MQP-BIO-DSA-8752

Determining the Localization of NCOA7-AS Through GFP Tagging and Fluorescence Microscopy

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

Andrew Camire

April 25, 2013

APPROVED:

Michael Volkert, PhD
Microbiology and Physio Systems
UMass Medical School
MAJOR ADVISOR

David Adams, PhD
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

In Multiple Sclerosis patients, previous experiments have determined the structure, potential function, and regulation of the oxidative damage resistance protein nuclear receptor coactivator-7 alternate start (NCOA7-AS) through induction with Interferon-β (IFN-β). However, the subcellular localization of NCOA7-AS remains unknown. In this project, the localization will be determined by fusing GFP to NCOA7-AS, expressing the fusion protein in HT1080 and HeLa cells, and using fluorescence microscopy. Cells will also be treated with IFN-β to see if this alters NCOA7-AS localization. The GFP microscopy data indicate that NCOA7-AS localization is diffuse in cells with no clear subcellular localization, and that addition of IFN-β does not alter the localization.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Volkert for allowing me the opportunity to work in his laboratory, as well as the direction, insight, and time offered throughout this year. I would also like to thank Lijian Yu for instructing me in various lab techniques and his assistance with my project. Finally, I would like to thank Dr. David Adams for the input and guidance he provided throughout the MQP. The experiments conducted for this project were performed in Dr. Volkert's lab in the Department of Microbiology and Physiological Systems at the University of Massachusetts Medical School. All cell lines, materials, and reagents were provided by Dr. Volkert's Lab.

BACKGROUND

Oxidation Resistance in Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory disease that occurs when the myelin sheathes surrounding the spinal cord and axons in the brain deteriorates, causing demyelination, and leading to a number of detrimental symptoms (Compston and Coles, 2008). MS causes the body's immune system to damage the myelin sheathes of axons, which leads to the loss of the ability of axons to send and conduct action potentials (Compston and Coles, 2008). This damage occurs when T cells that normally recognize foreign antigens enter the brain though disruptions in the blood brain barrier, recognize myelin as an antigenic agent, and begin to attack it (**Figure-1**).

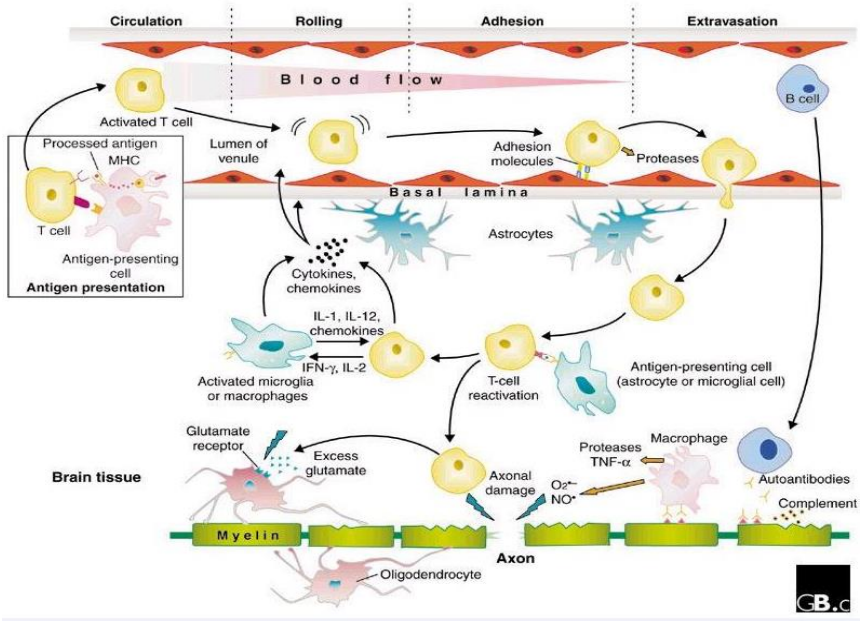

Figure-1: Pathogenesis of Multiple Sclerosis. (Baranzini and Hauser, 2002).

The process of immune cells reacting with self antigens is known as molecular mimicry (Wucherpfennig and Strominger, 1995). This triggers an inflammatory response, activating other immune cells and factors such as antibodies and cytokines, which leads to further damage to the blood brain barrier. In MS, possible targets of the immune response include myelin basic protein (MBP) and proteolipid protein (PLP), though recent data suggests myelin lipids may also be a target (Ho et al., 2012).

MS is responsible for a number of neurological symptoms, and physical and cognitive disabilities, such as ataxia, hypoesthesia, chronic pain, optic neuritis, and depression (Rosati, 2001). The cause of MS is believed to be a combination of genetic, environmental, and infectious factors. In the case of genetic factors, differences in the human leukocyte antigen (HLA) system, a set of alleles DR15 and DQ6 located on chromosome-6 that forms the major histocompatibility complex in humans, have been shown to increase the probability of contracting MS (Compston and Coles, 2008).

Another affliction caused by MS, as seen in many other neurological disorders, is oxidative DNA damage caused by oxidative free radicals. A healthy human body has a functioning antioxidant system that prevents damage or loss due to free radicals. However, imbalances of antioxidants or overproduction of oxidative free radicals due to neurodegeneration can lead to irreparable oxidative damage. This oxidative damage causes lesions in DNA and inactivation of proteins, inflammation, tissue damage, and possibly cellular apoptosis (Uttara et al., 2009). Recently, work has been conducted to identify a number of novel proteins that function to resist or repair oxidative damage caused by free radicals (Durand et al., 2007; Yu et al., submitted for publication). These oxidative resistance proteins show potential to possibly treat, or even prevent, oxidative

DNA damage resulting from neurodegenerative diseases, including MS. Results of this potential therapeutic work will be discussed further in other sections of this paper.

OXR Domain and Eukaryotic Oxidation Resistance Proteins

Reactive oxygen species (ROS) have been shown to cause oxidative damage to DNA. There are a number of cellular processes that prevent lethal effects of ROS. These protective proteins fall into two main categories, those that *prevent* oxidative DNA damage from occurring, and those that *repair* damage caused by ROS. One gene that encodes an oxidative resistance protein is OXR1. This gene can be induced by oxidative stress and heat, and its protein product localizes to mitochondria. OXR genes are conserved among all sequenced eukaryotes, ranging from yeast to humans (Durand et al., 2007).

Recently, experiments were performed to determine whether a second human gene, nuclear receptor coactivator 7 (NCOA7), that shares many similarities with OXR1, could function to prevent oxidative mutagenesis (Durand et al., 2007). This work also determined the structure of NCOA7 and its regulation (Durand et al., 2007). NCOA7 was able to reduce the DNA damaging effects of ROS. The oxidative resistance function of NCOA7 appears to reside in its carboxyl-terminal domain, which has similarities to OXR1. The experiments also determined that NCOA7 is constitutively expressed in human cells, and is not induced by oxidative stress, suggesting that the protein product of NCOA7 functions in oxidative resistance rather than repair (Durand et al., 2007). NCOA7 protein was also found to associate with the estrogen receptor, rather than be induced by stress, like OXR1. DNA damage due to oxidation has been shown to result as

a byproduct of estradiol metabolism (Seacat et al., 1997). This suggests that NCOA7 functions to lessen oxidative DNA damage that results from estrogen metabolism.

