Spring 5-2021

Determining Regulatory Sequences of the ATF3 Promoter within the Integrated Stress Response Pathway

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Determining Regulatory Sequences of the *ATF3* Promoter within the Integrated Stress Response Pathway

An honors thesis presented to the
Department of Biology,
University at Albany, State University of New York
In partial fulfillment of requirements
For graduation with Honors in Biology
and
graduation from The Honors College

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Research Advisor: Morgan Sammons, Ph.D.

May 2021

Abstract

Cells use multiple distinct pathways to respond to cellular stress depending on context and the particular stress. The integrated stress response pathway (ISR) controls the cellular response to numerous types of extrinsic and intrinsic stressors, such as exposure to environmental toxins and viral infection, through modulation of gene expression. Transcription factors of the ISR pathway promote the restoration of intracellular homeostasis or programmed cell death if homeostasis cannot be restored. These transcription factors act spatially and temporally to control the activity of regulatory regions such as enhancers and promoters. We aimed to study how promoters of genes activated by the ISR, such as the common stress response gene *ATF3*, are regulated in response to stress. To this end, we sought to define the most plausible promoter sequence of the *ATF3* gene and determine the transcription factors that regulate this sequence. The boundaries and important sequences within a promoter are often uncertain, therefore we used several techniques such as mutagenesis, molecular cloning, and reporter gene assays, to measure the transcriptional activity of various candidate promoter sequences. We determined a 1340 bp region (hereafter called "exon") to represent the entirety of the *ATF3* promoter as it had the most robust transcriptional response. Both shorter and longer regions lacked robust responses, including a previously published putative promoter region (Kilberg). Utilizing these two constructs, we additionally aimed to recognize the functions of several transcription factors such as ATF4 and TFAP2A/2B in response to cellular stress and their role within the ISR pathway. ATF4 and TFAP2A/2B's contribution to transcriptional activity of the *ATF3* promoter was measured and analyzed utilizing wildtype and mutant promoter variants in human colorectal cancer wildtype cells (HCT116WT). Results suggest that one ATF4 binding site, the critical amino acid response element (CARE), functions as an inducer of promoter activity when under stress conditions whereas ATF/CRE, as a common target site among the transcriptional ATF family, is responsible for basal levels of activation. Mutations to the TFAP2A/2B motif resulted in no significant change compared to the wild-type, suggesting that in this context, these transcription factors are unlikely to contribute to promoter activity. These results continue to highlight the importance of context, such as cell type and the particular cell stress, in the regulation of promoter and enhancer elements.

Keywords: *Promoter, Transcription factors, Cell stress, Regulatory gene expression*

Acknowledgements

I have been so lucky to have had such an amazing group of supporters behind me throughout all of my academic endeavors. The encouragement, enthusiasm, and dedication to my success has been overwhelmingly positive and has motivated me to set my goals high and achieve them through determination.

Firstly, I want to thank Dr. Morgan Sammons for giving me the opportunity to conduct biological research in his laboratory. Without any knowledge of research nor what it would entail, Dr. Morgan Sammons allowed me to come in and learn the wonders of research and biology as a whole. I was challenged to think critically and rewarded with my own experiments and completed data. Dr. Morgan Sammons served not only as my research advisor but as a mentor throughout my undergraduate career and has been a great influence in my overall educational experience.

Second, I want to thank Serene Durham, the PhD student I worked under, for always being there to answer my questions, challenge my thinking, expand my knowledge, and for being the person to come to, to talk about research or life. I also want to thank all of my colleagues within the Sammons Lab including Dana Woodstock, Jhos Dufflart, Abby McCann, Zenab Minhas, Kate Sazon, and Dan Koskas as well as former members Ally Catizone and Aparna Prasad for assisting me and encouraging me throughout my research experience and time in the Sammons Lab. Each and every one of the Sammons lab members have passed on and taught me valuable knowledge that helped me learn and ultimately succeed.

Third, I want to thank the collaborative lab of Dr. Juan Fuxman-Bass from Boston University for completing and sharing yeast-one hybrid data with the Sammons Lab which was used as a significant component to the completion and understanding of my own research project and thesis.

Fourth, I want to thank The Honors College and Dean Chang for providing me with an opportunity filled educational environment that allowed me to learn from my peers, form friendships, and engage with professors and the greater UAlbany community which set me up for success.

Lastly, I want to thank my support system at home, my family. The love and support received from them in conjunction with everyone at UAlbany allowed me to succeed in so many ways.

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Introduction

Organismal development and homeostasis depends on appropriately regulated gene expression. The first step of this process, known as RNA synthesis or transcription, is controlled through the use of DNA-encoded cis-regulatory elements (CRE) (Griffiths et al., 2000b). It is the vast array of genes and variety in expression that provokes diversity in cellular functions as well as visible organismal phenotypes. Both extracellular as well as intracellular environmental changes can alter and influence these differences. Whether beneficial or detrimental, cells tend to adapt through a variety of stress response mechanisms which play a vital role in not only cell survival but how genes are expressed due to different conditions (Wittkopp, 2007). Gene expression is controlled in a spatial and temporal manner by CRE which contribute to overall efficiency of transcription and translation (Sun et al., 1999). The specific genes that are used during various phases of development account for the changes in overall expression of such genes. Thus, regulation of genes and gene expression helps to maintain overall homeostasis throughout the cell and the organism as a whole and its mechanisms are seen to be predetermined in the genome (Seshasayee, 2014).

Coordinated expression of genes and their specific functions ultimately control the growth and activity of multicellular organisms. In early embryonic development, a zygote is formed after fertilization and has unlimited developmental potential. It is within this stage, exceedingly early in development, when transcription of the new genome first occurs (Assou et al., 2011). Following transcription, DNA sequences follow through the concept of the central dogma where mRNA is translated, ultimately forming a protein which can be used elsewhere for significant cellular functions. Alterations or mutations to DNA sequences can lead to various developmental or cellular effects by the potential formation of an unwanted protein and function

(Griffiths et al., 2000a). Thus, it is important for cells and the body as a whole to have ways to combat potential harmful mutational effects though regulatory processes or the induction of factors that will reverse adverse effects.

Cis-regulatory elements (CRE) such as enhancers are regions of DNA both 5 prime (5') and 3 prime (3') of a specific gene that regulates the levels of transcription of that gene. CREs play a significant role in controlling gene expression, and thus also control cell specificity through regulation of cell type-specific transcription. CREs act by coordinating transcription factor binding and the crosstalk between them (Jin et al., 2011). These highly complex elements are able to regulate multiple genes, often over great distances, and can be found within introns, exons, and within intergenic regions. The highly conserved sequences within enhancers allows them to contribute to approximately half of all coding DNA (Pennacchio et al., 2013). Enhancers are not always necessary for the initiation of transcription whereas promoters are absolutely required for gene transcription. Promoters are vital DNA regions adjacent to genes that are essential for recruitment of factors and provide the starting point for transcription (Landolin et al., 2010). Enhancers likely regulate transcription by directly communicating with promotors by chromatin looping (or other mechanisms) that triggers transcription through various means, often by facilitating pre-initiation complex formation (Nolis et al., 2009). The looping allows for distant enhancers to be brought within less than 200 nm of the downstream target gene (Pennacchio et al., 2013), often with the facilitation of histone modifications and other cofactors to organize the chromatin between both elements. Studies show that enhancers can transfer RNA polymerase II to the promoter, hence the formation of the pre-initiation complex. There are a number of other potential mechanisms allowing enhancers to regulate promoters. Although different from protein-coding mutations, mutations in the cis-regulatory regions, like enhancers

and promoters, also play a major roles in the ultimate activity of gene transcription and expression that leads to a variety of diseases in all ranges of complexity (Pennacchio et al., 2013). Non-coding mutations in enhancers illustrate the complexity and significance of these elements in regard to transcription and normal cellular function.

