

THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

THE CONSTRUCTION OF ATAXIN-1 VECTORS FOR ANALYSIS OF ATAXIN-1
FUNCTION IN DEVELOPMENT OF SPINOCEREBELLAR ATAXIA TYPE 1

TIMOTHY WANG
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Reviewed and approved* by the following:

Yingwei Mao
Assistant Professor of Biology
Thesis Supervisor

David Gilmour
Professor of Molecular and Cell Biology
Thesis Supervisor

Sarah Ades
Associate Professor of Biochemistry and Molecular Biology
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Ataxias are a group of disorders that are characterized by a progressive loss of body movement control that can eventually be fatal. Symptoms result from a degradation of neural cells throughout the central nervous system which can be caused from a wide array of clinical causes including multiple sclerosis, alcoholism, or brain tumors. Additionally, ataxias may be caused by a genetic mutation, and these types of ataxias can be inherited through generations. Inherited ataxias may be autosomal recessive or dominant. One of the most prominent autosomal dominant forms of ataxia is spinocerebellar ataxia, or SCA. While there are many causes of SCAs, the most common mutation leading to the development of SCA is an elongation of CAG triplet repeat sequence. The CAG triplet encodes glutamine (Q) and the mutation leads to a long polyQ segment in the resulting protein. This mutant protein is prone to aggregation and causes neurodegeneration through an unclear mechanism. Recently scientists have been investigating the normal functions and protein interactions of ataxin-1, the protein responsible for development of SCA1. The Mao lab in which I performed my honors research is also interested in investigating the protein interactions of ataxin-1 in order to better understand the mechanism for SCA1 pathogenesis. In order to perform further studies, I was tasked with creating six different ataxin-1 vectors. Three vectors were for the wild type 30Q ataxin-1 and three vectors were for the mutant 80Q ataxin-1. Each of the three vectors had a different tag for further protein purification and identification. Future considerations for experimentation with the vectors I created are provided, along with an analysis of some of the most recent scientific research into ataxin-1.

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Chapter 1

Introduction

Ataxia

Ataxia is a neurological disorder that causes a progressive loss of control of body movement. Symptoms can range from clumsiness and slurred speech to the loss of ability to breathe or swallow, which can lead to death. These symptoms are caused by neurodegenerations in the cerebellum, brain stem, and spinocerebellar tracts which can be caused by a variety of conditions including multiple sclerosis, brain tumors, or genetic defects. Ataxias resulting from genetic defects can be passed down through generations, and these ataxias can be inherited in either an autosomal recessive or autosomal dominant fashion. Common recessive forms of ataxia include ataxia telangiectasia and Friedreich ataxia, which affect the cell cycle regulation and mitochondrial function respectively. However, the focus of this paper will be on autosomal dominant ataxias called spinocerebellar ataxias, or SCAs (Cell Biology of Spinocerebellar Ataxia).

SCAs, like other forms of ataxia, are characterized by progressive worsening in control of body movement. Common symptoms include difficulties with balance, coordination, speech, and swallowing. In addition, SCAs can lead to spasticity in muscles and problems in controlling eye muscles and movement, such as nystagmus and ophthalmoplegia. In rare cases, SCAs can also lead to mental impairment, dystonia, and muscle atrophy. Symptoms typically manifest in adults, though there have been cases where the first signs of SCA began in childhood or late adulthood. The life expectancy is generally 10-20 years after the development of symptoms (Genetics Home Reference). There are around 30 identified forms of SCA, but causative mutations for each SCA

have not been identified. Some mutations that are known to lead to SCA development include mutations in protein kinase C or fibroblast growth factor 14. However, these mutations are only seen rarely in isolated types of SCA. For the most part, the most predominant mutation leading to SCA is a mutation leading to extended repeats of cytosine, adenine, guanine (CAG) triplets. In normal genes, the CAG sequence may be repeated up to 30 times, but in the mutant genes, the CAG sequence may be repeated greater than 100 times (Chung et al). This CAG triplet codes for the amino acid glutamine. Therefore, this mutation leads to the production of an abnormally long protein with an extended polyglutamine (polyQ) segment. These polyQ mutant proteins have been the hallmark for SCA diagnosis for many types of SCA (Cell Biology of Spinocerebellar Ataxia).

However, while it has been established that the presence of an abnormally elongated polyQ segment is required for SCA disease development, scientists have more recently begun investigating the mechanism by which these polyQ segments contribute to disease. Earlier on, it was hypothesized that the development of SCA was due to the toxicity of polyQ proteins. Studies by Marsh et al. showed that SCA may be caused by the proteolytic production of polyQ proteins (Marsh et al., 2000). However, studies by both Davies et al. and DiFiglia et al. provided evidence that showed that the proteolytic production of toxic polyQ proteins was required for pathogenesis in only Huntington's disease (Davies et al., 1997, DiFiglia et al. 1997). This seemed to disprove that the toxic polyQ proteins are sufficient to cause development of SCAs on their own.

Another trend seen in the development of SCAs was the tendency of mutant polyQ proteins to form protein aggregates. Scientists theorized that these aggregates may contribute to the pathogenesis of SCA. Studies by Orr and Zoghbi have shown that the mutant expansion of the polyQ gene segment renders the protein more prone to aggregating and forming inclusion bodies (Orr and Zoghbi, 2007). The formation of these polyQ protein aggregates and inclusion bodies is a widely seen phenomenon in polyQ mutation SCAs and there are differing theories as to the

extent to which these protein complexes actually contribute to the development of disease. While the presence of these polyQ protein complexes and inclusion bodies almost always accompany the development of SCA, there are some studies that show that the formation of these aggregates can be separated from the development of disease in mice (Klement et al., 1998, Saudou et al., 1998). Specifically, Klement et al. created comparable ataxic phenotypes in two different types of transgenic models. One model expressed an extended polyQ mutant protein. This model developed the typical SCA phenotype with aggregates of ataxin-1 in the nuclei of Purkinje neurons. The second model, however, carried a deletion mutation within the SARs. This model also developed SCA, but there was no aggregate formation. Furthermore, Bowman et al. found that in some cases, the formation of inclusion bodies can actually protect against the development of disease, perhaps by sequestering the mutant protein where it could not do harm (Bowman et al.).

Although the presence of mutant polyQ proteins and protein aggregates and inclusion bodies are hallmarks of SCAs, it is clear that the extent to which the polyQ segment or protein aggregates themselves contribute to SCA pathogenesis. Because of this, scientists have more recently turned their attention to discovering the normal functions of the mutated protein, the other proteins the mutated protein normally interacts with, and how mutation alters these functions or interactions to produce the SCA. With that, we turn our attention to SCA1 which is caused specifically by a polyQ mutation in the ataxin-1 protein.