The structure and regulation of an isoform of the NCOA7 gene was recently discovered (Yu et al., submitted for publication). This variant is known as nuclear receptor coactivator 7-alternate start (NCOA7-AS). NCOA7-AS is found to have homology with the C-terminal region of NCOA7 gene, thus sharing similarities with OXR1 (Yu et al., submitted for publication). NCOA7-AS contains exons 11-15 of full length NCOA7, but it begins with a new exon (10a) which is encoded between exons 10 and 11 (**Figure-2**). Exon 9 of NCOA7, like exon 8 of OXR1, is likely responsible for its antioxidant function. Therefore, exon 10a may replace the function of exon 9 for oxidative resistance. Previous work discussed later in this report demonstrates the antioxidant activity of NCOA7-AS, further supporting this hypothesis. NCOA7-AS also varies from NCOA7 in that it lacks the estrogen-binding domain found in exon 8 (Shao et al., 2002). This suggests that interacting with the estrogen-estrogen receptor complex is exclusive to NCOA7 and its longer forms, but not to NCOA7-AS (Yu et al., submitted).

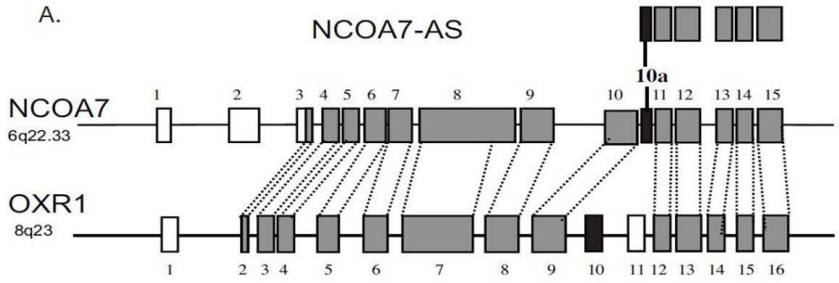

Figure 2: Structures of NCOA7, NCOA7-AS, and OXR1. (A) Gene alignments of NCOA7, NCOA7-AS, and OXR1. (B) Amino acid sequence of NCOA7-AS, underlined sequence indicates the sequence coded by exon 10a (Yu et al., submitted).

The experiments also determined that NCOA7-AS is regulated by, and dependent on, the Janus Kinase (JAK) and Signal Transducers and Activators of Transcription (STAT) pathway (Yu et al., submitted for publication). Mutations in key genes of the JAK-STAT regulatory pathway blocked the induction of NCOA7-AS. Induction of NCOA7-AS requires a functional IFN-receptor, JAK1, and STAT2 genes (Yu et al., submitted for publication). This differs from full length NCOA7, which is constitutively expressed and whose nuclear localization is stimulated by interactions with the estrogen receptor (Durand et al., 2007).

Protein Subcellular Localization

Cells of eukaryotic organisms are subdivided into functionally and morphologically distinct compartments. The compartmental location, or lack thereof, where a particular protein is found is defined as its subcellular localization (Schuler, 2004). Determining the subcellular localization of a protein is necessary to better understand its function.

One method that can be utilized to determine localization is immunostaining. This procedure involves endogenously tracking a protein in cells by inserting an antibody that identifies the protein of interest. A secondary fluorescent antibody that identifies the primary protein antibody is then added to observe the localization of the native protein (Coons et al., 1941). Bioinformatic models can also be used to predict localization (Scott et al., 2005). Another method that can be used to determine localization is ligating the gene that encodes the protein of interest with a fluorescent tagged expression vector,

inserting the fusion vector into cells, allowing the fusion protein to become expressed, and using fluorescence microscopy to observe protein localization (Huh et al., 2003).

The protein product of the OXR1 gene has been found to localize to the mitochondria of cells. Recently work has also been able to determine the localization of protein NCOA7 (Durand et al., 2007). Immunofluorescence assays showed that NCOA7 shows clear subcellular localization in the nucleus of human cells stimulated by estrogen (Durand et al., 2007). This differs from the mitochondrial localization of OXR1 (Elliot and Volkert, 2004), suggesting that NCOA7 may encode the nuclear counterpart of the OXR1 protein. The subcellular localization of NCOA7-AS, the alternate start form of NCOA7, is still undetermined.

Interferon-β Induction of NCOA7

Type I interferons are widely conserved proteins, and play an integral role in organizing antiviral responses (Mogensen et al., 1999). The common structure of interferons is a compact helical protein that reacts with heteromeric cell surface receptors present on mammalian cells (Stark et al., 1998). Over time, evolution has resulted in a defined family of IFNs, including IFNβ, that are only expressed in certain cell types. The reason IFNs have evolved is most likely due to the changes in adaptive immune responses, innate responses to cell proliferation, and proliferation of cells. Because of this, IFNs are able to induce a large number of varying genes (Stark et al., 1998).

IFNs are very useful in treating a number of human diseases, including Multiple Sclerosis (Croze et al., 2009). IFN works as a treatment for MS by shifting the Th1/Th2 response, stabilization of the blood brain barrier, and promoting the differentiation of

oligodendrocytes, brain cells that produce myelin to insulate axons (Arnason et al., 1996). Previous work showed that patients that suffer from neurological diseases often accrue neuronal damage due to increased reactive oxygen species (ROS). Thus, it is possible that the tissue damage occurring in MS patients by an inflammatory response results from an overabundance of reactive oxygen species (Melo et al., 2011).

One function of IFN is to repair the tissue damage caused by oxidative stress due to ROS (Croze et al., 2009). Recently, experiments showed that IFNβ-1b increased the expression of NCOA7-AS (discussed above) (Yu et al., submitted for publication). The experiments also determined the mechanism of regulation for NCOA7-AS, its structure, and its ability to function as an oxidative repair protein relative to the regular full length NCOA7 (Yu et al., submitted for publication).

NCOA7 exon expression measurements using exon microarrays were made using peripheral blood mononucleocytes (PBMCs) taken at 0, 4, and 18 hours from seven patients exhibiting the relapsing-remitting form of MS that were treated at time 0 with Betaseron, a form of IFNβ-1b used to treat MS patients (**Figure-3**). The results showed that exons 2, 7, 8, 9, and 10 are not expressed at elevated levels after Betaseron treatment, while exons 11, 12, and 15 show strong induction. This shows that three exons of NCOA7-AS are strongly induced by Betaseron (IFNβ-1b). PBMCs of three healthy volunteers and primary human fetal brain cells were also treated with Betaseron to examine the induction of NCOA7-AS. The combined results showed that NCOA7-AS is induced by IFNβ-1b in PBMCs of MS patients, healthy individuals, and cells obtained from the CNS (Yu et al., submitted for publication).

