Finding a Therapy for Wolfram Syndrome: Exploring a Calcium Signaling Pathway as a Target for a Disease Without a Cure

Introduction

Wolfram Syndrome is an autosomal recessive genetic disorder that is caused by endoplasmic reticulum dysfunction, affecting approximately 30,000 people worldwide, making it an orphan disease. Typical symptoms of Wolfram Syndrome include diabetes mellitus and diabetes insipidus, hearing loss, neurodegeneration, mood disorders, optic nerve atrophy, and urinary tract infections. Diabetes is typically diagnosed at age six, with optic nerve atrophy five years later, and the rest of the symptoms then follow. The majority of patients with the disease die between ages 25-49 with neurological disabilities such as bulbar dysfunction or organic brain syndrome, and the primary cause of death being respiratory failure due to brain stem complications. **There are no definitive cures for the disease**, with palliative care as the only option.

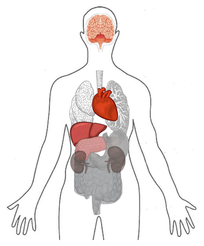


Figure 1: ***Organs affected by Wolfram Syndrome*** *The most commonly affected organs are shown. The ones in color indicate increased associated symptoms. Note the liver, pancreas, and brain. This figure and all subsequent figures were student made unless specified.*

The genetic form of Wolfram Syndrome that I studied was WFS1. Mutations in this gene cause Wolfram Syndrome. **The gene WFS1 encodes for the protein Wolframin and is highly active in the heart, brain, lungs, inner ear, and pancreas.** Neuronal Calcium Sensor 1 (NCS1) is a high-affinity, low-capacity, and calcium binding protein. NCS1 is important in regulating calcium signaling and maintaining calcium homeostasis within the cell. Additionally, **NCS1 has been found to be low in patients with Wolfram Syndrome.**

Calpain belongs to the family of calcium dependent proteins. When calpain cleaves NCS1, this calcium binding protein no longer works, causing reduced calcium signaling throughout the cell. **This loss of NCS1 activity appears to have an impact on Wolframin levels, suggesting that Wolframin and NCS1 are co-regulated.**

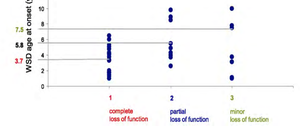
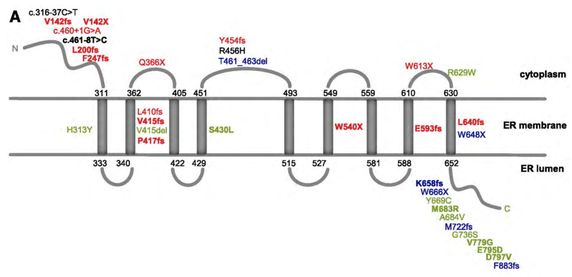


Figure 2: ***Mutations in WFS1 (below) categorized by type (left)*** *Mutations in the WFS1 gene in the characterized by relative loss of function (Rohayem et.al,, 2011). In this experiment, the N-terminus in the cytosolic domain was targeted as a binding site, which contains "complete loss of function" mutations.*



The dysregulation of the interaction between Wolframin and NCS1 was investigated to determine if this interaction explains the clinical implications of the disease. It was predicted that components of calcium signaling are impacted by WFS1 mutations, and the pathway underlying Wolfram Syndrome is due to the interaction between Wolframin and NCS1, where binding of the N terminal portion of Wolframin to NCS1 is disrupted.

This study could provide insight into the pathway underlying Wolfram Syndrome and could also provide an understanding into the function of Wolframin, which is presently unknown.

A proposed mechanism for the interaction between the two proteins will be complemented by:

- Molecular Docking and *in silico* binding techniques.
- The docking results will be tested using a glutathione S transferase tagged version of Wolframin to pull down NCS1 in a calcium dependent manner.
- A quantitative polymerase chain reaction will be performed to analyze RNA expression levels.

Working Hypothesis

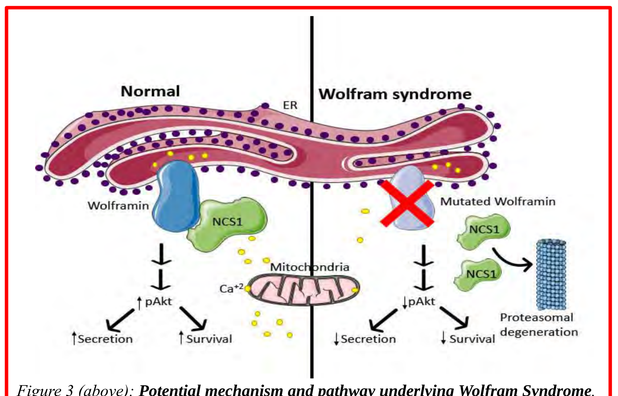


Figure 3 (above): ***Potential mechanism and pathway underlying Wolfram Syndrome****. Wolframin is able to maintain normal function, with increased secretion and survival, when bound to NCS1. When this interaction is disrupted due to the mutation of Wolframin, it causes NCS1 to degrade and lower secretion and survival of Wolfrmain.*