SCA1 and Ataxin-1

SCA1 is one of the more predominant types of SCA, affecting 1 to 2 people per 100,000 worldwide. SCA1 is caused by a mutation in the *ATXN1* gene which encodes the ataxin-1 protein. Ataxin-1 can be found throughout the body and its exact function is unknown. In the cells, ataxin-1 can be found in the nucleus or cytoplasm and may have roles in regulating protein production and processing RNA. The *ATXN1* gene mutation leads to an increased number of CAG triplets.

The wild type ataxin-1 protein contains anywhere from 4 to 39 CAG repeats, whereas the mutant ataxin-1 protein may contain anywhere from 40 to more than 80 CAG repeats. The extent of CAG repeat elongation also has a direct correlation with the severity of the SCA1. Generally, those with 40 to 50 CAG repeats show symptoms in mid-adulthood, while those with more than 70 CAG repeats show symptoms as early as mid to late adolescence (Genetics Home Reference).

Ataxin-1 is found throughout the neurons of the central nervous system, but immunofluorescence studies by Watase et al. have shown that ataxin-1 are found much more commonly in Purkinje neurons than in granule neurons (Watase et al.). In individuals with SCA1, the neurodegeneration takes place primarily in the brainstem neurons and cerebellar Purkinje neurons, and ataxin-1 is found concentrated in the nuclei of these cells (Zoghbi, 1995). Furthermore, studies by Klement et al. found that when they mutated the nuclear localization signal on a mutant ataxin-1 protein in mice, the mice never developed SCA1 (Klement et al., 1998). This seems to indicate that in addition to polyQ mutation and protein aggregation, localization to the nucleus is another hallmark of SCA1 that accompanies the development of disease.

Like other mutant polyQ proteins, the elongated forms of ataxin-1 have a tendency to form protein aggregates. As with all SCAs, the formation of these aggregates is tied to the development of the disease. Several scientists have studied the components of these aggregates in order to determine a possible link to the mechanism of SCA1 pathogenesis. Cummings et al. found that the aggregates contained both ubiquitin and components of the proteasome and the HDJ-2/HSDJ chaperone protein (Cummings et al., 1998). They used immunohistochemical staining of the ubiquitin, proteasome, and HDJ-2/HSDJ proteins to find that these proteins were found in the mutant ataxin-1 aggregates in the nucleus of cells in SCA1 infected patients. Addition of an excess of the chaperone protein actually led to a decrease in the aggregate

formation in the nucleus and reduced effects of the SCA1, indicating that aggregate formation may be important in SCA1 pathogenesis (Cummings et al., 1998).

However, as with before, there were several studies demonstrating cases where the absence of aggregate formation did not correlate to an absence of disease. The true purpose of the aggregate formation in disease development remains unclear and complex, so scientists have turned to studying the normal function of ataxin-1.

Ataxin-1 Function

Ataxin-1 normally is found in both the cytoplasm and the nucleus and has the ability to shuttle between both sites. Ataxin-1 also has the ability to become phosphorylated at serine 776 (S776) while in the cytoplasm (Emamian et al., 2003). This leads to binding of 14-3-3 (Chen et al., 2003). The binding of 14-3-3 overlaps the nuclear localization site and S776, which prevents nuclear import and dephosphorylation of ataxin-1 until some regulated time. At this time, 14-3-3 is dissociated and ataxin-1 is transported into the nucleus where in can either interact with either RBM17 when phosphorylated (Lim et al., 2008) or U2AF65 when dephosphorylated (de Chiara et al., 2009). Both RBM17 and U2A65 are RNA-splicing factors, indicating that ataxin-1 normally plays a role in RNA modification and protein synthesis (figure 3-1). To further complicate matters, ataxin-1 has also been known to interact with transcription repressor Capicua (Cic). Ataxin-1 has a large web of interactions with other proteins involved in important cellular processes, and it is these interactions that are being studied more recently instead of the mutant polyQ segment itself in order to determine the mechanism by which ataxin-1 causes neurodegeneration in SCA1.

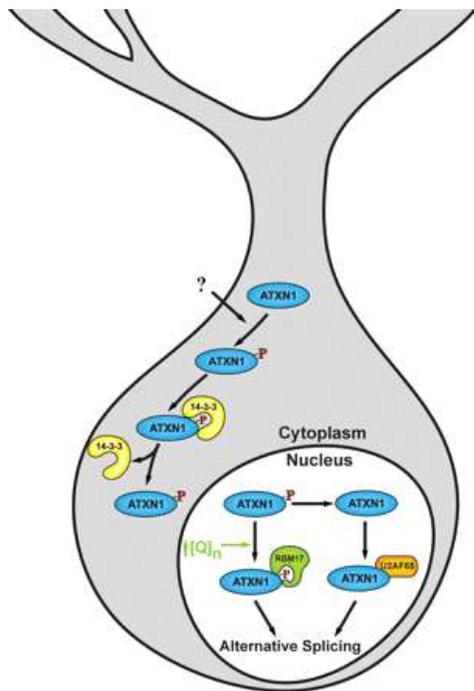


Figure 1-1. The ataxin-1 cellular pathway. Ataxin-1 is phosphorylated in the cytoplasm, which allows it to interact with 14-3-3. This blocks entry into the nucleus until a regulated time when 14-3-3 is dissociated. In the nucleus, ataxin-1 may serve as a regulator of alternative splicing through its interactions with either RBM17 or U2AF65.

The Mao lab wishes to learn more about the function of ataxin-1 and the proteins that it interacts with in order to learn more about the complex mechanism of SCA1 pathogenesis. In order to do this, I was tasked with the creation of six ataxin-1 vector constructs, three for the wild type ataxin-1 (30Q) and three for a mutant ataxin-1 (80Q). Each of the three vectors for each type of ataxin-1 were tagged with a His tag, FLAG tag, or GFP. In the future, the Mao lab will continue to use these constructs in order to perform experiments like yeast-2-hybrids or luciferase assays in order to further study ataxin-1 and its role in causing SCA1.

Chapter 2

Materials and Methods

Plasmids and Enzymes:

Original Plasmids. pCMV-3xFLAG-hATX1-30Q (262 µg/ml),
pCMV-3xFLAG-hATX1-80Q (269 µg/ml) provided by

Vectors. pENTR4-FLAG (empty), pENTR4-V5 (empty), pENTR4-GFP (empty)
provided by Invitrogen

Restriction Enzymes. Age1 (20,000 U/ml), Stu1 (10,000 U/ml), Xba1 (20,000 U/ml),
Xho1 (20,000 U/ml), Apa1 (10,000 U/ml)

Cells:

Competent Cells. Pick up colony from DH10b plate and transfer to tube with 1 ml LB without antibiotic. Let shake overnight at 37°C. In a 250 ml flask, add 20 ml LB, without antibiotic, and 200 µl of overnight culture. Let shake for at least 2-3 hours at 37°C. Cells were then pelleted by centrifugation at 15,000x g. The pellets were then stored in the freezer until they were ready to be resuspended and used for further experimentation.