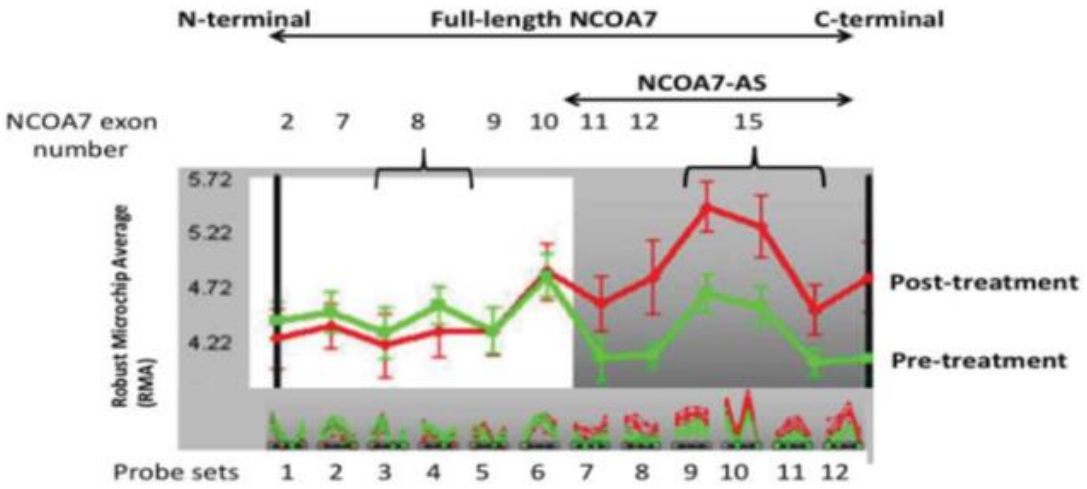

Figure 3: NCOA7 Exon Expression Measurements in IFN-Treated MS Patients Using Exon Microarrays. XRAY v2.63 Analysis of Human Exon 1.0 ST GeneChip® Data Using mRNA Collected from MS Patients Injected with Betaseron (IFNβ-1b). NCOA7-AS exons induced by Betaseron are shown from exons 10-16 (Yu et al., submitted for publication).

Human HT1080 fibrosarcoma cells express IFNAR1 and IFNAR2 receptors which interact with IFNβ-1b (Der et al., 1998). HeLa cells, a cell line used in this project but not in the work being discussed in this section, have been shown to interact with interferon as well (West and Balioni, 1979). NCOA7-AS shows strong induction in this cell line, as well as some neuroblastoma cell lines (**Figures 4A and 4B**). Previous work also shows that NCOA7 is not induced in response to oxidative stress (Durand et al., 2007). HT1080 cells were treated with varying doses of hydrogen peroxide, and levels of NCOA7-AS mRNA were measured after 4 hours. The data showed that NCOA7-AS is not induced by treatment with H_2O_2 (**Figure 4C**).

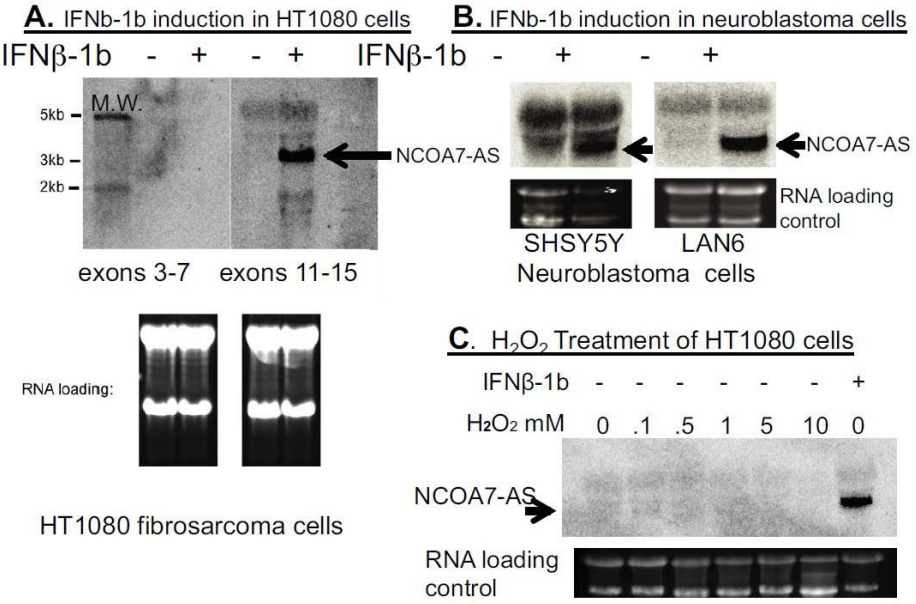

Figure 4: IFN Induction of NCOA7-AS in HT1080 and Neuroblastoma Cell Lines, and Non-Response to H_2O_2 Treatment. (A) Induction of NCOA7-AS in HT1080 fibrosarcoma cells. (B) A probe detecting NCOA7-AS in SHSY5Y and LAN6 neuroblastoma cells. (C) HT1080 cells treated with indicated concentrations of H_2O_2 or IFNβ-1b for 4 hours before mRNA isolation (Yu et al., submitted for publication).

Previous studies also showed that NCOA7-AS can serve as an oxidative damage resistance protein through expression in *E. coli* with mutations in *mutM* and *mutY*. Wild type *mutM* and *mutY* normally prevent GC->TA transversions (Michaels et al., 1992; Wang et al., 2004; Volkert et al., 2008). The strain also carries a lacZ allele, which reverts to Lac^+ in the presence of GC->TA transversions and acts as an oxidative DNA damage reporter (Michaels et al., 1992; Michaels and Miller, 1992; Wang et al., 2004; Volkert et al., 2008). Colonies carrying expression plasmid pTrc99A with either a full length NCOA7 or NCOA7-AS insert show suppression of oxidative mutagenesis, while colonies with the pTrc99A vector only show high levels of transversion mutations. This shows that both proteins exhibit strong oxidative damage prevention activity and thus have an antioxidant function (Yu et al., submitted for publication). As mentioned

previously, work also demonstrated that expression of NCOA7-AS is dependent on the JAK-STAT pathway (Yu et al., submitted for publication). However, the subcellular localization of the NCOA7-AS protein is still undetermined. In this project, GFP will be fused with NCOA7-AS to determine its cellular location and whether the localization is altered by treating the cells with IFNβ-1b.

PROJECT PURPOSE

This project was performed to determine the subcellular localization of NCOA7-AS protein in cells. Previous studies showed that NCOA7-AS can be induced by Interferon-β, and determined the localization of other forms of the NCOA7 gene. But the location of NCOA7-AS remains unknown. Determining the localization of NCOA7-AS would help to further understand the gene's role in cellular oxidative resistance, and provide further insight into its presence and function in patients with Multiple Sclerosis.