Note: (NCS1 = neuronal calcium sensor 1, ER = endoplasmic reticulum, Ca^{2+} = calcium ion, pAkt = phosphorylated protein kinase B)

The dysregulation of the interaction between Wolframin and NCS1 was investigated to determine if this interaction explains the clinical implications of the disease. **It was predicted that components of calcium signaling are impacted by WFS1 mutations, and the pathway underlying Wolfram Syndrome is due to the interaction between Wolframin and NCS1, where binding at the N terminal portion of Wolframin to NCS1 is disrupted.**

Crystal Docking Data Predicts Binding Site Between NCS1 and Wolframin

The predicted structures of NCS1 binding with Wolframin were generated by first submitting the sequence of residues 1-288, the N-terminus of Wolframin, to the Robetta server. Five predicted structures were examined using PyMOL, a molecular visualization system released under the Python license, and the top prediction was chosen for docking with NCS1 (Figure 4). A previously crystallized version of NCS1 (Figure 5) was submitted along with the predicted Wolframin structure to "PyDock," a software that predicts the interaction between two chosen proteins. **After preliminary analysis using PyMol, it was determined that focusing on the residues of NCS1 that were previously indicated to be cleaved by calpain, a calcium dependent protein would be most relevant. Therefore, the calpain cleavage site of NCS1, residues 36 and 37 of chain A, was selected as a critical region to bind with Wolframin.** These results were also analyzed using PyMol. The calpain cleavage site and the region where it interacts with Wolframin is shown with an arrow in Figure 6.

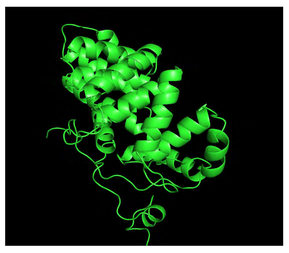


Figure 4 (above): ***Predicted structure of WFS1****, Robetta Server*

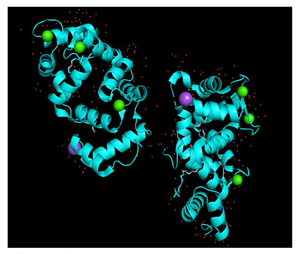


Figure 5 (above): ***Crystal structure of NCS1 chains*** *A and B, 1g8i (PDB).*

The two proteins are nested such that Wolframin protects the calpain cleavage site of NCS1

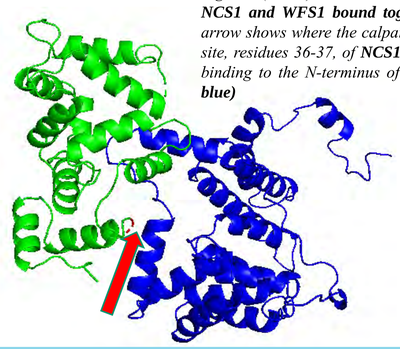


Figure 6 (below): ***NCS1 and WFS1 bound together.*** *Red arrow shows where the calpain cleavage site, residues 36-37, of* ***NCS1 (in green)*** *binding to the N-terminus of* ***WFS1 (in blue)***

GST-tagged Wolframin Pulls Down Protein Complex in a Calcium Dependent Manner

STEP 1: 1.5% gel with WFS1 N-terminus

1. Expressed the N terminus of WFS1 in *E.Coli*
2. Eluted the DNA by performing a miniprep
3. Sent this DNA for sequencing and designed primers for the N-terminus based on those results
4. Ran a PCR and used the resulting samples to run a 1.5% gel (right)

Figure 7: Student researcher preparing to run a western blot

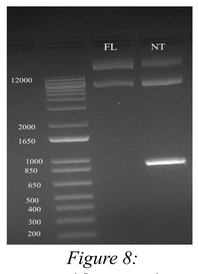


Figure 8: ***PCR with N-terminus*** *and FL of WFS1. Only the NT was necessary for purification*

STEP 2: PCR purification and extraction for IPTG induction

1. A PCR purification kit was used, and the samples were run on a gel
2. A gel extraction kit was used to extract the samples
3. A restriction digest was performed with BamHI ,EcoRI enzymes, and the N-terminus of WFS1.
4. An IPTG mixture was made with the bacteria (WT WFS1 and KO WFS1 previously expressed in *E.Coli*) and placed in the incubator for 16 hours
5. Resulting samples were run to show expression of Wolframin proteins and these were used in Step 3.