Promoters are regions of DNA that are necessary and required for transcription to occur. Core promoters encompass the transcriptional start site (TSS), short base pair binding motifs where RNA polymerase is recruited to initiate the process of transcription of DNA into a single RNA strand (Haberle & Stark, 2018). Not only does RNA polymerase bind, but a multitude of transcription factors (TFs) have sequence specific binding sites on the promoter that effect transcriptional activity of the downstream gene. These transcription factors can either act to recruit RNA polymerase or contribute to its activation once bound. The promoter acts as a scaffold for TFs, thus the overall activity depends on specific DNA sequences and homology for the binding of such factors. Mutations in the promoter sequence can alter transcription factor binding and may cause down regulation, cancer, or disease (Lee & Young, 2013). Similar effects occur when the mutation is within the transcription factor, preventing them from binding promoters or other regulatory elements. The importance of promoters is thus shown by the potential harmful effects of mutations leading to the decrease or halt in production of vital proteins and their functions all of which can disrupt cellular homeostasis and potential survival.

Both the enhancer and promoter regions of specific genes do not act alone; regulatory elements act as scaffolds for transcription factors. Transcription factors bind to these regulatory sites that alter gene expression activity by the promotion or inhibition of transcription. We study one of the most well-known transcription factors, p53. p53 binds to DNA within both enhancers and promoters of specific genes. As a tumor suppressor, p53 binds to important regulatory

elements controlling genes important in the response to DNA damage (Younger & Rinn, 2017). As part of its significant role in the response and prevention of cancer, p53 attempts to cease uncontrollable cell proliferation by prompting apoptosis or cell cycle arrest. Although p53 is seen to bind to promoters, it mainly binds enhancer elements (Younger & Rinn, 2017). When the cell undergoes particular stresses such as DNA damage, the p53 stress response pathway is initiated. Upon activation, p53 binds to and activates transcription of its target gene. *ATF3* is an example of a gene that p53 induces when DNA damage is present (Zhao et al., 2016). *ATF3* is downregulated with cancer and contributes to cellular homeostasis. *ATF3* also directly regulates p53 stability within the cell (Yan & Boyd, 2006), pointing towards an important role in tumor suppression. Through this pathway, p53 binds to specific binding motifs on the enhancer and regulates the transcription of the *ATF3* gene, apoptosis (cell death), and tumor suppression (Sammons et al., 2020). It has become a focus of recent study however, that these transcription factors binding affinity for either the promoter or enhancer changes depending on environmental factors, including stress.

Stressors in cellular environments can occur due to both extracellular or intracellular alterations. When stress is induced, the primary goal is to combat and overcome the stress to promote survival. For example, when DNA is damaged by exposure to ultraviolet (UV) radiation, the cell attempts to counter its deleterious effects by employing nucleotide excision repair or the activation p21 expression by p53 (Fulda et al., 2010). Additionally, when there is amino acid deprivation, the cell induces asparagine synthase (ASNS) or *ATF3* gene expression to resist adverse effects to the cell's survival (Y. Pan et al., 2003). Although the cell strives to stay alive prior to resorting to other stress response methods, if the stress applied is overwhelming or

too substantial for the cell to withstand, the cell can undergo apoptosis or autophagy (cell-eating) (Fulda et al., 2010).

To help impede harmful effects within the cell, specific stress response pathways are induced. The p53 stress response pathway involves the activation of genes through the binding of p53 to regulatory elements such as the enhancer. The integrated stress response pathway (ISR) on the other hand, involves specific TF binding to promoter elements. Both pathways involve intrinsic and extrinsic physiological differences that affect the binding of related transcription factors, the activity of transcription of stress induced genes, and the overall protein product and function. Inducers of the p53 response to cell stress include DNA damage, oncogene activation, ribosome dysfunction, and metabolic stress (Bursac et al., 2014). By contrast, the ISR pathway is induced by cellular stressors such as amino acid deprivation, endoplasmic reticulum stress, heme deprivation, or viral infection. These stressors pave the way for specific transcription factors to bind to stress response genes through a cascade of events. Transcription factors including ATF4, ATF6, and XBP1 are known to be major contributors to the ISR pathway by specifically binding regulatory elements that control downstream gene transcription (Pakos-Zebrucka et al., 2016). One such target appears to be the most common stress induced gene, *ATF3*. Transcription factors such as ATF4, ATF6, XBP1, and TFAP2A/2B all have homology and bind to the promoter region in response to cellular stress.

The *ATF3* gene is a stress response gene that is activated by the integrated stress response pathway. This regulatory gene is upregulated by both amino acid deprivation and endoplasmic reticulum stress through the binding of transcription factor ATF4 to the *ATF3* promotor region. Transcription factors have specificity to distinct binding motifs on regulatory elements including the promoter. ATF4 in particular has two binding sites on the *ATF3* promoter, the critical amino

acid response element (CARE) as well as activating transcription factors/cyclic AMP response element (ATF/CRE). Previous studies have revealed the possible significance of stress inducing treatments on the activity of the *ATF3* gene through ATF4 binding (Fu & Kilberg, 2013).

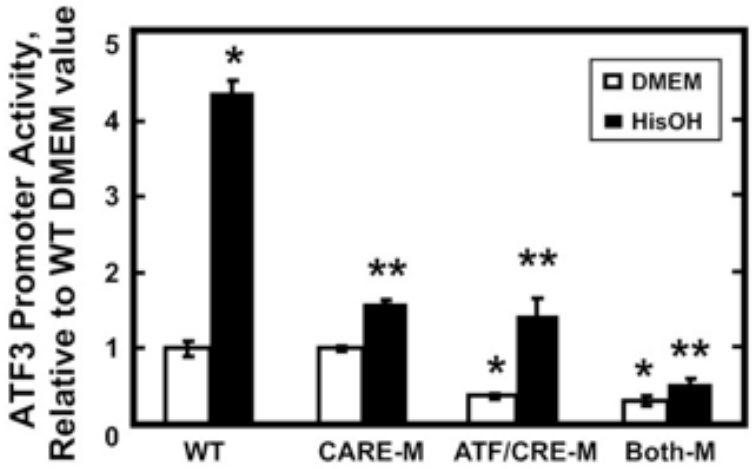

Figure 1
Lingchen Fu & Michael S. Kilberg – Stress induced CARE versus ATF/CRE luciferase results

Note. Results from "Elevated cJUN expression and an ATF/CRE site within the ATF3 promoter contribute to activation of ATF3 transcription by the amino acid response" by Lingchen Fu and Michael S. Kilberg paper comparing the ATF3 promoter activity relative to WT DMEM and Histidinol for both the ATF3 CARE and CRE sites. A forward transfection with histidinol treatment was experimented on HepG2 WT cells and a luciferase assay was completed to measure luminescence. Histidinol increased activity of WT cells significantly and had some effect on the mutant sites, specifically ATF/CRE.

Michael S. Kilberg and Lingchen Fu (2013) published several manuscripts regarding the *ATF3* promoter region and transcriptional responses through transcription factor binding. The authors used a 140 base pair section of the *ATF3* gene as their promoter sequence to specifically examine the effects of stress on both the ATF/CRE and CARE sites where transcription factor, ATF4, is proposed to bind. In doing so, Kilberg tested the wildtype version of both sites in HepG2 cells against the mutated versions of the ATF/CRE and CARE binding sites. The wild type ATF/CRE site, TTACGTCAG, was mutated via site directed mutagenesis to TT**G**CG**G**CAG and wild type CARE, TGATGCAAC to mutated TGAT**AT**AAC and effects

were compared against a histidinol treatment. Histidinol is a drug that depletes the amino acid histidine from the cells' environment, inducing amino acid starvation and cell stress. Through a luciferase reporter assay results concluded that transcriptional levels significantly increased with amino acid deprivation compared to the control and ATF/CRE is a potential effector of *ATF3* activity shown through the transcriptional reduction in non-treated cells (Fu & Kilberg, 2013).