Transformation. In a 1.5 ml tube, add plasmids or ligation products. Add 20-50 µl of competent cells. Incubate on ice for 30 minutes. Heat-shock the tube in 42°C water bath

for 30-45 seconds. Put back on ice for 1 minute. Add 200 μ l LB and shake for at least 1 hour at 37°C.

DNA Preparation:

DNA Purification. Use Invitrogen HiPure Plasmid DNA Purification Kit buffers.

Resuspend cell pellets with 400 μ l of R3 buffer with RNase A added. Add 400 μ l L7 buffer and mix gently by inverting tube. Incubate at room temperature for 5 minutes. Add 400 μ l N3 buffer and mix immediately by inverting the tube. Centrifuge at 12,000 x g for 10 minutes at room temperature. Load supernatant into column and centrifuge, discard flow-through. Add 700 μ l W8 buffer, centrifuge, and discard flow-through. Dry column by centrifuging an additional 3 minutes. Place column in new 1.5 ml microfuge tube. Add 20-50 μ l elution buffer and centrifuge.

DNA Digestion. Add 5 μ l of 10x NEB Buffer 4 to a 1.5 ml microfuge tube. Add the equivalent of approximately 40 U of the appropriate enzymes to the reaction tube. Add approximately 0.5 μ g of the desired DNA to the reaction mix. Add enough distilled water to bring the total volume of the reaction mixture to 50 μ l. Pipette mix the contents of the microfuge tube. Incubate for 1-2 hours in 37°C.

DNA Ligation. Mix together 2 μ l 10x T4 DNA ligase buffer with 1 μ l T4 DNA ligase in a microfuge tube. Add in the volumes necessary to obtain 50 ng of both vector DNA and insert DNA. Then fill the tube up to 20 μ l with distilled water. Leave the reaction mix overnight at room temperature.

Gel Purification. Use IBI Scientific Gel Purification Kit buffers. Excise the DNA from the gel, being sure to cut away excess gel around desired DNA fragment. Transfer up to 300 mg of gel to a 1.5 ml microfuge tube. Add 500 μ l DF buffer. Incubate at 55-60°C for 10-15 minutes or until the gel fragment is completely dissolved. Cool sample back to room temperature. Place a DF column into a collection tube and transfer up to 800 μ l of sample into column. Centrifuge at 14-16,000 x g for 30 seconds at room temperature and discard flow-through. Add 400 μ l of W1 buffer, centrifuge, and discard flow-through. Add 600 μ l wash buffer, centrifuge, and discard flow-through. Dry the column by centrifuging for 3 minutes, discard flow-through. Place DF column in new microfuge tube. Add 20-50 μ l elution buffer and centrifuge.

LR Reaction. The LR, or lambda recombination, reaction is an easy and effective way to transfer DNA from an entry vector to a wide variety of destination vectors. The technology is based upon the bacteriophage lambda site specific recombination system. Use Invitrogen Gateway Cloning kit. Mix desired amounts of entry vector and destination vector. Thaw LR clonase on ice and vortex briefly before adding to reaction mix. Spin down reaction mix briefly in centrifuge to ensure mixing. Add 50 μ l competent cells to mixture and incubate 30 minutes. Heat-shock mixture at 42°C for 30 seconds. Incubate with shaking for at least 1 hour at 37°C. Plate mixture on desired antibiotic plate.

Luciferase Assay:

Passive Lysis Buffer Preparation. The Passive Lysis Buffer, or PLB, is used to reduce any background autoluminescence. The PLB is supplied as a 5x concentrate. Prepare a sufficient quantity of 1x concentrate PLB by taking 1 volume of 5x PLB and adding 4

volumes of distilled water. The diluted PLB can be stored at 4°C for up to one month, but the PLB is best used right after it is prepared.

Passive Lysis of Cells. Cells should be no more than 95% confluent at the time of lysate preparation. Remove the growth medium from the cells and add a sufficient volume of PBS buffer to the surface of the culture vessel. Swirl briefly and remove the rinse solution. Put 100 µl of the 1x PLB into each cell culture. Put the culture plates on a shaker and rock at room temperature for 15 minutes. Transfer 50 µl into each well of a 96 well plate.

Running the Biotek Luminometer. Wash out the 96 well plate and dry gently with a cotton swab. Turn on the luminometer and open the Gen5 software to 24 well plate experiment. Follow the instructions on screen to be tailored to the specific assay needed. Prime the reader by taking the cover off the injectors and place the syringes into the correct tubes. Click on the dispenser button and make sure dispenser 1 is selected and click prime. Place the 96 well plate into the reader with the A1 position oriented to the top left corner. Click read plate and wait until the read is complete.

Chapter 3

Results

The pENTR vector is extremely easy to clone into a variety of different destination vectors using the Invitrogen Gateway Cloning Technology kit. Therefore, it would make the ideal system to express and create a wide variety of different ataxin-1 vectors in order to further study the different protein interactions and normal functions of ataxin-1. Specifically, I was tasked with taking the pCMV-3xFLAG-hATX1-30Q and pCMV-3XFLAG-hATX1-80Q vectors that were given to us and creating three pENTR vectors for ataxin-1-30Q and three pENTR vectors for ataxin-1-80Q.

The first step was to digest the two starting vectors, pCMV-3XFLAG-hATX1-30Q and pCMV-3XFLAG-hATX1-80Q, and the pENTR-FLAG empty vector to yield the ataxin-1 30Q and 80Q inserts along with the pENTR-FLAG backbone. AgeI and StuI restriction enzymes were used with 10x NEB Buffer 4. Afterwards, gel purification was performed in order to purify and isolate the desired DNA fragments. Next, a ligation was performed to create pENTR-FLAG-hATX1-30Q and pENTR-FLAG-hATX1-80Q vectors. I then created competent cells and transformed the two product pENTR vectors into the cell culture and plated them on kanamycin plates. Colonies that grew successfully on the kanamycin plates were cultured overnight and then the DNA was isolated and purified from the cells. A digestion was performed in order to determine which colonies had successfully taken up the pENTR-FLAG-hATX1-30Q or pENTR-FLAG-hATX1-80Q vectors.

