two minutes and then the cells were re-suspended in the media they were originally grown in. An appropriate dilution was made and placed in new plates or flasks.

Fluorescent Microscopy

Cells were plated into a 24-well plate with small coverslips in each well. The cells were washed in phosphate-buffered saline (PBS) and then fixed to the coverslips with 4% paraformaldehyde in 1x PBS for 20 minutes. After washing the fixation solution off with PBS, the cells underwent permeabilization and blocking for an hour in PBS with 5% BSA and 0.1% triton-X100. The primary antibody for AIMP2 was applied at a concentration of 0.001 µg/mL in PBS with 3% bovine serum albumin (BSA) and 0.1% triton-X100 and incubated at 4°C overnight in a humidity chamber. After the primary incubation, the cells were washed with PBS before the secondary was added at a concentration of 1:500. The cells were incubated in the secondary for an hour and a half in the dark at room temperature before being washed with PBS

followed by water. The coverslips were mounted to slides using ProlongGold and imaged on an epifluorescence microscope.

Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The various plates of cells were lysed using an M-cell lytic solution with protease and phosphate inhibitors. The resulting solution was pelleted to remove unnecessary cellular debris. To determine the protein concentration of the various cell solutions, a Bradford assay was performed using standards of 2000 $\mu\text{g/mL}$, 1500 $\mu\text{g/mL}$, 1000 $\mu\text{g/mL}$, 750 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, and 125 $\mu\text{g/mL}$ of BSA⁶. Depending on the concentration of protein in the cell lysates, either 15 μg or 10 μg were used to run on the gel; however, each gel was run with a consistent amount of protein. Before loading the gel, 4x SDS loading dye containing 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 1% β -mercaptoethanol, 12.5 mM EDTA and 0.02% bromophenol blue was added to the samples and they were boiled for ten minutes at 100°C. The samples were loaded onto a 10% polyacrylamide gel with a 5% stacking gel and run in a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The gel was run at 100 V for about ten minutes until the samples were through the stacking gel and then the gel was run at 200 V for about 45 minutes.

Western Blotting

Fiber pads, filter papers, and the nitrocellulose membrane were soaked in the cold transfer buffer for ten minutes before being placed in the cassette with the gel. The assembly of the gel and membrane in the cassette was performed in a container with cold transfer buffer to ensure there were no air bubbles and that each piece was soaked. Once the cassette was placed into the running container, an ice block was placed in the container along with a stir bar to

prevent localized areas of heat. Ice was then packed around the container to keep the buffer cold. The transfer was run at 80 V for one hour. To ensure the transfer was successful, the membrane was briefly stained with ponceau stain. This ensures the lanes ran straight and that no bubbles occurred throughout the transfer.

The completed transfer membrane was blocked with 5% BSA in TBST overnight in a cold room. The block was removed and the membrane was washed with TBST for about an hour. A primary, anti-AIMP2 antibody in 3% BSA with Tris buffered saline with Tween-20 (TBST) containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20 was placed on the membrane and incubated for about an hour. Following incubation, the membrane was again washed for an hour with TBST and then incubated in an HRP goat secondary antibody for an hour. This was followed by a wash as described above. Proteins on the blot were visualized by briefly incubating with Clarity ECL Western Substrate. Protein levels were quantified by performing densitometry in ImageJ.

Puromycin Assay

Cells were plated onto small coverslips as described in the fluorescence microscopy methods. The cells were then treated with puromycin for 30 minutes before being fixed to the coverslips with paraformaldehyde. A 'click-chemistry' reaction was then performed to attach Alexa 594, a fluorescent probe, to the puromycin⁷. The relative intensity of fluorescence was measured using a fluorescent microscope to determine how much puromycin was incorporated into nascent polypeptides in order to determine the rate of protein synthesis.