The ideas experimented and results discovered by Kilberg as well as the influence of the ISR pathway as a whole, drove the interest in research regarding these transcriptional gene regulatory regions and elements. Initially, to understand how effectors of the ISR pathway are regulated and influenced through the use of the *ATF3* gene, determining what the promoter region of this gene is and what sequences would indicate and function as the most probable promoter in vivo, was top priority. As a stress induced gene, the binding of transcription factors including ATF4 and TFAP2A/2B regulate transcription of *ATF3*. Thus, determining where these factors are binding and analyzing the effects of environmental and internal stressors will aid in the discovery of the ISR pathway mechanisms in regard to regulatory promoter sequences.

Materials and Methods

1. Cell Culture

HCT116, human colorectal cancer, wildtype (WT) cells were used in all experiments and cultured using McCoy's 5A, 1X (Iwakata & Grace Mod.) with L-glutamine media where they were kept to grow in a 37 degree incubator. Corning 0.25% Trypsin, 2.21mM EDTA, 1X [-] sodium bicarbonate was used to resuspend cells and cells were counted using Life Technologies Countess II FL. During transfection, cells were treated with a cell stress inducing treatments such as histidinol, using controls of RNase free H_2O and DMSO accordingly, all concentrated at 1000X; treatment administration ranged from 4-12 hours depending on the experiment and cells were washed with PBS.

2. Site Directed Mutagenesis

Mutant plasmid promoter constructs were made by site directed mutagenesis. The backbone DNA was cut using appropriate restriction enzymes and the mutated base pairs were inserted using a primer with the associated mutation (Table 1; see Appendix, p. 39). All primers were ordered from Integrated DNA Technologies (IDT) and the reaction was completed as per New England Biolabs Q5 Site-Directed Mutagenesis Kit Protocol.

3. Luciferase Gene Reporter Assay

Luciferase gene reporter assays were completed using Promega Dual-Glo Assay kit. Samples were experimented using reverse transfection through a two-day seeding and transfection process with HCT116 wild type cells. Similar experiments were completed using a forward transfection encompassed by a three-day process including seeding of HCT116WT cells, transfection, and

treatment of cells with stress inducing drugs such as histidinol. The BioTek Hi Synergy luminometer/plate reader provided luminescent data for both the firefly and Renilla buffers. All data was analyzed through the normalization of firefly to Renilla and appropriate data tables and graphs were created.

4. Polymerase Chain Reaction (PCR)

DNA was amplified through 25-30 cycles of PCR and were compared against a no 5Q master mix negative control. The double stranded linear DNA backbone was denatured at 95°C and the DNA constructs were annealed using their associated forward and reverse primers homologous to the backbone as seen in Table 1. The PCR product is run through a 1% agarose gel against a 100kb or 1000kb ladder, dependent on sample DNA size. Samples were run at 120V and DNA was retrieved though cutting out each corresponding band. Gel purification produced pure DNA and all concentrations were calculated by nanodrop or qubit.

5. Molecular Cloning

Using fast digest with both pGL4.11 and pGL4.10 parental backbones for respective experiments and restriction enzymes, KpnI and HindIII, linear vectors were made; the positive control was the cut version whereas the negative was uncut due to the lack of Kpn1 and HindIII. Mixes were incubated at 37°C overnight and the product was obtained by purifying only the positive control band from a 1% agarose gel.

6. Gibson Assembly

Gibson assembly is an isothermal reaction used to assemble multiple linear fragments and is a form of cloning method. DNA concentrations were calculated in picomoles and the NEB Gibson Assembly protocol was followed using vector, inserts, NEB 2x, and H_2O with separate negative controls of excluding master mix and excluding DNA insert. The mixture was placed in the thermocycler for 15 minutes at 50°C with a lid temperature of 55°C.

7. Bacterial Transformation

Experimental plasmids were transformed into *Escherichia coli* (E.coli). The bacteria were heat shocked by the transfer from an ice bath to a heated water bath allowing the plasmid to enter through denaturation. LB broth was added and centrifuged for 4.5 minutes at 500G creating a pellet. For a greater concentration, 900µL was aspirated, the pellet was resuspended, and the remaining sample was plated on 15mL ampicillin resistant nutrient agar plates. After 12-16 hours, colonies from each plate were added to 5mL of LB broth in falcon tubes along with 5µL of ampicillin and were rotated at 37°C overnight. The bacteria were centrifuged and miniprepped following E.N.Z.A Plasmid Mini Kit protocol to isolate the pure DNA. The concentrations were found using nanodrop and 10µL of each sample were sent out for sequencing via Eurofins Genomics.

8. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

HCT116 WT cells were seeded in a 12 well plate overnight and treated with various cell stress inducers on day 2 for 4-6 hours. RNA was extracted using Quick-RNA Miniprep Kit and manufacturing protocol was followed. Concentrations of RNA were used to create cDNA added

to both a reverse transcriptase (RT) master mix and a no RT master mix, placed in thermocycle, and stored at -4°C. qPCR master mixes are made using BioRad iTaq SYBR Green Supermix along with standards and plated into 384 well plate with corresponding primers compared against a GAPDH control (Table 1). Using the ABI 7900HT real-time PCR instrument, the plate was read and analyzed.

9. Data Analysis

Preliminary in-silico experiments, plasmid constructs, and sequencing comparisons were made using SnapGene. All luciferase reporter assay and qPCR data were analyzed using Microsoft Excel. Both graph construction and statistical analysis were completed via Graphpad Prism.

Results

We sought to determine what transcription factors bind to the *ATF3* promoter and thus regulate its transcription. In order to do this, we determined the best promoter sequence in this context and studied transcriptional responses to cellular stress through validation experiments and comparison against previous literature.

To study the function of the *ATF3* promoter, we first wanted to create a transcriptional activity reporter system. We selected a standard luciferase reporter assay to use, where a putative promoter sequence was cloned upstream of a luciferase gene. If the inserted sequence is a promoter, luciferase RNA will be translated into a functional enzyme that produces luminescence to be measured. The increased measurement of this enzyme's activity corresponds to increased transcription.