The pENTR-FLAG-hATX1-30Q and 80Q vectors were then used to generate the other pENTR vectors. Originally these two vectors were digested with XbaI and XhoI in 10x NEB

Buffer 4. However, these enzymes cut the plasmid in such a way that the pENTR-FLAG backbone fragment and the ataxin-1 fragment were too close in size. Therefore, the bands could not be distinguished from each other to perform a gel purification. To solve this problem, I digested the pENTR-FLAG-hATX1-30Q and 80Q vectors with Xba1, Xho1, and Apa1. This third enzyme cut the pENTR-FLAG backbone into two pieces so that the fragments containing the ataxin-1 could easily be distinguished from the fragments containing the vector backbone pieces. This made it possible to perform a gel purification of the desired ataxin-1 DNA. Afterwards, the pENTR-GFP and pENTR-V5 empty vectors were digested with Xba1 and Xho1 and the open vectors were then purified from the gel. Lastly, all of the gel purification products were ligated together to form the six desired pENTR ataxin-1 vectors. From here, both wild type (30Q) and mutant (80Q) ataxin-1 can be shuttled to a variety of destination vectors, including pLenti vectors, through the Invitrogen Gateway Cloning Technique. Ataxin-1 can then easily be expressed in a wide array of hosts and can readily be purified via its FLAG or V5 tag and visualized via its GFP tag. This makes it much easier to study the function of ataxin-1 and its role in the development of SCA1. Below, find an overall diagram for the experimental procedure for the creation of the pENTR ataxin-1 vectors (figure 3-1).

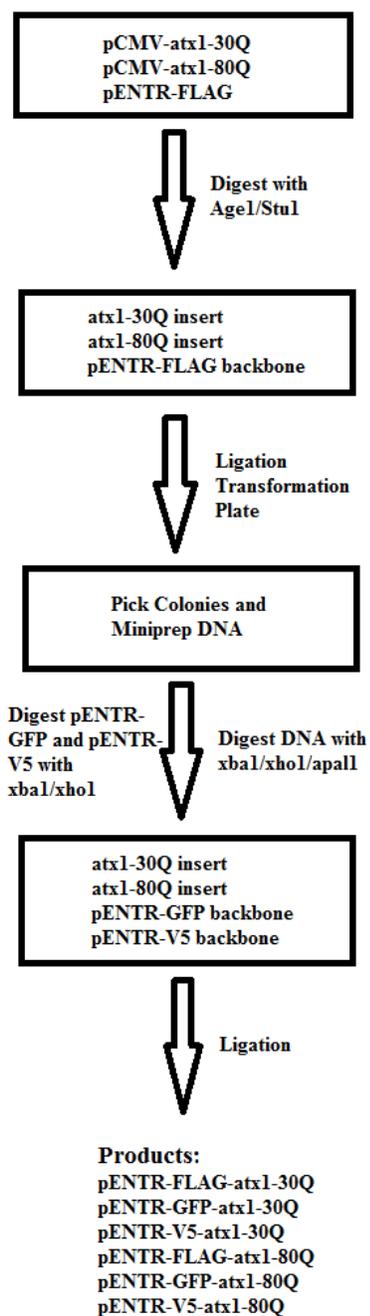


Figure 3-1. Overall experimental procedure diagram. This diagram provides a brief overview of the experiments performed to transform the starting plasmids and empty vectors into the six desired ataxin-1 pENTR vectors.

Digestion with Age1/Stu1:

The digestion of the original ataxin-1 plasmids yielded two bands for the ataxin-1 plasmids as expected. The smaller fragment contains the ataxin-1 30Q or ataxin-1 80Q and the larger fragment contains the vector backbone. The digestion of the pENTR-FLAG vector yielded one band on the gel because the fragment cut out by the restriction enzymes was very small and migrated off the gel. The single band contains the open pENTR-FLAG vector (figure 3-2).



Figure 3-2. Digestion with Age1/Stu1. From left to right, the lanes contain the pENTR-FLAG vector, a 10 kb DNA marker, and the original ataxin-1 30Q plasmid. The appearance of one band post digestion for the pENTR-FLAG vector and two bands post digestion for the ataxin-1 30Q plasmid is consistent with what we expected to see. The band in the square is the ataxin-1-30Q fragment after digestion with Age1/Stu1.

Gel Purification:

After the digestion, a gel purification was performed in order to purify the ataxin-1 30Q and 80Q fragments, as well as the open pENTR vector for future ligation. After the gel purification was done, another gel was run in order to verify the purity of the DNA. The gel showed that the purification was successful (figure 3-3).



Figure 3-3. Gel Purification. The DNA from the gel purification was run on a gel. From left to right, the lanes contain the ataxin-1 30Q fragment, the ataxin-1 80Q fragment, the pENTR-FLAG backbone, and the 10 kb DNA marker. The last two lanes contain the purified pENTR-GFP and pENTR-V5 plasmids.

Transformation:

After ligation of the purified DNA fragments, the pENTR-FLAG-hATX1-30Q and 80Q vectors were transformed into competent cells via the procedure listed above. The cells were then plated on kanamycin plates. DNA was purified from successful colonies and digested with Age1/Stu1 to verify that they had taken up the vector successfully (figure 3-4).



Figure 3-4. Screening for successful colonies. DNA from 10 colonies (labeled 1-10) was digested with AgeI/StuI and run alongside the digested original ataxin-1 80Q plasmid. Colonies that were successfully transformed showed a band corresponding to the 80Q fragment in the square from the ATX1-80Q control run in lane 11.

Digestion with XbaI/XhoI:

The pENTR-FLAG-hATX1-30Q and 80Q vectors, along with the empty pENTR-GFP and pENTR-V5 vectors were first digested with XbaI/XhoI. For the empty pENTR vectors, one band showed up on the gel, which was expected because the fragment cut by the restriction enzymes was very small and ran off the gel. The digestion of the pENTR-FLAG-hATX1-30Q vector showed two bands, but they were not the two bands we expected to see. The heavier band may have been created inadvertently because the DNA was supercoiled at the time of digestion. The smaller band may have been either the ataxin-1 30Q segment or the cut vector backbone

because the two segments were extremely close in size and difficult to distinguish on the gel.

Another method of digestion was needed (figure 3-5)



Figure 3-5. Digestion with Xba1/Xho1. From left to right, the lanes contain pENTR-GFP, pENTR-V5, and pENTR-FLAG-hATX1-30Q. The digestions for the empty pENTR vectors yielded the expected results, but the digestion of the pENTR-FLAG-hATX1-30Q yielded a band that could have been either the ataxin-1 30Q fragment or the cut vector backbone.