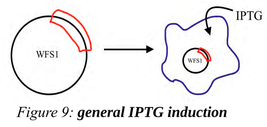


Figure 9: ***general IPTG induction***

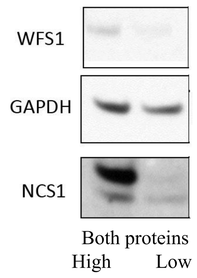


Figure 10: ***Protein levels*** *– WT WFS1 (left), KO WFS1 (right) using a standard western blot.*

When NCS1 is high, WFS1 is high, and when NCS1 is low, WFS1 is low.

STEP 3: Making the GST-Wolframin Complex

1. GST (glutathione S-transferase) buffer, beads, and other necessary reagents were prepared
2. Spun down GST-Wolframin lysate to remove precipitate
3. Washed with EGTA buffer
4. Ran western blot to confirm GST-Wolframin complex (see Figure 11)

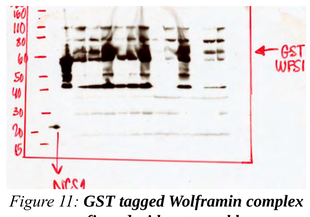
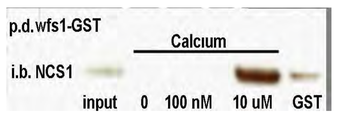


Figure 11: ***GST tagged Wolframin complex confirmed with western blot***

STEP 4: Performing the pull-down at varying Ca^{2+} concentrations

1. Added appropriate buffers to individual tubes and varying concentrations of Ca2+ - NCS1 into each tube (along with GST and input)
2. Left to rotate in cold room for 12 hours
3. Eluted with 50mM GHS
4. Ran a western blot with an antibody probed against NCS1

Figure 12 (right): ***NCS1 and Wolframin bind in a calcium dependent manner.*** *NCS1 and the cytoplasmic domain of Wolframin (wfs1-GST) were expressed in bacteria and NCS1 was purified. The cytoplasmic domain of Wolframin had a GST tag that was used to pull down the protein complex.*



Note: p.d. = pull down, i.b. = immunoblot

Increased free calcium means an increased pull down of NCS1.

qPCR Shows NCS1 and WFS1 are Co-Regulated

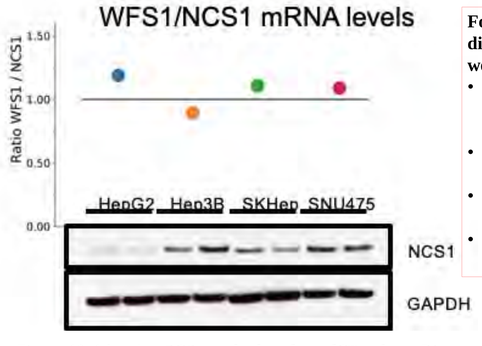


Figure 13 (above): ***qPCR results based on dCT values.*** *Direct ratios of WFS1/NCS1 mRNA levels were taken, (lower):* ***Relative expression of NCS1*** *(note the varying amounts) in HCC cell lines with a GAPDH loading control.*

Four different cell lines derived from different hepatocellular carcinomas were quantified:

- **HepG2** (well differentiated hepatocellular carcinoma of human origin with low NCS1)
- **Hep3B** (human cell line of epithelial origin)
- **SKHep** (human cell line of endothelial origin)
- **SNU475** (human cell line of epithelial origin)

NCS1 to WFS1 ratio is constant, suggesting co-regulation

The dCT values of WFS1/NCS1 yielded a ratio close to 1, independent of if NCS1 was high (SNU475, Hep3B) or low (HepG2, SKHep).

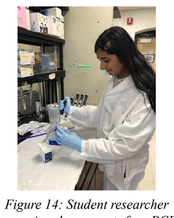


Figure 14: Student researcher preparing the reagents for qPCR with HCC cell lines and CYBR green reagent.

Preparation of reagents and running quantitative real-time PCR

1. To make the reagents for testing the coregulation of NCS1 and WFS1 using qPCR, Human WFS1-specific primers were obtained from a published paper (Plass M, et.al).
2. The cDNA was isolated from hepatocellular carcinoma cell lines (Note: I received cell lysates, as I was unable to directly handle the cells before this point due to my age).
3. In the qPCR mastermixes. 18s primers were used as a control/housekeeping gene, along with NCS1 and WFS1 primers, resulting in three separate mixes but with identical cDNAs from the four cell lines.
4. The well plate was covered and centrifuged, and then put in the qPCR machine with the pre- determined settings.
5. Python was used to make graphs from the raw excel data to do standard dCT and ddCT analysis.
6. These results were validated by repeating the experiment three times.
7. In order to show the relative expression of NCS1 in each of the cell lines, a western blot was performed to visualize the levels of NCS1 with a GAPDH loading control.