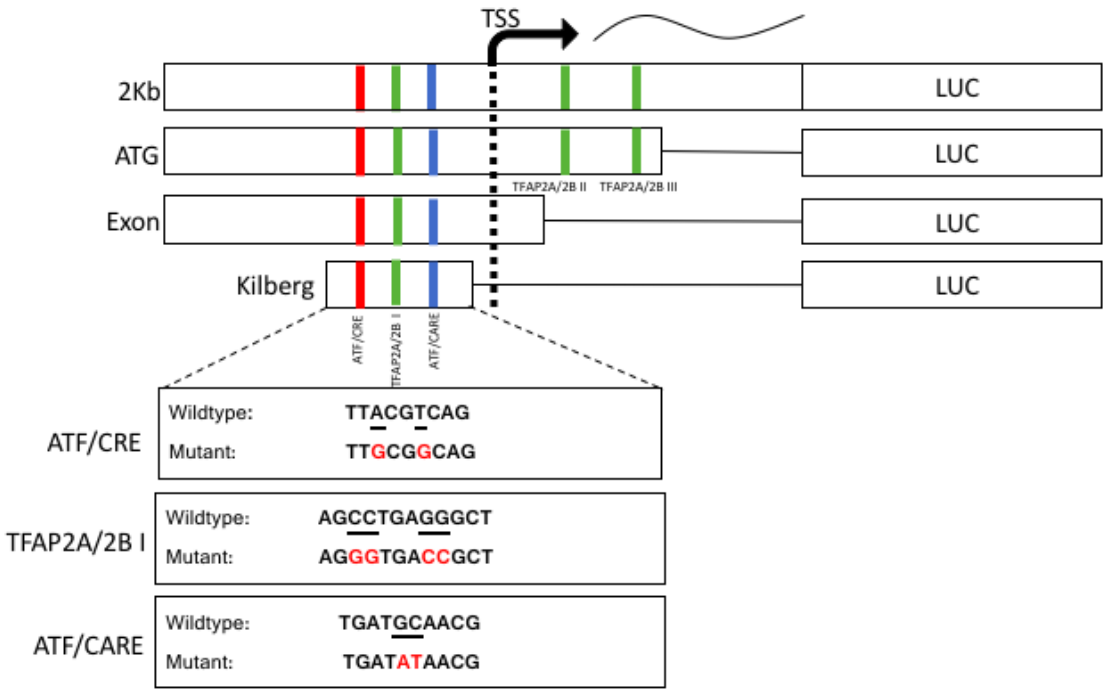

Figure 2
Sequence mutations of the ATF3 promoter constructs

Note. Four promoter constructs were created by Gibson Assembly using a 2 kilobase portion of the *ATF3* stress response gene and inserted into a luciferase reporter plasmid, pGL4.11. The 2 kilobase region was 1000 base pairs (bp) upstream and 1000 bp downstream of the transcriptional start site (TSS). The ATG and exon regions begin 1000bp upstream of the TSS and end after the first ATG and the first exon, respectively. The Kilberg construct was a 140bp region encompassed within all four constructs. The wildtype ATF/CRE, ATF/CARE, and TFAP2A/2B I transcription factor binding sites were within all constructs and were mutated via site directed mutagenesis in the exon and Kilberg promoter regions.

We began with a 2 kilobase region of genomic DNA that spanned -1000 and +1000bp from the *ATF3* transcriptional start site (TSS). This region was also used in a related enhanced Yeast 1-hybrid (eY1H) assay that will be discussed below. From the 2kb fragment of the *ATF3* gene, smaller, more specific, and biologically relevant regions that are proposed to incorporate the *ATF3* promoter were created. A region 1000bp 5' of the TSS until the first ATG in the sequence was denoted the ATG construct. A second construct began at the same 5' position and ran until the end of the first exon and was denoted the exon construct. Lastly, the Kilberg construct was 140bp within all of the other constructs, positioned just downstream of the TSS

(Figure 2). All of these constructs were completed by Gibson Assembly using sequence specific primers for each (Table 1) and were inserted into a luciferase reporter plasmid, pGL4.11.

With the design and creation of the four *ATF3* promoter constructs, we aimed to determine which construct would be the most conducive promoter *in vivo*. To do this, we conducted a luciferase gene reporter assay by transfecting HCT116WT cells with cloned plasmids containing the respective promoter constructs. The activity and effectiveness of each promoter in respect to the luciferase reporter was measured by the normalized luciferase value which is the ratio of normalized Renilla to luciferase plasmid (Carter & Shieh, 2015).

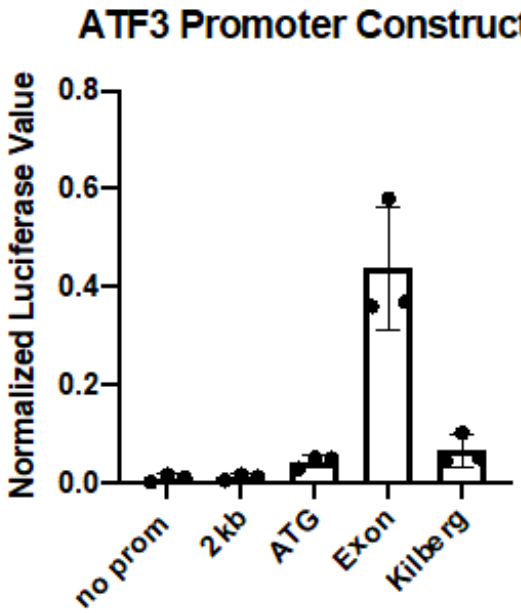

Figure 3
Luciferase comparison of ATF3 promoter constructs

Note. A luciferase assay was conducted to determine the overall activity of each promoter sequence against a no promoter negative control. Fragments were inserted into luciferase plasmid pGL4.11. The negative control showed little to no activity, followed by the 2kb region. The sequence that stopped after the first exon produced the most transcriptional luciferase activity of approximately 0.4 with both Kilberg and ATG producing less.

To deduce the preeminent promoter construct to use in further experiments, we sought to discover the construct with the highest level of luciferase activity thus, transcriptional activity.

As seen in Figure 3, the promoter construct with the highest detection of luminescence was the exon region having approximately an 8.5-fold increase in luciferase activity from that of the Kilberg construct, having the next highest normalized luciferase value. The 2kb region showed little to no activity and produced results very similar to that of the no promoter negative control. Thus, all further experiments were completed using the exon and Kilberg promoter constructs and were the basis toward a thorough investigation of the *ATF3* promoter region.

Aiming to understand how the *ATF3* promoter is contributing to gene transcription, overall expression, and eventually in response to stress conditions, the exon and Kilberg promoter constructs were used for future experiments. The 140bp Kilberg construct revealing its activity was previously published however, the larger exon fragment had increased transcriptional activity (Figure 3) thus, we proposed that the Kilberg promoter sequence may not encompass all of the significant portions of the *ATF3* promoter or the promoter in its entirety. Therefore, both the exon and the Kilberg promoter regions were compared to each other in regard to activity and functionality as regulatory regions.

Transcription factors such as ATF4 bind to regulatory regions such as the promoter. Specifically, ATF4 is known to bind to both the CARE and ATF/CRE sequence specific binding motifs within all of the promoter constructs created (Fu & Kilberg, 2013). To further understand these promoter constructs, mutations were made within the CARE and ATF/CRE binding sites to analyze the effects of transcriptional activity compared to the wildtype versions. Using site-directed mutagenesis, two base pairs were exchanged for others that would potentially disable ATF4 to bind (Figure 2). The effects of these mutations on the activity of the exon and Kilberg constructs were examined through a luciferase gene reporter assay.