Digestion with Xba1/Xho1/Apa1:

The pENTR-FLAG-hATX1-30Q and 80Q vectors were digested with an extra restriction enzyme. This enzyme cut the backbone of the vector, resulting in three bands on the gel corresponding to the ataxin-1 insert and the two backbone fragments. This allowed the ataxin-1 insert to be distinguished easily from the vector backbone on the gel so that a gel purification could be performed (figure 3-6).

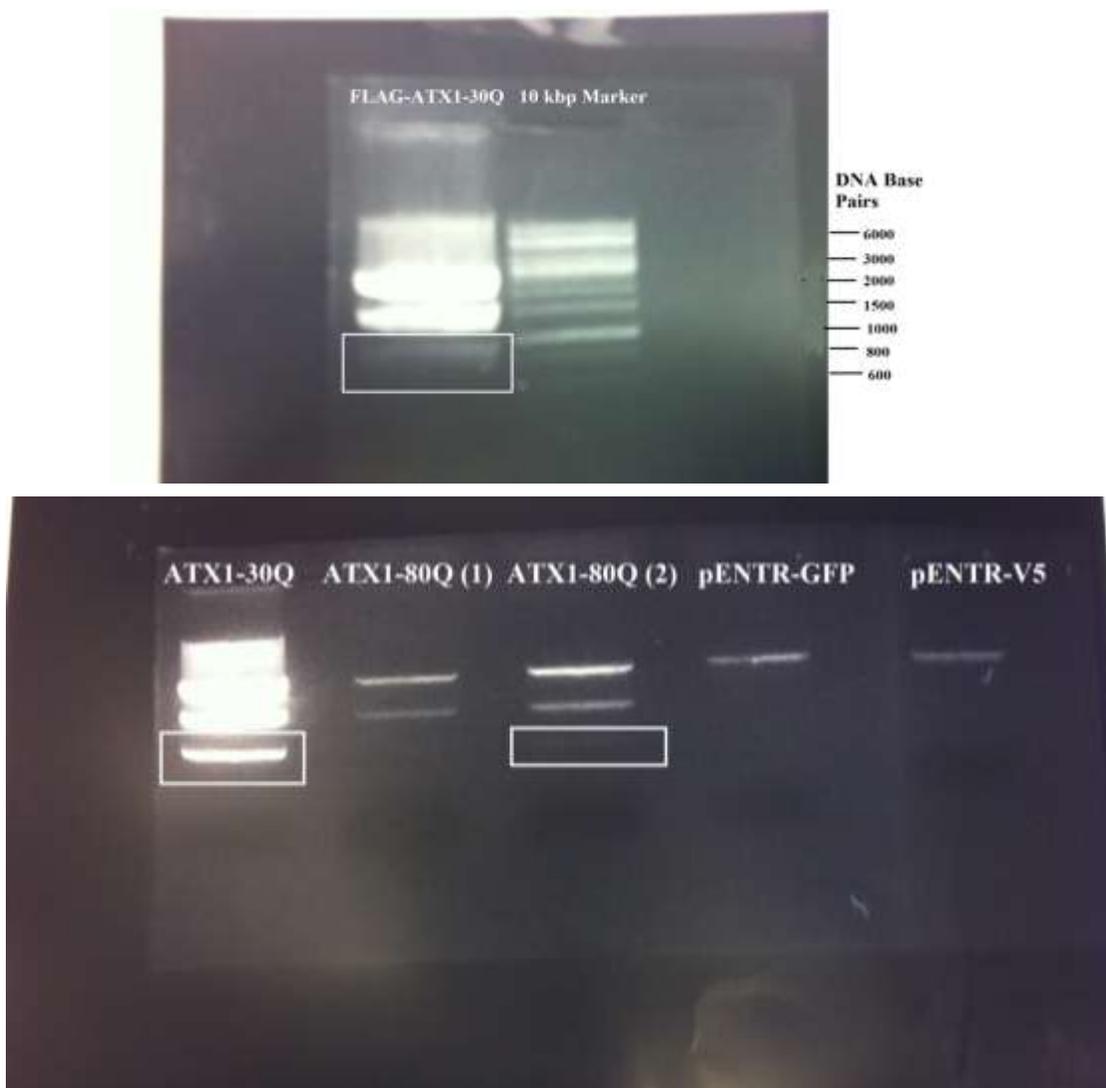


Figure 3-6. Digestion with Xba1/Xho1/Apa11. (Top) Digestion of the pENTR-FLAG-hATX1-30Q vector with the three restriction enzymes allowed the ataxin-1 30Q fragment (boxed) to be distinguished from the cut vector backbone so that gel purification could be performed. (Bottom) From left to right, the lanes contain pENTR-FLAG-hATX1-30Q, pENTR-FLAG-hATX1-80Q from two different colonies, pENTR-GFP, and pENTR-V5. The pENTR-FLAG vectors were digested with three enzymes and the empty pENTR vectors were digested with the original two. The boxed bands indicate the ataxin-1 30Q and ataxin-1 80Q (weak band) fragments as a result of digestion.

LR Reaction:

The LR reaction was performed using the produced pENTR vectors to transfer the ataxin-1 gene to a new destination vector, the pLenti vector, for expression and further experimentation. pLenti vectors are used because they offer a number of advantages for expression in mammalian cells. The pLenti vector uses a lentiviral expression system that is able to deliver DNA to both dividing and non-dividing mammalian cells, and can work either in vitro or in vivo. Thus, pLenti vectors are effective for expression in a wide range of mammalian cells. This is perfect for delivering the ataxin-1 gene to human neural cells. The vector schematic for both the pENTR vector and pLenti vector are shown below (figure 3-7).