Messenger RNA Analysis

Cells were cultured in the appropriate media as described above. Some flasks and plates, containing patient fibroblasts or ATCC cells respectively, were treated with cycloheximide while others were kept as controls. RNA was isolated from cells using the QIAGEN RNeasy® Mini column. Once the RNA was isolated, the samples were put through reverse transcription in order to create a cDNA library of the genes. A high-capacity cDNA reverse transcription kit from ThermoFisher Scientific was used and the reaction was run with an RNase inhibitor. A polymerase chain reaction (PCR) was utilized to amplify the cDNA corresponding to the mRNA of AIMP2. This was performed using Q5® High-fidelity DNA Polymerase. The reaction was run with 1x Q5 Reaction Buffer, 200 µM dNTPs, 0.5 µM of both the JTV11 and JTV13 primers, 1 µL of sample DNA, 0.02 U/µL of Q5 High Fidelity DNA Polymerase, 1X Q5 High GC Enhancer, and nuclease-free water. Samples from the patient fibroblasts and the ATCC cells both with and without the presence of cycloheximide were used as samples for this process

Cell Cycle Analysis

Patient and ATCC cells were re-suspended in a minimal amount of media. Each type of cell was mixed one to one with trypan blue dye and then placed into a cell counting chamber slide. A Countess™ (Invitrogen) machine was used to calculate the number of cells contained in a milliliter of the re-suspension along with determining the percent viability. Both ATCC and patient cells were plated onto a six well plate with 2.66×10^5 cells for the entire plate. The cells were allowed to grow for a few days until they were adherent. Media was then removed and reduced serum media was added to synchronize cells. This was left on for approximately 40 hours. Media was then returned to the cells at intervals so that when they were lysed, the cells had been in full media for 10, 9, 8, 7, 6 hours, and 30 minutes. The cells were once again lysed in

M-cell lytic. The resulting lysates underwent an SDS-PAGE and a western blot as described above; however, the antibody used was for cyclin D1.

Results and Discussion

AIMP2 mRNA and Protein Levels

While previous studies into the MSC and the AIMP molecules that help the complex come together have shown that some mutations that occur within the complex cause neurodevelopmental disorders, research into mutations in the AIMP2 gene is sparse. It was determined through genome sequencing that patients exhibiting a neurodevelopmental phenotype had homozygous nonsense mutations, Y35Ter, in the gene coding for AIMP2³. Through the collection and analysis of the mRNA, it is clear that mRNA expression is altered in patient cells (Figure 1A). While the full length AIMP2 mRNA level seems to remain consistent, the splice variant which does not contain exon two, along with an unknown form are dramatically decreased in patient cells (Figure 1A). To further understand what was occurring with the mRNA, the cells were treated with cycloheximide, which is known to inhibit the degradation of mRNA⁸. Treatment with cycloheximide appeared to normalize the mRNA expression pattern in patient cells, suggesting that nonsense-mediated decay may be occurring (Figure 1A).

After identifying differences at the mRNA level, the next step was to analyze the level of AIMP2 protein within the cells. Through western blot analysis, we determined that AIMP2 protein levels were dramatically decreased in patient cells compared to ATCC control fibroblasts (Figure 1B). The protein levels were normalized to a loading control and t-test analysis ($p < .05$) confirmed a significant reduction of AIMP2 in patient fibroblasts (Figure 1B). Fluorescence microscopy was also used to study the level and localization of AIMP2 within the cell. The

fluorescence imaging confirms that AIMP2 levels are reduced in patient cells and shows that wild-type AIMP2 is localized diffusely throughout the cytoplasm with a slight enrichment around the nucleus (Figure 1C).

Protein Synthesis

Once it was determined that both the mRNA expression and the protein level of AIMP2 were altered, it was important to study how this affected overall protein synthesis. AIMP2 is known to be an integral part of the formation of the MSC which is heavily involved in mammalian protein synthesis¹. To study protein synthesis, cells were treated with puromycin. The puromycin is incorporated into nascent polypeptides⁹. This, along with a fluorescent probe that binds to the puromycin, allows protein synthesis rate to be analyzed⁹. When this was performed on patient fibroblasts and ATCC cells, there was a lower rate of protein synthesis in patient cells (Figure 2). This decrease cannot be considered statistically significant until this experiment is replicated and concludes consistent results. If the results of the replications are consistent, it would support the idea that this mutation significantly impacts protein synthesis. This leads to the idea that even this slight decrease in the rate of protein synthesis could dramatically impact how the rest of the cell functions.

Levels of the MSC associated leucyl-tRNA synthetase

While the nonsense mutation in AIMP2 seems to cause a decrease in the rate of protein synthesis, it does not actively have a catalytic function. The enzymes that are associated with AIMP2 are the ARS which charge tRNAs with the correct amino acid². Due to the role AIMP2 plays within the MSC, when it is mutated, the MSC either falls apart or cannot form in the first place¹. To further understand how the MSC and its individual parts are impacted by the AIMP2

mutation, another component was analyzed through western blotting. Leucyl-tRNA synthetase (LARS) is a component of the MSC that appears to be closely associated to AIMP2¹. When the protein level of LARS was analyzed in patient fibroblast, there appeared to be a slight decrease when compared to ATCC cells (Figure 3). This is an important result because it could mean that the inability to form the MSC leads to ARS degradation in patient fibroblast. Further research is needed to determine if this is an isolated incident or if it happens among all of the ARS that are located within the MSC.