METHODS

DH5α Strains, Plasmids, and Primers

Table 1: Table of Strains Used in this Project

E. coli Strain Number	Cell Type and Plasmid Insert
MV7034	DH5α + pMV1559
MV7035	DH5α + pMV1560

Table 2: Table of Plasmids Used in this Project

Plasmid Name	Vector	Insert
pMV1559	pEGFP-N1	NCOA7-as
pMV1560	pEGFP-N1	NCOA7-as

Table 3: Table of Primers Used in this Project

Primer Number	Primer Name	Sequence (5'-3')	Use
609	609 N1 prF	GCGCGAATTCATGAGAGGCCAAA GATTACCCTT	PCR Product restrictions of NCOA7-as and pEGFP-N1
610	610 N1 prR	CGCGACCGGTCGATCAAATGCCCA CACCTCCAG	
611	611 C1 prF	GCGCTCCGGAAGAGGCCAAAGAT TACCCTTGGA	PCR Product restrictions of NCOA7-as and pEGFP-C1
612	612 C1 prR	GCGCGAATTCTCAATCAAATGCCC ACACCT	
613	613 pEGFPN1 seq	TACCGGACTCAGATCTCGAGCTCA	Clone Sequencing of plasmid pMV1559
614	614 pEGFPN1 seq	CAGCTCCTCGCCCTTGCTCACCAT	
615	615 pEGFPC1 seq	GATCACTCTCGGCATGGACGAGCT	Clone Sequencing of plasmid pMV1560
616	616 pEGFPC1 seq	GGATCCCGGGCCCGCGGTACCGTC	

Ligation of NCOA7-AS and pEGFP

PCR

The NCOA7-AS gene used for this project was procured from a variant of the gene with a silent mutation at codon 40 (base pair 120) that is routinely used in the Volkert lab. The silent mutation (AGC → AGT) does not affect the protein sequence, as both sequences encode serine at that location. The plasmid expression vector used was pEGFP, isolated from *E. coli* strains using Qiagen's QIAprep® Miniprep kit. Features of these vectors include a constitutive CMV promoter, a kanamycin resistance gene, and an EGFP tag.

PCR products were created to have two copies of NCOA7-AS to ligate with pEGFP, one which attached NCOA7-AS to the N-terminus, and the other at the C-terminus of EGFP. The N-terminus product was created using primers 609 (GCGCGAATTCATGAGAGGCCAAAGATTACCCTT) and 610 (CGCGACCGGTCGATCAAATGCCCACACCTCCAG). The PCR product contained an EcoRI restriction site at the beginning of the coding strand of the gene and an AgeI site at the end of the template strand. These restriction sites were also found in the pEGFP-N1 vector. The C-terminus product was created using primers 611 (GCGCTCCGGAAGAGGCCAAAGATTACCCTTGGA) and 612 (GCGCGAATTCTCAATCAAATGCCCACACCT). This PCR product contained a BspEI restriction site at the beginning of the coding strand, and an EcoRI site at the end of the template strand. These restriction sites are also present in the pEGFP-C1 vector, at an appropriate position to produce a EGFP fusion protein. All PCR reactions were performed using Taq Polymerase (New England Biolabs).

The N-terminus PCR product and pEGFP-N1 were double-digested with EcoRI and AgeI in New England Biolabs' reaction buffer 1 at 37°C for 3 hours. The C-terminus PCR product and pEGFP-N1 were double-digested with BspEI and EcoRI in New England Biolabs' reaction buffer 3 at 37°C for 3 hours. Samples of each restriction digestion were then run on an electrophoresis gel, and band lengths were observed to determine the effectiveness of the digestions. Since the gel showed bands of expected sizes for each product, the restriction products were then purified using Qiagen's QIAprep® Miniprep kit.

Ligations and Transformations

The NCOA7-AS and pEGFP digests were ligated using New England Biolabs' Quick Ligase kit. The ligation created two clones, one that was a C-terminus fusion (EGFP-NCOA7-AS) and the other was an N-terminus fusion (NCOA7-AS-EGFP). DH5α cells were transformed with the resulting ligation products, plated on LB+Kan plates, and incubated for 24 hours. The transformations yielded five colonies containing the C-terminus fusion, and five with the N-terminus fusion. These colonies were inoculated into LB-KAN broth, and the plasmid DNA was purified after 24 hours of incubation. The C-terminal fusion vector DNA was then digested using BspEI and EcoRI, and the N-terminal fusion vector was digested with AgeI and EcoRI. Restriction digestion determined that there were four potential transformants that could be used for experimentation: three N-terminus clones and one C-terminus clone. They were purified and named strains MV7033, MV7034, MV7035, and MV7036. Purified plasmid DNA from these strains was sent to GeneWiz Boston (Cambridge, MA) for sequencing to

determine if the fusion contained NCOA7-AS. Primers used for sequencing were 613, 614, 615, and 616.

Lactose Papillation Plating

To further test potential positives and to determine whether fusion to EGFP interfered with NCOA7-AS's antioxidant activity, *E. coli* with mutations in *mutM* and *mutY* were transformed with the 4 clones purified in the ligation step. Wild type *mutM* and *mutY* prevent GC->TA transversions by repairing 8-oxoG. This strain also carries a *lacZ* gene that changes to Lac+ when GC->TA transversion mutagenesis occurs, which functions as a reporter of spontaneous oxidative DNA damage. For each clone, 30 µl of cells were combined with 20 µl of PCM buffer and 5µl of DNA on ice, the reactions were incubated at 37°C for 2 minutes, and then incubated at room temperature for 10 minutes. After this incubation, 2 ml of LB were added to each of the transformation mixes, and the cultures incubated in a 37°C shaker for 1 hour. All cultures were plated at 10^{-5} and 10^{-6} dilutions on Lac+ Lactose papillation plates with Kan and incubated for 24 hours at 37°C.

Cell Lines and Culture

The cell lines utilized in this study were HT1080 and HeLa. HT1080 is a fibrosarcoma cell line created from a biopsy of a fibrosarcoma present in a 35 year old human male that had not undergone radiation or chemotherapy (Rasheed et al., 1974). HeLa cells are an immortal cell line derived from cervical cancer cells originally taken from a patient in 1951 (Scherer et al., 1953). The cells were cultured at 37°C in 1X

DMEM supplemented with 10% Fetal Bovine Serum, 4.5 g/L of glucose and L-glutamine, in the absence of sodium pyruvate.

Transfection and Fluorescence Microscopy

Transfection of HeLa and HT1080 Cells

Cell lines used for transfection were split into 6 well plates, with each well containing 2 ml of media and cells. Cells were maintained at 50-80% confluency in DMEM with 10% FBS and no antibiotics. This setup was completed for both HeLa and HT1080 cells. For each transfection, 4 µg of DNA were diluted in 250 µl of Opti-MEM® Reduced Serum Medium, and mixed gently. Then 10 µl of Lipofectamine™ was diluted in 250 µl of Opti-MEM® and incubated at room temperature for 5 minutes. The solution containing the diluted DNA was then combined with that of the diluted Lipofectamine™ and incubated for 20 minutes at room temperature. Growth medium of the cells was removed and replaced with 2 ml of fresh medium, and the 500 µl of the DNA / Lipofectamine™ complexes were added to each well. The cells were incubated for 3 hours, and media was replaced after this incubation period. There were 20 transfections performed in total: 2 HeLa transfections containing a fluorescent control (M Cherry), 2 HT1080 transfections containing a fluorescent control (M Cherry), 4 HeLa transfections containing the plasmid isolated from strain MV7034, 4 HT1080 transfections containing the plasmid isolated from strain MV7034, 4 HeLa transfections containing the plasmid isolated from strain MV7035, and 4 HT1080 transfections containing the plasmid isolated from strain MV7035.