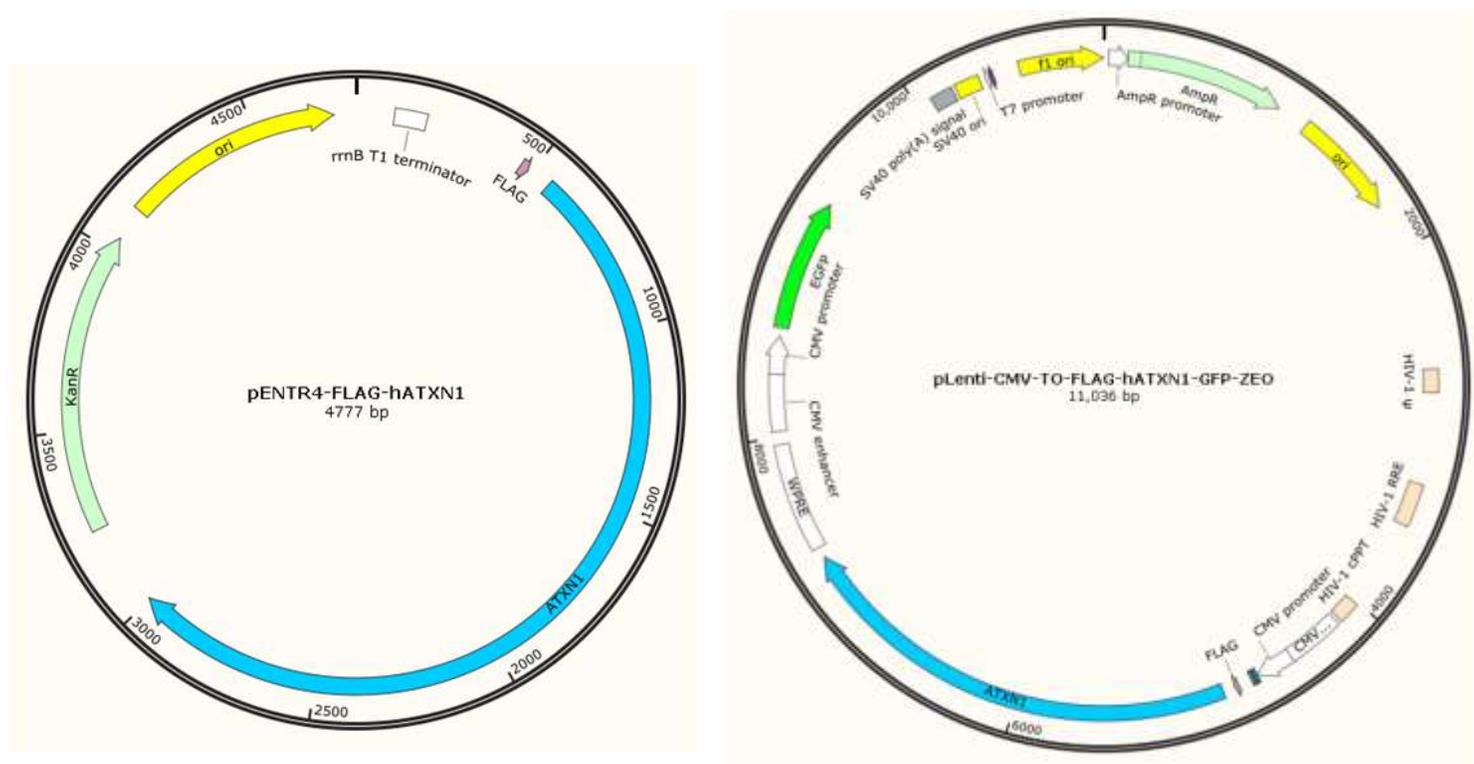


Figure 3-7. Ataxin-1 vectors. (Left) The pENTR-FLAG vector that was produced following the above experimental procedure. (Right) The pLenti-FLAG vector that was produced by performing an LR reaction using the pENTR vectors. Vectors drawn using software Plasmidomics 0.2.

Preliminary Luciferase Assay Results:

We have begun experimenting with the pLenti ataxin-1 vectors to investigate the connection between the ataxin-1 protein and the Wnt signaling pathway. The Wnt signaling pathway has previously been shown to have a role in regulating neurogenesis and neuronal differentiation (Goold et al., 2007). Disturbances in the Wnt signaling pathway have led to the development of neurodegenerative diseases that are similar to SCA1. In a study by Goold et al., the connection between ataxin-1 and the Wnt signaling pathway was investigated. These scientists found that mice that lacked normal ataxin-1 showed deficiencies in the expression of proteins involved in the Wnt signaling pathway. This led the scientists to hypothesize that ataxin-1 may have a role in the genetic programming for neuronal differentiation through interaction with Wnt signaling proteins (Goold et al., 2007).

Our lab wanted to further investigate the connection between ataxin-1 and the Wnt signaling pathway using a luciferase assay. Human neural cells were transfected with luciferase reporter genes that encode proteins involved in Wnt signaling in addition to the ataxin-1 vectors that I produced. Our preliminary data shows a drop in luminescence from the Wnt signaling proteins when the cells are co-transfected with mutant 80Q ataxin-1 vectors, indicating that the mutant ataxin-1 is having a negative effect on the expression of Wnt signaling proteins (figure 3-8). This could explain a new mechanism for the development of SCA1 in cells with mutant ataxin-1. However, the data curiously also shows a drop for the normal 30Q ataxin-1. Since this data is preliminary, more experimentation is necessary.