Cell Cycle

One important idea as to how the mutation in AIMP2 causes the neurodevelopmental phenotype relates to defects in cell cycle progression. It was hypothesized that the mutation altered the cell's ability to progress through the cell cycle which could in turn cause microcephaly. To study the progression from G1 to S phase in the cell cycle, cyclin D1 levels were analyzed in an unsynchronized cell population along with cells that were synchronized through serum starvation. Cyclin D1 is one of the first cyclin molecules to be upregulated in the cell cycle and this molecule determines the cell's progression into the proliferation process¹⁰. When the levels of cyclin D1 in patient fibroblasts and ATCC cells was measured, there seemed to be very little difference between the two groups. For the unsynchronized study, there was a trend towards decreased cyclin D1 levels in patient cells, but no statistically significant difference between the patient fibroblasts and ATCC cells (Figure 4B). When the cell cycles were synchronized, the resulting expression of cyclin D1 was inconclusive. The protein levels clearly increased after serum administration at the different time points, but there was no significant difference between the patient fibroblasts and ATCC cells in either of the trials (Figure 4A).

These results do not support the hypothesis that the cell cycle is being impacted by this mutation; however, it does not exactly refute it either. The cells that were studied in this experiment were skin fibroblasts. These are fairly easily obtained from patients and can be easily cultured once the correct conditions are determined. For some experiments, such as analyzing protein levels and protein synthesis, patient fibroblasts can be extremely useful. The downfall, in this case however, is that the mutation in AIMP2 causes neurodevelopmental phenotypes which means that this mutation is specifically causing something to occur in neurons. Neurons are different from fibroblasts in many ways, so it is hard to fully understand what is occurring in the neurons by studying fibroblasts. So, while the fibroblasts are not showing that a mutation in AIMP2 impacts the cell cycle, it is very possible that neuronal cell proliferation is affected in some way.

Future Studies and Significance

While studying patient permits the determination of how the AIMP2 mutation impacts protein expression and synthesis, it is important to further study how the mutation affects neurons. Obtaining patient neurons is not an option so creating a mutant line of neurons is necessary. This was attempted in PC12 cells which are rat pheochromocytoma cells¹¹. The transfection of previously studied ARS mutations was attempted with no success. Further studies into the AIMP2 mutation would require the development or identification of a neuronal cell line that can be easily transfected for study.

This nonsense mutation in AIMP2 causes a severe neurodevelopmental phenotype in the patients who have it. While the specific mechanism as to how the mutation causes microcephaly and the other neurological symptoms is unknown, these results start to pave the way for determining the pathophysiology of this disorder. These results support the hypothesis that the

mutation causes decreased AIMP2 protein levels which impacts the function of the MSC and its associated ARS. These changes lead to the neurodevelopmental phenotype seen in patients.

Hopefully, by understanding the change in protein level and the rate of synthesis, future studies can build upon this research and work towards a cure or treatment. Without knowing how the mutation causes the phenotype, treating or curing the resulting disorder is nearly impossible.

There is still a lot of work to be done before a treatment can even be thought about, but with current technological advances in all areas of research, along with these initial results, further studies can be done to understand the mutation and possibly alleviate the symptoms.