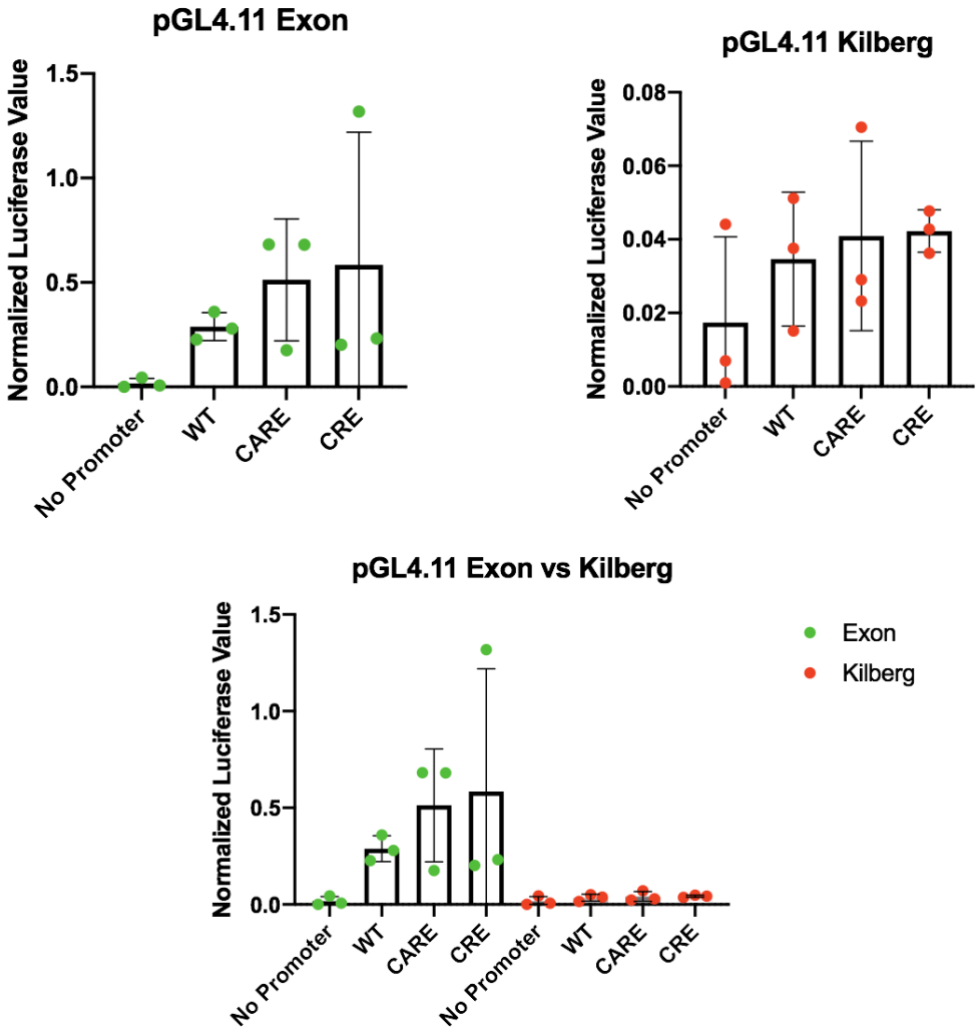

Figure 4
pGL4.11 luciferase comparison of exon and Kilberg promoter constructs

Note. The activity of *ATF3* is compared against the exon and Kilberg promoter constructs between both wild type and mutant versions using luciferase plasmid pGL4.11 and transfected into HCT116 WT cells in which the transcriptional activity of the various constructs can be determined. Rep 2 constitutes the DMSO component of treatment while the remaining replicates received no treatment

Three biological replicates each with three technical replicates, were reverse transfected into HCT116WT cells and transcriptional activity was measured through a luciferase gene reporter assay. The exon region produces significantly more firefly luciferase activity than that of the Kilberg promoter construct (Figure 4). However, looking at each experiment individually, all results are shown to have little to no correlation and are not consistent with each other as seen by

discordant error bars and data points. This introduced confusion as well as additional questions as to why the data showed discrepancies compares to literature.

From previous literature, it was realized that experiments similar to the one completed in Figure 4 utilized several different techniques and reporter plasmids that may have contributed to the cause of discrepancy. The first major difference was the type of reporter system being used. Our system, called pGL4.11, contains a PEST tag on the luciferase coding sequence, whereas previous work used a plasmid without such tag (Fu & Kilberg, 2013). The PEST tag is sequence of amino acids leads to rapid degradation of proteins containing that tag (Rechsteiner & Rogers, 1996). Thus, when fused to luciferase, it measures the amount of luciferase activity at the particular moment in time at which it is measured and allows the protein product to be continuously degraded. Conversely, pGL4.10, lacking a PEST tag, is a reflection of protein buildup overtime, which is directly correlated with transcriptional activity over that time period. To determine whether the presence of the PEST tag was influencing our results, pGL4.10, a luciferase reporter plasmid that does not contain a PEST tag was tested against the previous, pGL4.11 with a PEST tag through a luciferase assay.

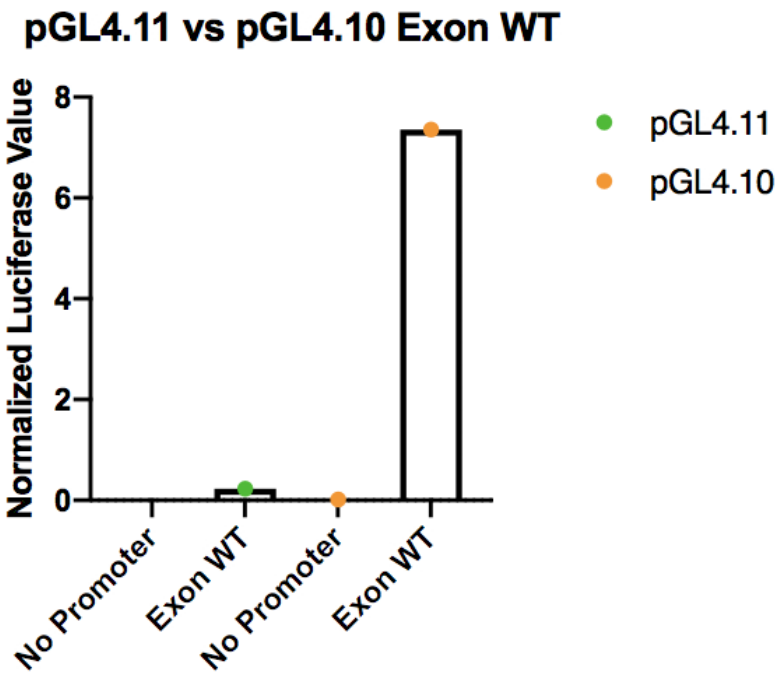

Figure 5
Comparison of PEST tag versus no PEST tag in exon WT promoter plasmids pGL 4.11 and pGL4.10

Note. The wildtype exon promoter construct of both pGL4.11 and pGL4.10 were compared against a no promoter negative control to study the effects of a PEST or no PEST tag on luciferase activity. The exon WT of pGL4.10 is significantly increased. The data point for pGL4.11 no promoter negative control is not revealed within this graph.

The comparison of pGL4.11 and pGL4.10 helped to determine how the PEST tag influences the data output and thus, which reporter plasmid was more biologically relevant and useful in further research experiments. By looking at the normalized luciferase value in Figure 5, pGL4.10 wildtype exon produced significantly more luminescence than that of both pGL4.11 as well as the no promoter control. Although a plasmid that includes a PEST tag is a better representation of transcription, for the purposes of this research and the questions we were aiming to solve, a plasmid that produces greater activity (no PEST tag) was more advantageous. Furthermore, a plasmid lacking a PEST tag was used in previous literature so shifting to this reporter plasmid acted as a meaningful step to recapitulating literature's results.

Moving forward with utilizing the pGL4.10 reporter plasmid for all subsequent experiments, we sought to determine if there were any other experimental factors that differed between our work and prior literature. We thus tested additional contrasting factors such as moving from a 96 well cell culture plate, 4 hour drug treatments, and reverse transfections to using a 12 well cell culture plate, 12 hour drug treatments, and forward transfections. The goal was to mimic the experimental parameters from literature as closely as possible. After making the above changes, the exon and Kilberg promoter constructs, WT and mutants, were tested via a luciferase gene reporter assay. HCT116WT cells transfected with various plasmids were treated with either a H_2O negative control or a stress inducing drug histidinol (HisOH).