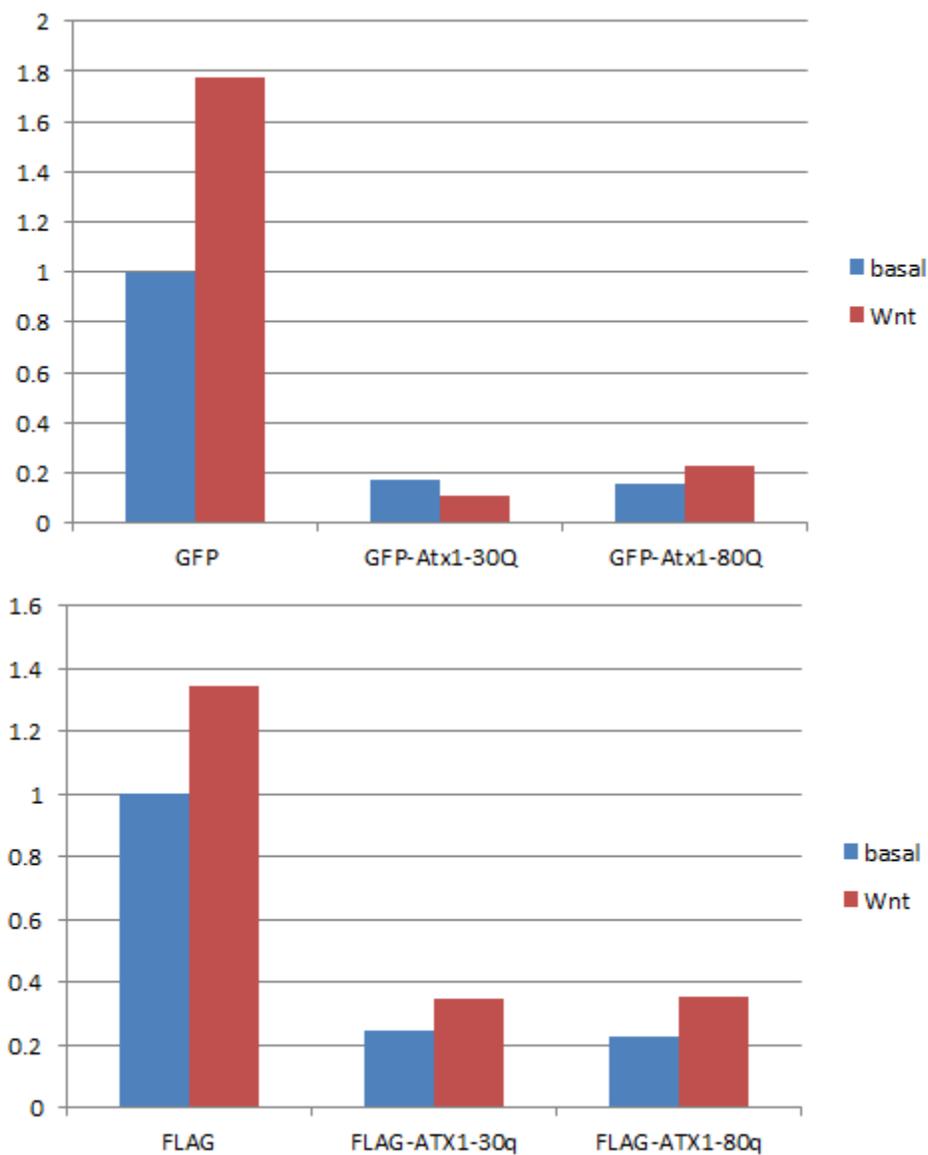


Figure 3-8. Preliminary luciferase assay data. Preliminary data from the luciferase assay shows that luminescence from luciferase reporter Wnt proteins is reduced in the presence of mutant ataxin-1 for both the GFP (top) and FLAG (bottom) ataxin-1 vectors. The data presented in these tables is based off of single data points.

Chapter 4

Discussion

Now that the lab has six ataxin-1 constructs that are ready to use, we can begin more experimentation to determine the proteins that ataxin-1 interacts with and affects which may lead to more breakthroughs on the exact mechanism of SCA1 pathogenesis. There have been a number of recent studies into ataxin-1 and the proteins it interacts with that the Mao lab will be building upon. One such study by Lim et al. examined the interactions between ataxin-1 and RBM17 and Cic and the effect that polyQ elongation has on those interactions (Lim et al., 2008). The scientists first verified the RBM17/ataxin-1 interaction by using a co-affinity purification assay with GST-tagged ataxin-1 and myc-tagged RBM17. Antibodies to the GST-tagged ataxin-1 brought down the RBM17 in addition to the GST-ataxin-1, which was verified using an antibody to the myc tag on the RBM17. After this interaction was verified, the scientists tested the effect that the mutant polyQ elongation had on this interaction. To do this, the scientists performed a co-affinity purification with myc-tagged RBM17 along with GST-tagged ataxin-1 containing a 2Q, 30Q, or 82Q segment. They found that RBM17 interacted preferentially with the elongated mutant polyQ protein because the amount of RBM17 that was co-purified along with the ataxin-1 was greatest with the 82Q mutant form of ataxin-1.

Additionally, the scientists were able to prove that the interaction between RBM17 and mutant ataxin-1 is crucial in the development of disease by using a *Drosophila* model. The mutant ataxin-1 and RBM17 were injected into the eyes of the *Drosophila*, which caused SCA1-like degeneration of retinal cells. This degeneration was lessened when either levels of RBM17 were knocked down or wild-type ataxin-1 was used instead of mutant ataxin-1 (Lim et al., 2008).

Lastly, Lim et al. investigated native ataxin-1 and its ability to form protein complexes. By performing co-elutions and gel filtration chromatography, the scientists were able to determine that native ataxin-1 forms separate complexes with RBM17 and transcription repressor Cic. Formation of complexes with RBM17 enhanced the toxicity of the ataxin-1 protein while formation of complexes with Cic seemed to have the opposite effect. Furthermore, RBM17 complexes reduced the formation of protective nuclear inclusion bodies against SCA1 while Cic complexes enhanced the formation of protective nuclear inclusion bodies that sequestered mutant polyQ proteins. Lim et al. hypothesized that RBM17 and Cic compete for complex formation with native ataxin-1, which may serve to regulate the balance between these proteins. In the case of SCA1, the ataxin-1 is elongated, and the longer polyQ enhances ataxin-1/RBM17 complex formation, which in turn could enhance toxicity of the ataxin-1 and lead to development of SCA1 (Lim et al., 2008).

Another ataxin-1 protein interaction that is being investigated by di Chiara et al. is the interaction with splicing factor U2AF65. The interaction between ataxin-1 and U2AF65 was verified using a co-immunoprecipitation assay. FLAG-tagged ataxin-1 was detected with anti-FLAG antibodies. The precipitated complexes were then transferred to a nitrocellulose membrane which was then probed with anti-U2AF65 antibody to detect presence of U2AF65 in the complexes with the ataxin-1. The scientists found that U2AF65 was found whenever the ataxin-1 was pulled down, indicating that the two proteins interacted together to form protein complexes.

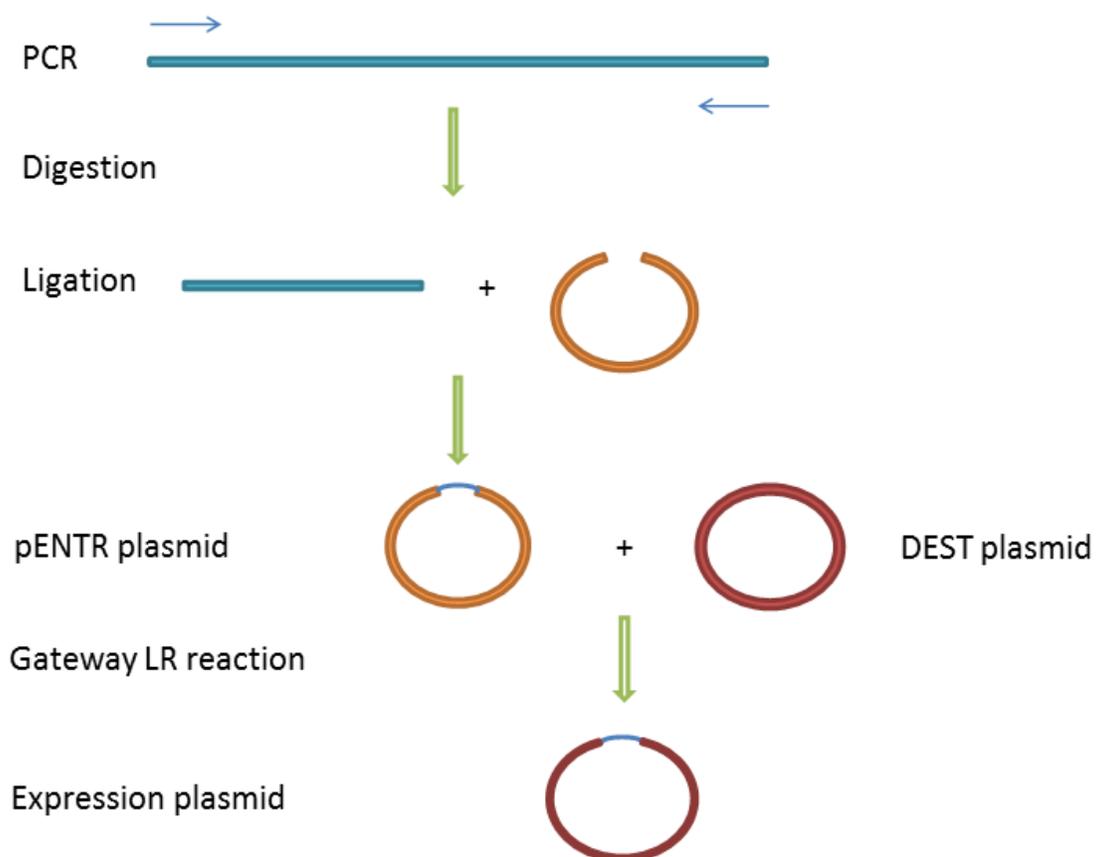
The scientists then studied the effect that ataxin-1 binding had on the splicing mediated by U2AF65. Using a splicing assay with the pyPY reporter mini-gene, the scientists were able to find that ataxin-1 binding had a positive effect on splicing mediated by U2AF65. They also found that phosphorylation of ataxin-1 almost completely loses its ability to bind splicing mediator U2AF65, and instead binds preferentially to previously mentioned splicing mediator RBM17. di Chiara et al. hypothesized that in addition to its many other roles, ataxin-1 may serve as a