Fluorescence Microscopy

All wells containing transfected cells were observed under fluorescence microscopy at 100X magnification to verify M. cherry (red) or GFP (green) fluorescence, and photographs of each transfection were taken. After observation, half of each type of transfection was treated with 32 µl of 50 mg/ml of G418 to ensure selection of NCOA7-AS clones. These G418 selected transfections were incubated for 4 days, then observed under fluorescence microscopy at 100X magnification and photographed. The remaining half of the transfections were treated with 2.2 µl of a mixture of 2 µl of Interferon-β, and 200 µl of DMEM with 10% FBS. The treated transfections were incubated for 24 hours, then observed under fluorescence microscopy at 100X magnification and photographed.

RESULTS

The overall purpose of this project was to determine the localization of NCOA7-AS using a GFP fusion approach, and determining whether treatment of the cells with IFNβ-1b would alter NCOA7-AS localization.

Creation of an NCOA7-AS-GFP Fusion Plasmid

Two PCR products were created using primers 609, 610, 611 and 612, and a stock plasmid sample of NCOA7-AS as the template. Primers 609 and 610 were designed to produce an NCOA7-AS product that had EcoRI and AgeI restriction sites to be fused to the N-terminus of EGFP. Primers 611 and 612 were designed to produce an NCOA7-AS product that had BspEI and EcoRI restriction sites to be fused to the C-terminus of EGFP. These PCR products, pEGFP-N1 and pEGFP-C1 were all digested using their corresponding restriction enzymes, then both of the digested PCR products were ligated into their respective pEGFP vectors, and transformed into competent DH5α. Ten total transformants were obtained, five with the N-terminus fusion and five with the C-terminus fusion. To determine whether these transformants contained the NCOA7-AS insert, plasmid DNA was purified, restricted, and run on a 5% agarose electrophoresis gel (**Figure 5**). Analysis of the gel showed that three N-terminus fusions (circled in the figure) appeared to contain the NCOA7-AS insert (665 bp) (lanes 8, 9, and 10, in Figure 5). The inserts appeared to have the correct size, as measured against a 1kb ladder. None of the C-terminus fusions appeared to contain the insert (lanes 2-6, Figure 5).

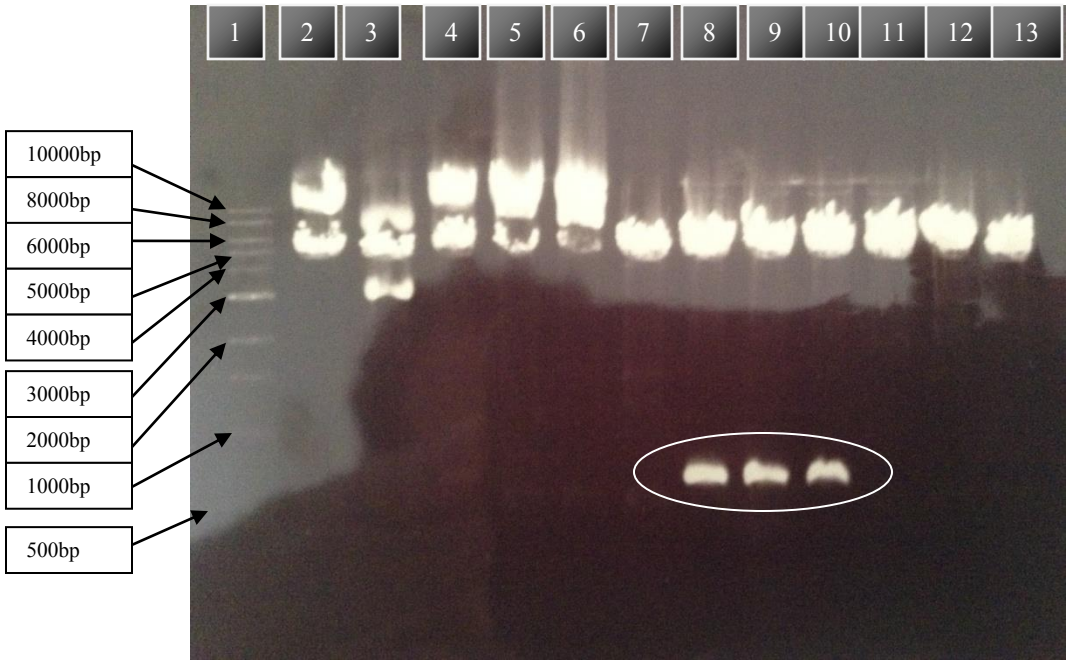

Figure 5: NCOA7-AS-GFP Fusions Created using Primers 609, 610, 611, and 612. Gel electrophoresis of purified and restricted DNA from DH5α transformants. Positive plasmid DNAs pMV1559 and pMV1560 were isolated from transformants MV7034 and MV7035 respectively (Lane 1: 1kb DNA ladder; Lane 2: EGFP-NCOA7-AS from C1 transformant 1 cut with BspEI and EcoRI; Lane 3: EGFP-NCOA7-AS from C1 transformant 2 cut with BspEI and EcoRI; Lane 4: EGFP-NCOA7-AS from C1 transformant 3 cut with BspEI and EcoRI; Lane 5: EGFP-NCOA7-AS from C1 transformant 4 cut with BspEI and EcoRI; Lane 6: EGFP-NCOA7-AS from C1 transformant 5 cut with BspEI and EcoRI; Lane 7: pEGFP-C1 vector cut with BspEI and EcoRI; Lane 8: NCOA7-AS-EGFP from N1 transformant 1 cut with EcoRI and AgeI; Lane 9: NCOA7-AS-EGFP from N1 transformant 2 cut with EcoRI and AgeI; Lane 10: NCOA7-AS-EGFP from N1 transformant 3 cut with EcoRI and AgeI; Lane 11: NCOA7-AS-EGFP from N1 transformant 4 cut with EcoRI and AgeI; Lane 12: NCOA7-AS-EGFP from N1 transformant 5 cut with EcoRI and AgeI; Lane 13: pEGFP-N1 vector cut with EcoRI and AgeI) (Circled bands indicate possible presence of NCOA7-AS) Expected length of NCOA7-AS insert: 665bp.

To determine whether the apparently negative C-terminus fusions analyzed were actually the result of a partial digestion, samples of four C-terminus fusions were again digested and run on a 5% agarose electrophoresis gel. Analysis of this gel showed that only one transformant possibly contained NCOA7-AS in the fusion (lane 8 circled, **Figure 6**).