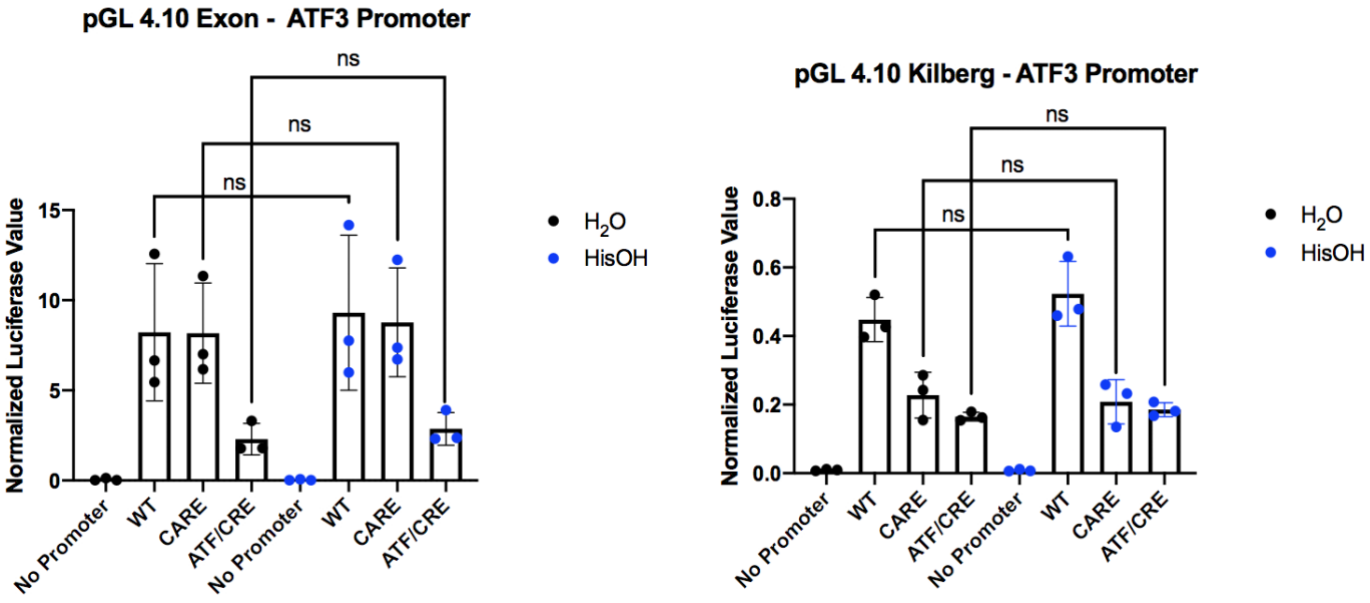

Figure 6
Luciferase comparison of exon and Kilberg with histidinol treatment

Note. The WT and mutant versions of the exon and Kilberg promoter regions within pGL4.10 were forward transfected into HCT116WT cells and studied through a luciferase gene reporter assay. Cells were treated with a H_2O negative control and stress inducing drug, histidinol for 12 hours in a 12 well cell culture plate. Each experiment conducted three biological replicates and three technical replicates within each.

Compared to literature, both the exon and Kilberg promoter constructs within the negative control condition revealed results similar to that of the control results seen in Figure 1 with a drop in transcriptional activity with the ATF/CRE ATF4 mutant binding motif. However, between the control and the stress induced environment (HisOH), there is no significant effect, unlike what is seen in previous literature (Figure 6). Histidnol did not seem to create any effect on promoter activity within any of the replicates completed. This result leads to further questions regarding the success of drug treatments in relation to luciferase reporter assays and its stress inducing response of *ATF3* within the ISR pathway.

In order to test whether the *ATF3* promoter is non-responsive to histidinol in our system or whether histidinol is non-functional in HCT116 cells, we performed a RT-qPCR reaction to quantitatively determine the accumulation of hASNS and hATF3 mRNA after treatment with histidinol. ASNS and *ATF3* are both validated histidinol-responsive genes controlled by the ISR. After a forward transfection of the WT exon pGL4.10 plasmid and a four-hour treatment on HCT116WT cells, qPCR analysis of all data was normalized to hGAPDH.

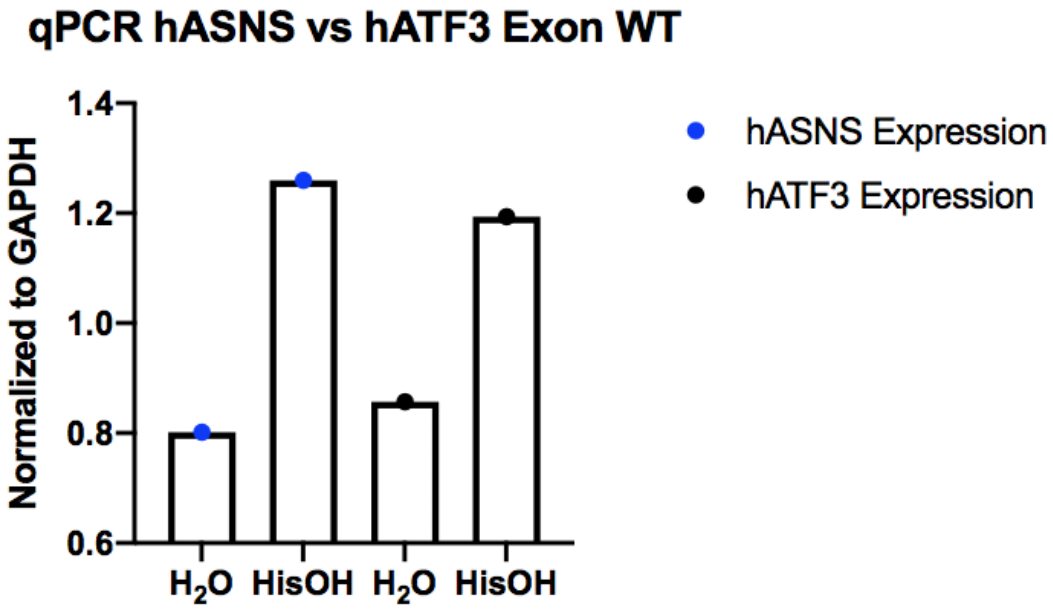

Figure 7
RT-qPCR analysis of hASNS and hATF3 gene expression with histidinol treatment

Note. The WT exon was forward transfected into HCT116WT cells in which RT-qPCR was completed to determine the levels of mRNA production through a histidinol treatment. Primers, hASNS and hATF3 were used to detect the mRNA production. HisOH increased production by a small margin with both primers.

Against a water control, Figure 7 shows that HisOH increased mRNA expression of both hASNS and hATF3. This suggests that HisOH was in fact working as it was intended to by inducing these gene's expression. To further ensure that our treatments were going to behave as desired a new bottle of histidinol was purchased and new aliquots were made.

A second round of RT-qPCR analysis was conducted using the new aliquot of histidinol. We also wanted to run this experiment again not only to enhance validation but to complete it without the cells being transfected. In the previous RT-qPCR experiment, the HCT116WT cells were transfected with the exon WT plasmid. We wanted to directly see the functional outcome of this treatment however, the transfection may have had some effect on results. In the new

experiment, we reused the hASNS gene and added p21and the RNA that was extracted was from untransfected cells. The application of this experiment was simply to observe through mRNA quantification, if HisOH was completing its job by comparing known effects on gene expression of p21(no effect) and hASNS (upregulation).

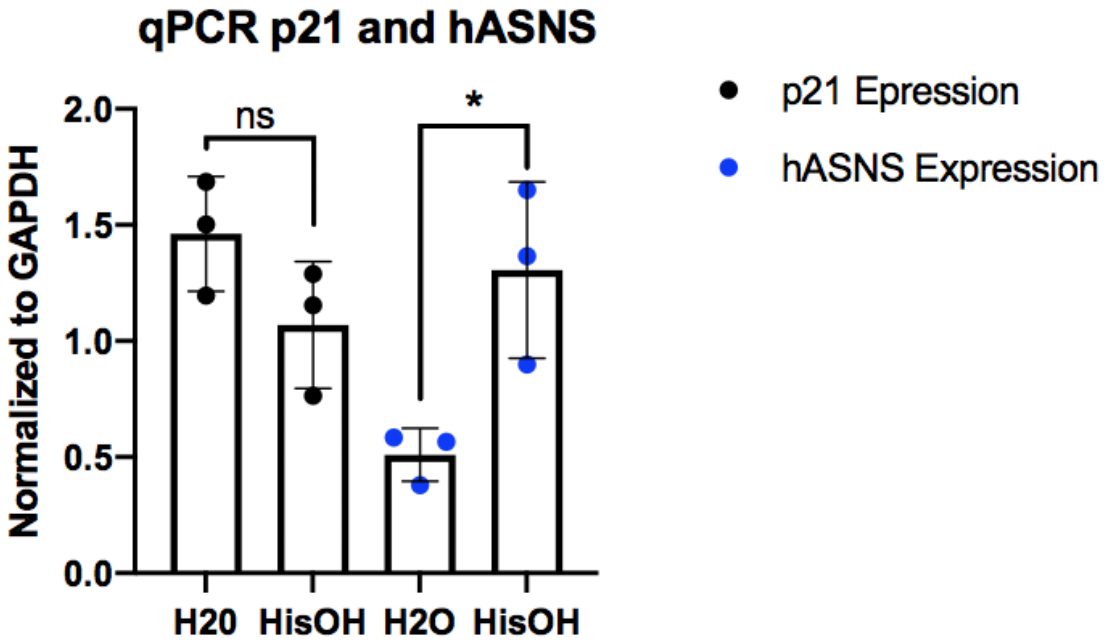

Figure 8
RT-qPCR analysis of p21 and hASNS gene expression with histidinol treatment

Note. qPCR was completed on HCT116WT cells to compare the effects of HisOH treatment on mRNA production. Levels of mRNA for hASNS increased significantly and p21 expression saw no statistical effect.