regulator between these two different splicing paths in the nucleus. Phosphorylation of ataxin-1 may serve as the switch between RBM17 or U2AF65 splicing. However, as previously noted, mutant elongated polyQ ataxin-1 preferentially interacts with RBM17, which disturbs the balance between these splicing pathways. di Chiara et al. hypothesize that this may contribute to the pathogenesis of SCA1.

The exact mechanism by which mutant ataxin-1 causes the development of SCA1 is still unclear, but scientists are beginning to make headway into this investigation. While the exact function of native ataxin-1 is still unknown, it is apparent that ataxin-1 is involved in a number of processes and interacts with a number of different proteins, and that it might serve as the key to balance between a great number of different cellular pathways. Recently, scientists have been discussing the effects that the mutant ataxin-1 may have on ataxin-1's normal functions and protein interactions instead of focusing solely on the potential toxicity of the polyQ segment itself. It is my hope that the various ataxin-1 constructs that I have created will allow the Mao lab to perform further investigation into ataxin-1 function in the same vein as Lim and di Chiara.

Appendix A

Visual Schematic of Overall Experimental Process



Appendix B

Luciferase Assay Preliminary Data Spreadsheets

		average	sd	fold change
gfp-	0.59761	0.694135	0.106123	1
gfp-	0.807775			
gfp-	0.677019			
gfp+wnt	1.118329	1.181091	0.188853	1.77956
gfp+wnt	1.031609			
gfp+wnt	1.393333			
g-hatx1-30q	0.172973	0.186944	0.018112	0.170671
g-hatx1-30q	0.180451			
g-hatx1-30q	0.207407			
g-hatx1-30q+wnt	0.226994	0.233432	0.011807	0.111256
g-hatx1-30q+wnt	0.226244			
g-hatx1-30q+wnt	0.247059			
g-hatx1-80q	0.187302	0.182375	0.016673	0.157115
g-hatx1-80q	0.19603			
g-hatx1-80q	0.163793			
g-hatx1-80q+wnt	0.162679	0.183867	0.023888	0.225095
g-hatx1-80q+wnt	0.179167			
g-hatx1-80q+wnt	0.209756			

FLAG	1.428571	1.418176	0.032947	1
FLAG	1.381285			
FLAG	1.444672			
FLAG+wnt	1.802247	1.903434	0.167365	1.342171
FLAG+wnt	1.811437			
FLAG+wnt	2.096618			
FLAG-ATX1-30q	0.441696	0.352111	0.078253	0.248284
FLAG-ATX1-30q	0.297101			
FLAG-ATX1-30q	0.317536			
FLAG-ATX1-30q+wnt	0.470862	0.490377	0.100928	0.34578
FLAG-ATX1-30q+wnt	0.400631			
FLAG-ATX1-30q+wnt	0.599638			
FLAG-ATX1-80q	0.349794	0.324184	0.037589	0.228592
FLAG-ATX1-80q	0.28103			
FLAG-ATX1-80q	0.341727			
FLAG-ATX1-80q+wnt	0.407692	0.50054	0.082004	0.352946
FLAG-ATX1-80q+wnt	0.563063			
FLAG-ATX1-80q+wnt	0.530864			

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ACADEMIC VITA

Timothy Wang

1100 Dorset Drive

West Chester, PA 19382

Education

B.S., Microbiology, 2013, The Pennsylvania State University, University Park, PA

Honors and Awards

- Braddock Scholarship, The Pennsylvania State University Eberly College of Science, August 2009
- Academic Excellence Scholarship, The Pennsylvania State University Schreyer Honors College, August 2009
- National Merit Scholarship, National Merit Scholarship Corporation, August 2009
- Foster Memorial Scholarship, The Pennsylvania State University Department of Biochemistry and Molecular Biology, August 2011
- Microbiology Student Marshal, The Pennsylvania State University Department of Biochemistry and Molecular Biology, February 2013
- Phi Beta Kappa Honors Society, Phi Beta Kappa, Lambda of Pennsylvania Chapter, March 2013

Professional Experience

- Fundraising and Wheelchair Skills Intern, Whizz-Kidz Charity, London, United Kingdom, June-July 2012

Research Experience

- Undergraduate Research Assistant, Neuroscience Department—The Pennsylvania State University, University Park, PA, January 2011-Current
- Undergraduate Research Assistant, Kinesiology Department—The Pennsylvania State University, University Park, PA, January-May 2010

Research Interests

I have an interest in performing biomedical research that will aid in the development of new drugs or therapies to fight disease. Specifically, I have a strong interest in researching the underlying causes and mechanisms for development of neurological diseases like schizophrenia or ataxia. Besides neurological disorders, I also have a strong interest in other medical areas. In the past, I have worked with children with cerebral palsy and cancer. I would like to perform medical research with the aim of developing new drugs or therapies to aid children suffering from chronic or terminal illnesses. Lastly, I have an interest in the links between nutrition, exercise, stress, and health. I would like to perform research into the reported health benefits current fad diets, workout trends, and stress relaxation activities such as tai chi or yoga.