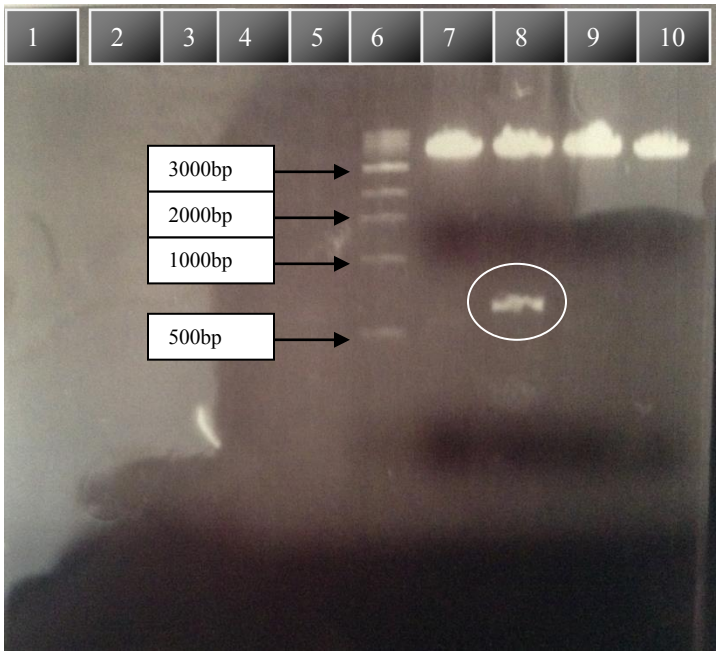

Figure 6: NCOA7-AS-GFP C-terminus fusions using primers 609 and 610. Gel electrophoresis of second digested sample of purified DNA from DH5α transformants (Lane 1-5: empty, Lane 6: 1kb DNA ladder, Lane 7: EGFP-NCOA7-AS from C1 transformant 1 cut with BspEI and EcoRI; Lane 8: EGFP-NCOA7-AS from C1 transformant 3 cut with BspEI and EcoRI; Lane 9: EGFP-NCOA7-AS from C1 transformant 4 cut with BspEI and EcoRI; Lane 10: EGFP-NCOA7-AS from C1 transformant 5 cut with BspEI and EcoRI) (Circled band indicates possible presence of NCOA7-AS). Expected length of NCOA7-AS insert: 665bp.

This C-terminal fusion plasmid, and the three N-terminus fusion plasmids, were sequenced by GeneWiz using primers that overlapped the beginning of the coding strand and end of the template strand of the expected NCOA7-AS insert. The sequencing confirmed the insert in two of the N-terminus fusions, which were saved as strains MV7034 and MV7035.

To again try to obtain a C-terminus fusion, DH5α cells were transformed with a sample of a new C-terminus ligation, as well as the remainder of the previous C-terminus ligation. This resulted in only two transformants from the new ligation. DNA from these colonies was purified, restricted, and run on a 5% agarose electrophoresis gel (data not shown). Analysis of the gel revealed that the insert was again not present in the C-terminal fusion plasmid.

Oxidative Resistance Confirms the NCOA7-AS Insert

In order to determine whether the NCOA7-AS-EGFP fusion protein retained oxidation resistance activity, *E. coli* containing mutations in *mutM* and *mutY* were transformed with the two confirmed N-terminus fusion plasmids. As previously described, GC->TA transversion mutations in colonies of these cells are indicators of spontaneous oxidative damage, and such cells become Lac+. *MutM mutY E. coli* transformed with one of the failed C-terminus ligations (**Figure-7A**) shows an elevated level of transversion mutations, indicating a high level of oxidative damage, as seen by the dark blue Lac^+ microcolonies within the white Lac^- parent colonies. *E. coli* containing the sequenced, purified N-terminal plasmids (**Figures 7B and 7C**) shows that the presence either of the two vectors expressing NCOA7-AS-EGFP suppresses oxidative mutagenesis (fewer Lac+ colonies). Thus, oxidation resistance activity appears to be contained within the expressed NCOA7-AS-EGFP protein, indicating that fusion with the GFP did not hinder its oxidative resistance activity, and that NCOA7-AS is present in both vectors.

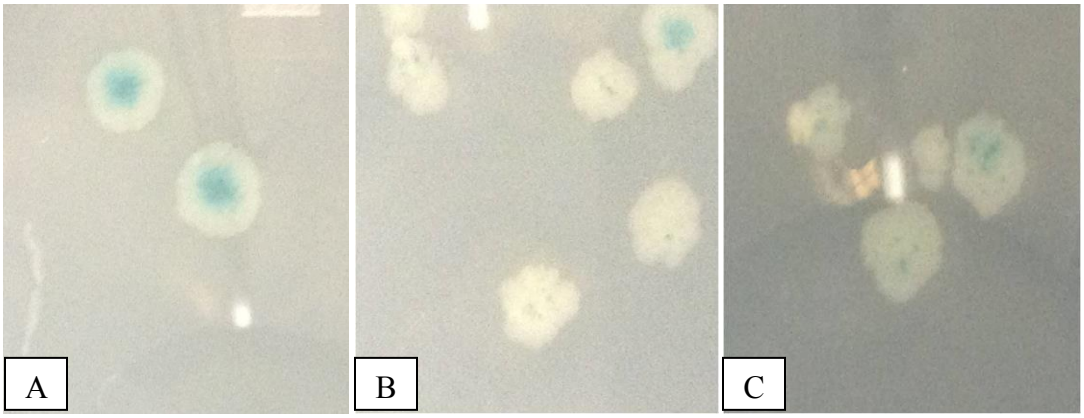

Figure 7: (A) *MutM mutY E. coli* transformed with failed NCOA7-AS-GFP C-terminus fusion. (B) *MutM mutY E. coli* transformed with purified plasmid from strain MV7034. (C) *MutM mutY E. coli* transformed with purified plasmid from strain MV7035.

Fluorescence Microscopy of Transfected HeLa and HT1080 Cells

In order to determine the subcellular localization of the NCOA7-AS fusion, HeLa and HT1080 cells were transfected with purified DNA from strains MV7034 and MV7035, and examined using fluorescence microscopy. Transfections were also treated with IFNβ-1b to determine whether interferon altered localization of the fusion protein. HeLa transfections containing the M. Cherry control show red fluorescence (**Figure 8A**), indicating the transfections worked correctly. Observing HeLa cells transfected with DNA from strains MV7034 and MV7035 showed that most of the cells expressing the fusion protein were dead cells (**Figure 8**), but a few live cells were found to express the NCOA7-AS fusion protein as diffuse throughout the cell with no specific subcellular localization (**Figures 8B and 8E**). Stable selection of some of the transfections also showed the same result that most cells expressing the protein were dead, but those that were alive showed diffusion of the protein throughout the cell (**Figures 8C and 8F**). HeLa transfections treated with IFNβ-1b showed the same result, suggesting that interferon does not alter the localization of the fusion protein (**Figures 8D and 8G**). All transfections performed on HT1080 cells with purified DNA from strains MV7034 and MV7035 showed no expression of the fusion protein.