The extent of normalized expression was further determined to provide reassurance that the HisOH treatment was functioning as expected. HisOH is proposed to increase both hATF3 and hASNS expression and conversely, not affect p21 expression. Figure 8 reveals promising results as hASNS expression increased when treated with HisOH. However, p21 expression is statistically unchanged, as expected. All experimental data combined, including those

encompassing HisOH treatments, suggests that histidinol is capable of activating the endogenous ISR pathway, but that it is unable to increase activity of the *ATF3* promoter in our system.

The transcription factor ATF4 binding to the *ATF3* promoter in response to cellular stress is not the only transcription factor contributing to the activity of the *ATF3* gene. Wanting to look further into other potential players with binding motifs within the promoter of this ISR mechanism, an enhanced Yeast-one hybrid assay (eY1H) was conducted by a collaborative lab, the lab of Juan Fuxman-Bass of Boston University to quantifiably determine the transcription factors associated with and potentially involved in the functionality and activity of the *ATF3* promoter region. An eY1H is a high throughput technique used to study protein-DNA interactions within the yeast nucleus. Thus, it allows for the determination of proteins such as transcription factors which act as the prey, that bind to a particular region of DNA, in this case, the 2kb fragment of *ATF3* acting as the promoter which is called the bait sequence. Downstream of the bait are two genes such as LacZ and HIS3. Additionally, Gal4's activation domain from the yeast is attached to the prey which allows for gene expression of both LacZ and HIS3 when the prey binds to the bait sequence (Reece-Hoyes et al., 2011). The strength of the transcription factor depends on its affinity for the bait sequence, its activity, as well as the number of binding motifs and their location (Shrestha et al., 2019).

Through analysis of yeast-one hybrid data (Table 2; Appendix, p. 40), results suggest that the transcription factor TFAP2A/2B, binds to the *ATF3* promoter within the 2kb region and could be a potential candidate for study. Using Genome Browser to observe where TFAP2A/2B has a promoter binding motif, three sequence specific binding sites were indicated (Figure 2). The focus of further research will be on the motif denoted TFAP2A/2B site I and hereafter referred to as TFAP2A/2B. This site was found to be located directly between both the

ATF/CRE and CARE sites of the ATF4 transcription factor used in all previous experiments. Utilizing the same question, what is TFAP2A/2B's role in *ATF3* activity through the ISR pathway, allows for greater understanding of the cell's response to stress as a whole. To acknowledge and recognize its potential significance, a mutant promoter construct was created by site-directed mutagenesis and transcriptional activity was analyzed through a luciferase gene reporter assay.

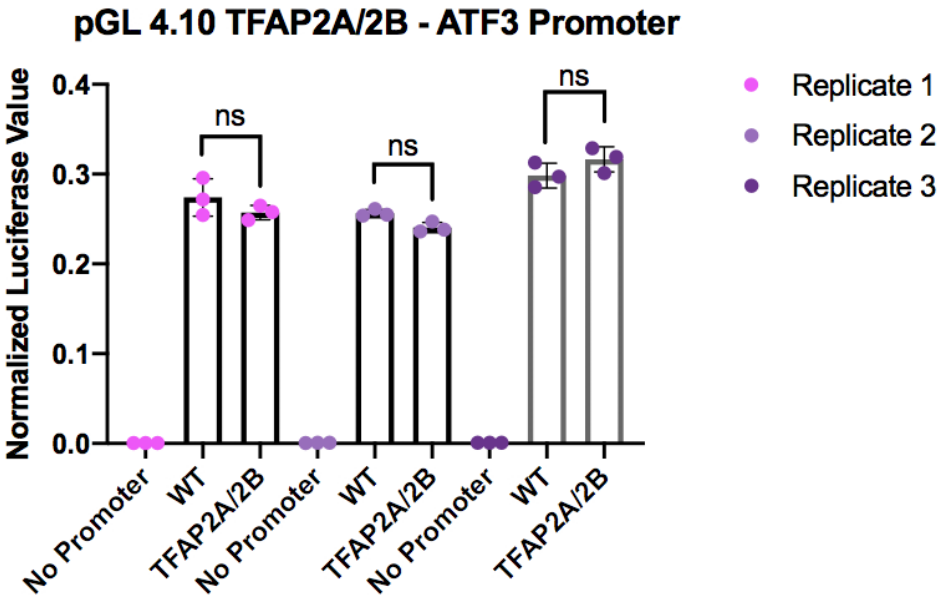

Figure 9
Luciferase interpretation of TFAP2A/2B mutant promoter motif

Note. Transcription factor, TFAP2A/2B, is proposed to bind to the promoter of the *ATF3* gene through analysis of yeast-one hybrid data. Promoter constructs with WT and mutant TFAP2A/2B binding motifs were forward transfected into HCT116WT cells and gene activity was quantified through normalized luciferase values. Three technical replicates were incorporated within three biological replicates. Low luciferase value is explained by a dilution factor of 7. All replicates show no significance between WT and mutant.

Data suggests that both the WT and the TFAP2A/2B mutant constructs produced greater *ATF3* activity than that of the no promoter negative control. However, results reveal that there was no significance in activity between WT and TFAP2A/B mutants across all three replicates. Although results do not show great significance within this experiment alone, TFAP2A/2B is not

ruled out in terms of potential importance within the ISR pathway as a whole, or even at the *ATF3* promoter. Besides TFAP2A/2B, there are many other possible factors given by the eY1H data that may potentially contribute to the activity of the *ATF3* gene and the overall functioning of the ISR that could be studied.

Discussion

The aim of dictating what the *ATF3* promoter is and its role, was studied through the introduction of cellular stress and its effects on the integrated stress response pathway. Looking at what transcription factors were involved was integral in determining how the promoter functions in response to these stresses. Specifically, ATF4 binding to both the ATF/CRE and CARE sites plays a major role in *ATF3* activation. Inducing cellular stresses through the use of histidinol was used to study their effects on transcription and additional work was done to validate those results. Ultimately, the functions of the *ATF3* promoter is significant in the integrated stress response pathway and further studies will increase understanding of this process.

The answers to the questions above began with four various promoter constructs made from the 2kb region. Each with different potential properties, the most probable promoter sequence was suggested by the quantitative luciferase activity levels. The exon region provided a significant fold increase in transcriptional activity followed by that of the Kilberg construct due to the content of each construct and how they were made for the reporter. The 2kb region being 1000bp 5' and 1000bp 3' of the TSS and occupying a position directly upstream of luciferase, made it so the start of translation to read the mRNA sequence and form the protein that produces luminescence, out of frame. Due to this aspect, the correct protein was not produced and in turn little to no luciferase activity was shown through the assay. Although having increased activity as compared to the no promoter negative control and the 2kb region, the activity produced using the ATG construct was not to the extent of exon. The ATG construct had a 5' untranslated region (UTR) after the transcriptional start site (TSS). A 5' UTR sequence is involved in regulation of both translation and overall RNA stability and can bind both translation repressors and inducers

(van der Velden & Thomas, 1999). In this case and in this context, the 5' UTR that was encompassed within the ATG promoter construct had a deleterious effect on luciferase production. As for the exon construct, it began 1000bp 5' of the TSS and spanned the entirety of the first exon. Due to the fact that the exon construct produced the greatest luciferase activity, we speculate that the partial UTR sequence that was within the exon promoter did not have a negative effect on transcriptional activity. Perhaps this portion of the UTR did not include the sequence necessary for specific repressors to bind. Additionally, the exon construct did not have an issue with framing, further explaining its response in the assay. Finally, the 140bp Kilberg construct had similar results as the ATG construct with induced transcriptional activity compared to the no promoter negative control and 2kb construct but not the extent of the exon construct. Like the exon however, it does not have issues regarding framing or UTRs due to its sequence location relative to the TSS but, the probable reason for its deduction in luciferase activity can be due to the lack of extra sequence 5' of the TSS. Due to this reduction in sequence length, it lacks binding sites for transcription factors that potentially have significance in the *ATF3* promoter's activity. It is likely that if 1000 bp or so was added 5' of the Kilberg construct, it would have similar results as the exon region. Overall, this does not conclude that the 2kb, ATG, or Kilberg are necessarily "bad" promoters in general, but they are not efficient with the ability to receive data regarding promoters by using reporter assays. Thus, moving forward with the exon and Kilberg constructs for additional experiments was best within this context due to significant activity levels and an aim of recapitulating results from literature.