Discussion of Results

Wolfram Syndrome is a complicated disease characterized by diabetes mellitus, optic nerve atrophy, mood disorders, and neurodegeneration. There is no cure or treatment for Wolfram Syndrome and the mechanism through which the disease manifests is still unknown. Thus, the goal of these experiments was to explore a potential underlying pathway. **The primary objective was to investigate the interaction between two proteins, Wolframin and NCS1, and to determine whether this interaction can explain the clinical implications of the disease.** It was demonstrated that components of calcium signaling are impacted by WFS1 mutations and that the pathway underlying Wolfram Syndrome is related to the interaction between Wolframin and NCS1, in particular the N-terminus of WFS1. The N-terminus of WFS1 and the critical residues of NCS1 were determined using PyMol and the binding software PyDock. These resides, 36-37, are also the calpain cleavage site which is important because when this site is cleaved by calpain, NCS1 no longer works. **Because NCS1 and WFS1 are codependent, when NCS1 no longer functions, WFS1 is affected. The codependence of these two proteins was demonstrated in the qPCR showing that the ratio of the mRNA for the two proteins is constant, even when the level of the protein expression is examined over a large range.** The GST-tagged pull down showed that NCS1 and Wolframin bind in a calcium dependent manner. In addition, increased free calcium lead to an increased pull down of NCS1. **These findings further support the molecular docking binding results and the involvement of calpain.** These results suggest that the interaction between Wolframin and NCS1 is a critical one that when disrupted could be the path underlying Wolfram Syndrome. This disease is caused by mutations in WFS1, and the reaction of NCS1 to these disruptions is a factor in the

Sources of Error and Limitations

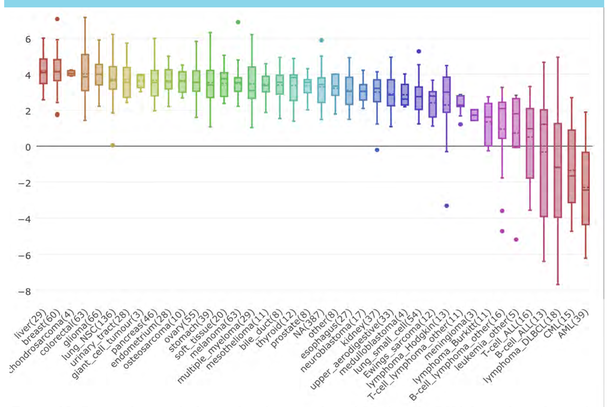


Figure 15: ***Regression analysis results of expression of Wolframin RNA in various tissues by copy number.*** *Note the high levels in liver and pancreas. Data courtesy of the CCLE database from the Broad Institute at MIT.*

Although this experiment was successful in various ways, there were other aspects that could be improved. The cDNA used in the qPCR was from a hepatocellular carcinoma cell (HCC) line, which expresses WFS1, but not at as high of a level as other cell lines such as the pancreas (see Figure 15, above). Although using the HCC line yielded viable results, a next step to this would to isolate the cDNA from a pancreatic cell line, or another cell line with high expression of WFS1, and repeat the experiment. In addition, there were some complications with the qPCR at first. The SYBR green mix that was used in the first attempt was old and contaminated, so it had to be replaced. In addition, the reverse sequence that I designed at first was incorrect and had to be redesigned. Once these two mistakes were resolved, the experiment ran smoothly but meant that the initial experimental timeline was set back. Running out of time meant that I was unable to observe the effect of Wolframin on calcium signaling, which was originally part of the plan. Due to my age and institutional protocols, there are certain procedures I am unable to perform, so I was limited to the *in silico* and *in vitro* models for the experiment. Finally, since Wolframin has not been crystallized yet, predicted structures were used. Although these models are reasonably accurate, there is a higher margin of error compared to the crystallized form of the protein.

Key Conclusions

- **WFS1 and NCS1 are co-regulated**
- **The N-terminus of Wolframin protects the calpain cleavage site of NCS1**
- **The interaction between Wolframin and NCS1 is calcium dependent**

Future Directions

Future directions regarding the interaction between NCS1 and Wolframin could be to:

- Redo the crystal docking between the N-terminus of NCS1 and Wolframin once there is an established crystal structure of Wolframin
- Express the mutant and wild type WFS1 in cells and determine their effect on calcium signaling. A further extension includes obtaining INS-1 cells (rat pancreatic beta cells) and growing them at 3 different levels of glucose (normal, high, and toxic) to measure cell survival and motility.
- A further step with the INS-1 cells would be to extract the cDNA and perform qPCR experiments to determine changes in NCS1, Wolframin, ER calcium pump, and voltage gated calcium channel.

Finally, knowing more about the function of Wolframin and the role that WFS1 plays in other diseases can provide insight into the function of and potential therapeutics for other diseases such as diabetes and mood disorders.