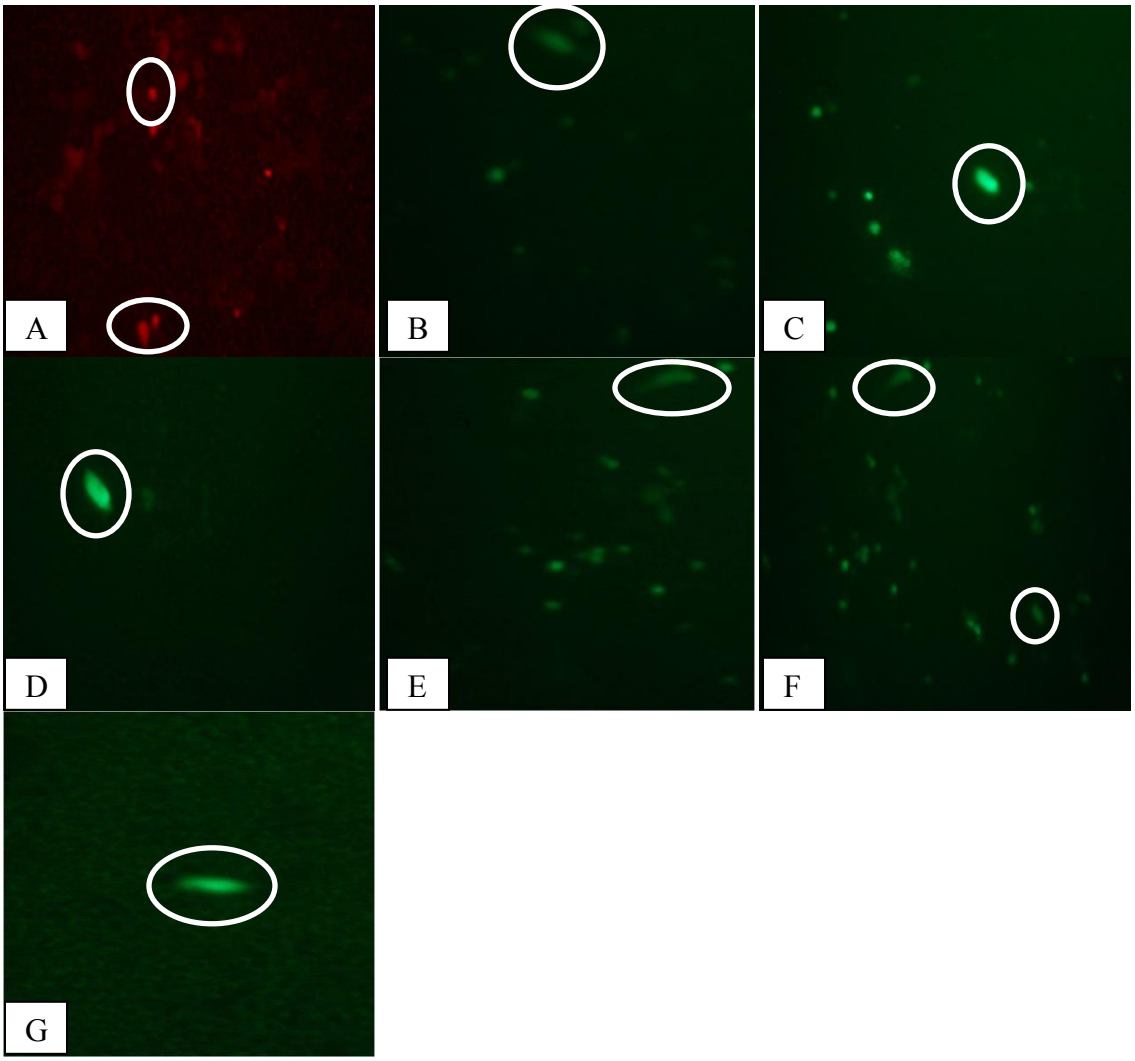

Figure 8: Fluorescence Microscopy of Transfected HeLa Cells at 100x Magnification: (A) HeLa transfected with M. Cherry. Circled cells indicate live cells expressing red fluorescing M. Cherry. (B) HeLa transfected with purified DNA from MV7034 with no treatment. (C) HeLa transfected with purified DNA from MV7034 treated with G418. (D) HeLa transfected with purified DNA from MV7034 treated with IFNβ-1b. (E) HeLa transfected with purified DNA from MV7035 with no treatment. (F) HeLa transfected with purified DNA from MV7035 treated with G418. (G) HeLa transfected with purified DNA from MV7035 treated with IFNβ-1b. B-G: Circled cells indicate live cells expressing the NCOA7-AS-GFP fusion protein.

DISCUSSION

The main goal of this project was to determine where protein NCOA7-AS localizes within cells. This goal was accomplished by creating an NCOA7-AS-GFP fusion protein, transfecting it into two different human cells lines, and observing where the fusion protein localized in the cells using fluorescence microscopy.

In order to determine whether the ligations resulted in the fusion of NCOA7-AS and EGFP genes, purified DNA from DH5α transformants was restricted to identify plasmids that had inserts of the appropriate size. Plasmids that appeared to have the insert were then sequenced to confirm in-frame fusion of the two proteins. Sequencing confirmed the clone construction was correct for two strains MV7034 and MV7035.

In order to determine whether the fusion protein exhibited the antioxidant activity present in the normal, non-fused protein, *E. coli* containing mutations in *mutM* and *mutY* were transformed with purified DNA from strains MV7034 and MV7035. These transformations showed oxidative damage prevention (measured by a reduced dark blue LacZ+ expression and thus fewer DNA transversions). This demonstrates that the fusion NCOA7-AS-EGFP proteins retained the oxidation resistance activity of normal NCOA7-AS.

Fluorescence microscopy examination of HeLa cells transfected with the plasmids showed that most of the cells expressing it were dead. However, a few live cells were found to express the NCOA7-AS fusion protein as diffuse green throughout the cell with no specific subcellular localization. This suggests that NCOA7-AS has no specific subcellular localization. HT1080 cells however were unable to express the fusion protein after transfection, so no results could be obtained from those cells.

The other goal of this project was to determine whether induction of the cells with IFNβ-1b altered the location of the NCOA7-AS fusion protein. It was observed in HeLa cells treated with IFNβ-1b that the fusion protein was again diffuse throughout the cells, with no clear subcellular localization, which suggests that IFNβ-1b treatment does not alter the localization of NCOA7-AS.

Previous studies have determined the structure of NCOA7-AS, its function as an oxidative damage prevention protein, its regulation via the JAK-STAT pathway, and its induction by treatment with IFNβ-1b (Yu et al., submitted for publication). However, localization of the protein was previously undetermined. This project helps to further this work by suggesting that NCOA7-AS has no specific subcellular localization, based on the results obtained from fluorescence microscopy of the fusion protein.