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Figures

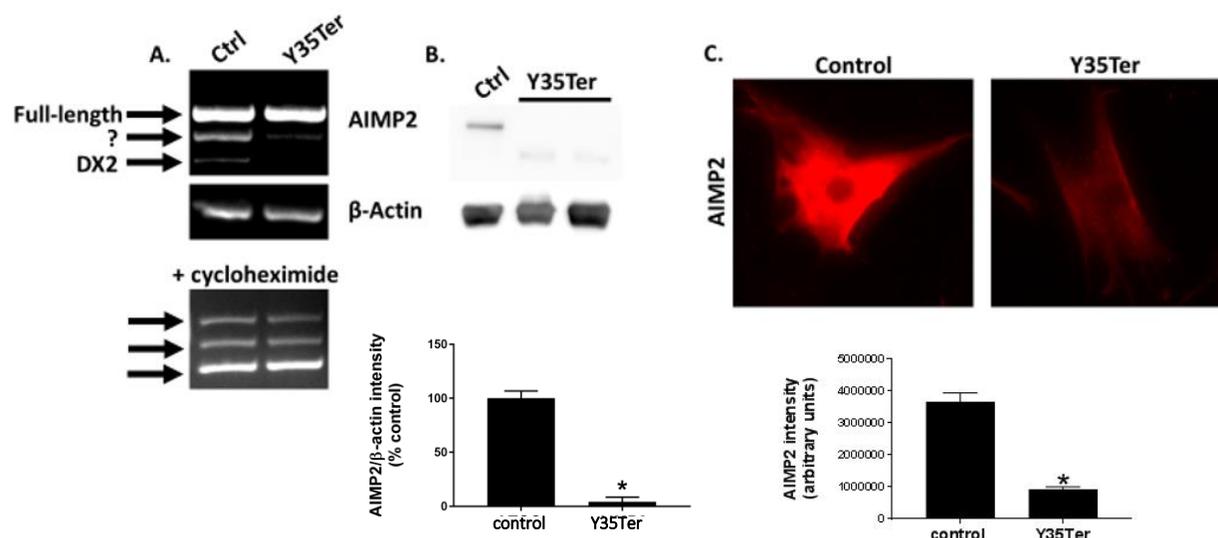


Figure 1. Analysis of AIMP2 protein levels in patient fibroblasts and ATCC cells. **A.** RT-PCR of AIMP2 mRNA along with a β -Actin loading control. The same was performed on cells treated with cycloheximide. **B.** Western blot analysis comparing the amount of AIMP2 in ATCC cells and patient fibroblasts. β -Actin was used as a loading control in order to normalize the data. The resulting graph shows the comparison between the normalized intensity of AIMP2 along with the statistical significance of the difference between the two experimental conditions (*). **C.** ATCC cells and patient fibroblasts were tagged with an antibody for AIMP2 (0.001 μ g/mL) and then imaged using fluorescence microscopy. The relative intensity of the fluorescence of the cells was measured across numerous cells and averaged to determine statistical significance (*).