Unfortunately some problems arose throughout the course of the project. One problem that occurred was the inability to produce a C-terminal fusion of NCOA7-AS and pEGFP. Multiple ligations were performed, but none resulted in a usable C-terminus fusion. A likely explanation for this is that the fusion was too unstable, and the bacterial cells selected the parental plasmid pEGFP against the fusion protein plasmid, removing the NCOA7-AS insert after ligation, thus deleting any NCOA7-AS sequences. Another problem was that many of the transfected HeLa cells that expressed the NCOA7-AS-EGFP fusion protein were dead. The high number of dead cells expressing the protein indicates that the fusion may be toxic to the cells due to high expression of the gene caused by the CMV constitutive promoter in the fusion. The biggest problem that occurred was that HT1080 cells were unable to express the fusion protein in any of the transfections. Transfecting the HT1080 cells was not a problem, as they demonstrated M.

cherry red signals equivalent to HeLa cells with the transfection marker plasmid. The lack of transfection with the NCOA7-AS plasmids most likely resulted from the HT1080 cells being more sensitive than HeLa to the toxicity of the fusion, so they could not successfully express it.

There is still much to learn about NCOA7-AS. Because the fusion of NCOA7-AS to a vector with a strong constitutive promoter somewhat limited the project. For example, a EGFP fluorescent clone with an NCOA7-AS insert and a promoter that can be induced with IFNβ-1b could be created. This would allow the experimenter to control the level of NCOA7-AS expression, hopefully to limit its toxicity while allowing its location to be determined. It would also allow a method for determining whether IFNβ alters the localization of NCOA7-AS. Another method of confirming localization would be to conduct experiments that examine the location of the endogenous protein. For example, immunostaining could be performed with an NCOA7-AS-specific antibody and a fluorescent secondary antibody to examine localization of the native protein to determine if it shows a similar diffuse localization as the fusion protein in this project.

BIBLIOGRAPHY

Arnason BG, Dayal A, Qu ZX, Jensen MA, Genc K, Reder AT (1996). Mechanisms of action of interferon-beta in multiple sclerosis. *Springer Seminars in Immunopathology,* 18: 125-148.

Baranzini SE and Hauser SL (2002). Large-scale gene-expression studies and the challenge of Multiple Sclerosis. *Genome Biology*, 3(10): 1027.1-1027.5.

Compston A, Coles A (October 2008). Multiple sclerosis. *Lancet*, 372(9648): 1502–1517.

Coons A, Creech HJ, Jones RN (1941). Immunological properties of an antibody containing a fluorescent group. *Proc Soc Exp Biol Med,* 47: 200–202.

Croze E, Veichko S, Tran T, Yamaguchi KD, Salamon H, Reder AT, Kanppertz V, Paz P (2009). Betaseron induces a novel alternate start transcript in cells obtained from relapsing-remitting multiple sclerosis patients and human brain that is associated with control of oxidative resistance. *Multiple Sclerosis,* 15: 475.

Der SD, Zhou A, Williams BR, Silverman RH (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proceedings of the National Academy of Sciences of the United States of America,* 95: 15623-15628.

Durand M, Kolpak A, Farrell T, Elliott NA, Shao W, Brown M, Volkert MR (2007). The OXR domain defines a conserved family of eukaryotic oxidation resistance proteins. *BMC Cell Biology,* 8: 13.

Elliott NA and Volkert MR (2004) Stress induction and mitochondrial localization of OXR1 proteins in yeast and humans. *Molec Cell Biol*, 24: 3180-3187.

Ho PP, Kanter JL, Johnson AM, Srinagesh HK, Chang EJ, Purdy TM, Van Haren K, Wikoff WR (2012). Identification of naturally occurring fatty acids of the myelin sheath that resolve neuroinflammation. *Science Translational Medicine,* 4(137): 137ra73.

Huh W, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003). Global analysis of protein localization in budding yeast. *Nature,* 425: 686-691.

Melo A, Monteiro L, Lima RM, Oliveira DM, Cerqueira MD, El-Bacha RS (2011). Oxidative stress in neurodegenerative diseases: mechanisms and therapeutic perspectives. *Oxidative Medicine and Cellular Longevity,* 2011: 467180.

Michaels ML, and Miller JH (1992). The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). *J. Bacteriol.,* 174: 6321-6325.

Michaels ML, Cruz C, Grollman AP, Miller JH (1992). Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proc. Natl. Acad. Sci. USA,* 89: 7022-7025.

Michaels ML, Tchou J, Grollman AP, Miller JH (1992). A repair system for 8-oxo-7,8-dihydrodeoxyguanine. *Biochemistry,* 31: 10964-10968.

Mogensen KE, Lewerenz M, Reboul J, Lutfalla G, Uze G (1999). The type I interferon receptor: structure, function, and evolution of a family business. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research,* 19: 1069-1098.

Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB (1974). Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer*, 33(4): 1027–1033.

Rosati G (April 2001). The prevalence of multiple sclerosis in the world: an update. *Neurological Science,* 22(2): 117–139.

Scherer WF, Syverton JT, Gey GO (1953). Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exp. Med.,* 97(5): 695–710.

Schuler D (2004). Molecular analysis of a subcellular compartment: the magnetosome membrane in Magnetospirillum gryphiswaldense. *Arch Microbiol.,* 181: 1-7.

Scott MS, Calafell SJ, Thomas DY, Hallett MT (2005). Refining Protein Subcellular Localization. *PLoS Comput Biol,* 1(6): 66.

Seacat AM, Kuppusamy P, Zweier JL, Yager JD (1997). ESR identification of free radicals formed from the oxidation of catechol estrogens by Cu2+. *Arch Biochem Biophys,* 347(1): 45-52.

Shao W, Halachmi S, and Brown M (2002). ERAP140, a conserved tissue-specific nuclear receptor coactivator. *Molecular and Cellular Biology,* 22: 3358-3372.

Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD (1998). How cells respond to interferons. *Annual Review of Biochemistry,* 67: 227-264.

Uttara B, Singh VA, Zamboni P, Mahajan RT (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol*., 7(1): 65-74.

Volkert MR, Wang JY, Elliott NA (2008). A functional genomics approach to identify and characterize oxidation resistance genes. *Methods Mol Biol,* 477: 331-342.

Wang JY, Sarker AH, Cooper PK, Volkert MR (2004). The single-strand DNA binding activity of human PC4 prevents mutagenesis and killing by oxidative DNA damage. *Molecular and Cellular Biology,* 24: 6084-6093.

West DK and Baglioni C (1979). Induction by interferon in HeLa cells of a protein kinase activated by double-stranded RNA. *Eur. J. Biochem.,* 101: 461-468.

Wucherpfennig K, Strominger J (1995). Molecular mimicry in T cell-mediated autoimmunity: Viral peptides activate human T cell clones specific for myelin basic protein. *Cell,* 80(5): 695–705.

Yu L, Croze E, Yamaguchi KD, Tran T, Reder AT, Volkert MR (submitted). Induction of a unique isoform of the NCOA7 oxidation resistance gene by interferon β-1b in mononuclear cells and Multiple Sclerosis patients.