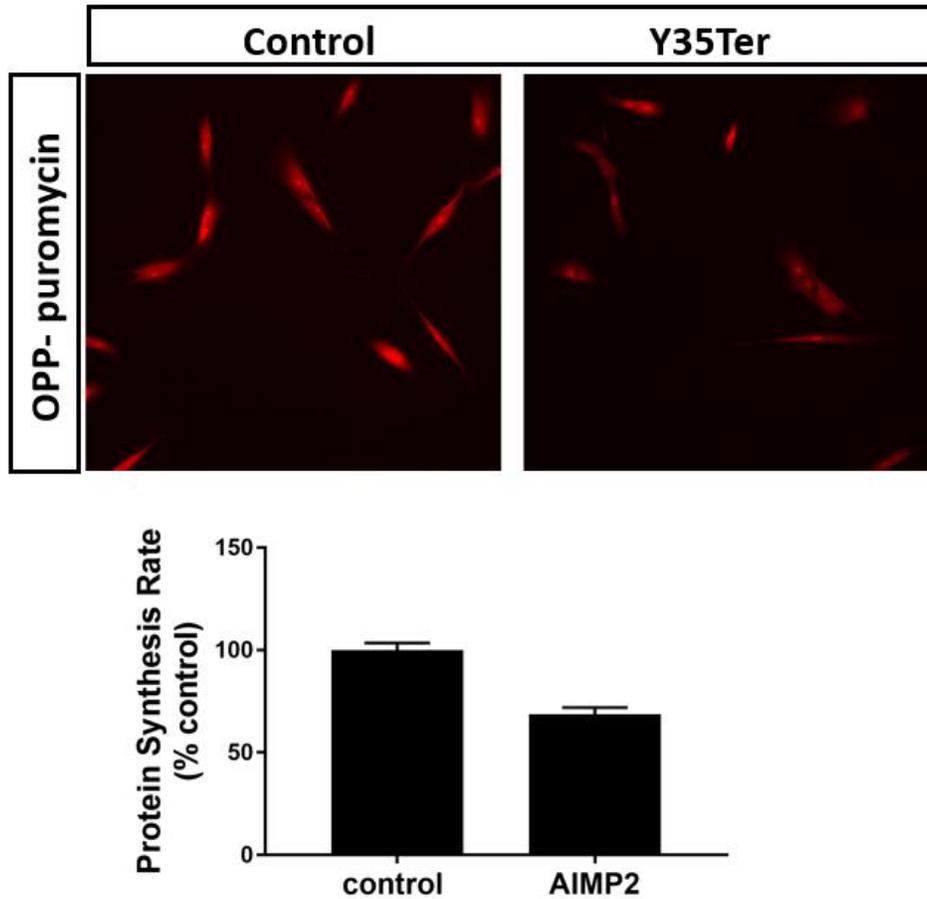


Figure 2. Puromycin treated cells with a ‘click-chemistry’ fluorescent probe, Alexa 594, attached to the puromycin. The relative intensity of the fluorescence of numerous cells was measured and normalized. Puromycin is incorporated into forming polypeptides so the fluorescence shows relative protein synthesis.

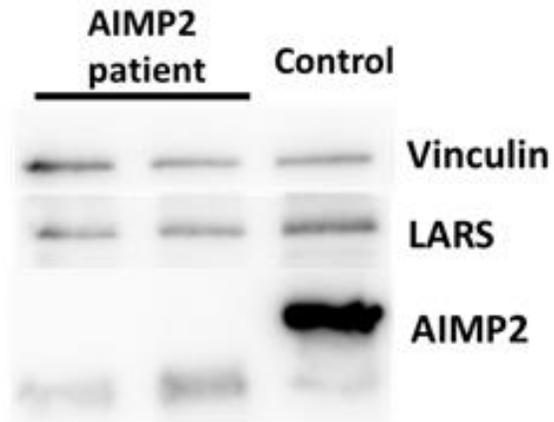


Figure 3. Western blot analysis comparing the amount of AIMP2 and LARS in ATCC cells and patient fibroblasts. Vinculin was used as a loading control in order to normalize the data. Clarity ECL Western Substrate was used to visualize these proteins.

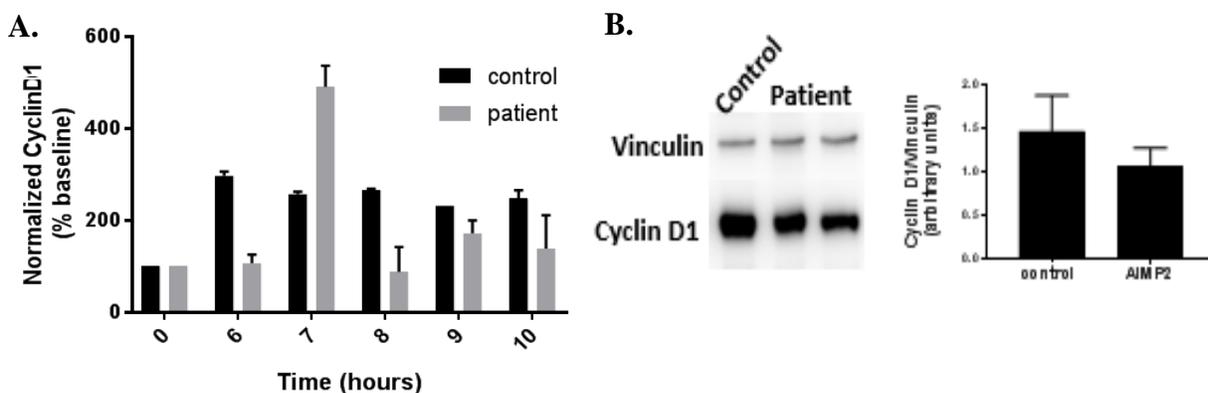


Figure 4. Cell cycle analysis of western blots with an antibody to Cyclin D1 to determine how AIMP2 impacts cell cycle progression. **A.** The cell growth and starvation were synchronized in order to determine the cells ability to make Cyclin D1 at certain points in the cell cycle. The relative intensity of the Cyclin D1 was normalized to either vinculin or β -Actin depending on how well the antibody worked. The data were then normalized to the zero time point. **B.** Western blot analysis comparing the amount of Cyclin D1 in ATCC cells and patient fibroblasts that had unsynchronized cell cycles. The resulting graph shows the comparison between the Cyclin D1 levels of the two groups when compared to vinculin as a loading control.

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