Previously published literature such as the Fu and Kilberg paper, provided data to compare to and we intended to see if results were replicable in conjunction with completing similar experiments using the exon construct. The transcriptional regulation of *ATF3* focuses on

the binding of transcription factor, ATF4, to both the CARE and ATF/CRE sites, with the ATF/CRE site having more significance in control environments. Mutations were made in both the exon and Kilberg *ATF3* promoter constructs and it was proposed that due to the mutations, ATF4 would not be able to bind and *ATF3* activity would significantly decrease with the ATF/CRE mutation (Fu & Kilberg, 2013). Beginning the study of the exon and Kilberg regions without the use of drugs for the induction of cell stress, the results were proposed to be comparable and have the same trends as those of literature however, data received from the luciferase reporter assay using the pGL4.11 reporter plasmid, revealed discrepancies as compared to literature (Figure 3). The trends of the biological replicates did not match each other, nor did they match previous studies. Instead of having both the WT and CARE mutants with similar elevated luciferase values and the ATF/CRE mutant significantly lower, results showed the WT promoter to have the least amount of activity and ATF/CRE to have the highest, excluding the no promoter trials. For the ATF/CRE mutant ATF4 binding site, activity is presumed to decrease due to the inability of ATF4 binding, however in many replicates of both the exon and Kilberg constructs, the activity increased which was cause for confusion. In all luciferase experiments, each promoter resulted in luminescence levels that were greater than that of the no promoter which indicates that the constructs are acting as promoters by inducing transcription. Nevertheless, this does not explain the reasons behind the discrepancies in luciferase date against literature. After questioning whether there were issues pertaining to my techniques in conducting the assays, it was realized that there were many experimental parameters between my experiments and literature's that differed.

Trying to recapitulate results observed from literature, several experimental parameters were not controlled for that may have caused a significant effect in results. Literature used

HepG2 cells, a reporter plasmid that lacked a PEST tag, seeded cells within a 12 well plate, used a forward transfection, and treated cells with stress inducing drugs for 15 hours whereas I used HCT116WT cells, a reporter plasmid that contained a PEST tag, seeded cells in a 96 well plate, used a reverse transfection, and treated cells over 4 hours. Due to these deviations in procedure, I began my trial and error process with the reporter plasmid, predicting that this factor may have had major effects on the results as a PEST tag directly contributes to the output of luciferase activity. A PEST tag is a protein sequence motif that induces degradation and promotes short-lived proteins after formed (Meyer et al., 2011). Thus, when a luciferase assay is conducted, transcriptional levels may present to be lower than that of a plasmid with no PEST tag. Whereas, a plasmid with no PEST tag measures the activity produced by the accumulation of transcripts overtime. Adding to the discrepancies, transcription is stochastic (Raj & van Oudenaarden, 2008) which may cause differences in results due to the fact that when measured, data is collected on what is occurring at that moment in time. In some cases, the measurement may occur at a time period when transcription is paused, ultimately producing lower luminescent values. Although both are accurate ways to measure transcriptional activity, the use of a reporter plasmid without a PEST tag will be able to present values that incorporate the accumulation of protein over time hence, the indication of significantly larger activity in the pGL4.10 exon WT when directly compared to pGL4.11 (Figure 5) which is most useful in the context of the questions being asked and the experiment being performed.

Other experimental factors were altered as well including the use of a forward transfection rather than a reverse transfection, with results seen to have somewhat higher luminescence values. The experiments were completed by seeding and transfecting HCT116WT cells in 12 well culture plates as opposed to 96 well plates and histidinol treatments were

implemented for 12 hours instead of 4 hours, all of which contribute to the understanding of data received and validation of each result. Considering that results were altered when comparing several components, the use of pGL4.10, a forward transfection, and all other alterations were valuable in determining the parameters that would be beneficial to use with further experiments and for the recapitulation of literature's data.

Using all of the new experimental techniques, the same luciferase reporter assay was repeated with the addition of cell stress induction as an independent variable. Histidinol is a drug that deprives the cell of the essential amino acid histidine, which is cause for amino acid deprivation, a type of cellular stress. The effects of this withdrawal are characterized by the ISR pathway and it is suggested by previous literature that it promotes ATF4 binding to induce transcription of the *ATF3* gene (Pakos-Zebrucka et al., 2016). Adding histidinol into a cell's environment experimentally will be a major indicator of the how the ISR pathway works in regard to ATF4 binding and the effects it has on transcription as a whole. From previously published literature on histidine limitation and effects on the *ATF3* gene, it gives the expectation that activity will increase significantly in wildtype cells treated with histidinol and that transcription is temporally regulated through the binding of ATF4 (Y.-X. Pan et al., 2007). It can be inferred further that mutant versions will have lower activity than wildtype but increased compared to the control environment, H_2O (Fu & Kilberg, 2013).

By the employment of all new parameters, excluding cell type, results from the luciferase reporter assay revealed data similar to that of literature. The exon data matches that of results seen by Fu and Kilberg where the activity of the WT and CARE mutant have no significant difference and the activity of the ATF/CRE mutant was significantly reduced within the control environment. This suggests that the ATF/CRE binding site may be more responsible for basal

levels of transcription as it showed significance when ATF4 was not readily available in the cell's environment. Due to the similar activity of the CARE mutant to the WT in the control environment in addition to its respective name, critical amino acid response element, it is suggested that this binding site may be more responsible for activity when under stress, specifically amino acid withdrawal. However, when comparing the control environment (H_2O) to that of a histidinol (HisOH) treatment, results disclosed data with no significant effect on *ATF3* activity. Wanting to confirm that our stress inducing drug was in fact functioning as it was intended to, two validation experiments were conducted.

A RT-qPCR analysis was performed to decipher the amount of mRNA produced by inducing genes with known effects of HisOH on expression. Both hASNS and hATF3 gene expression are proposed to significantly increase with the introduction of histidinol (Y. Pan et al., 2003). Results of mRNA production on cells that were transfected with WT exon plasmid and treated with H_2O and HisOH, matched predictions by showing an increase in expression normalized to GAPDH when treated with HisOH. This result validated the question of whether or not the histidinol was functioning correctly, however it is not effective in the context of a luciferase reporter assay.

For further validation, a second qPCR analysis was completed comparing hASNS and p21 expression, normalized to GAPDH using an aliquoted sample of newly ordered HisOH. p21 is a gene that is expressed with DNA damage in a p53 dependent manner (Shamloo & Usluer, 2019) thus, it showed no significant effect, as predicted. Conversely, hASNS expression was predicted to increase with the addition of histidinol to the cells' environment (Y. Pan et al., 2003). Results followed these anticipated predictions by significantly increasing expression of hASNS The main cause of concern, histidinol's proper functioning, was validated through this

qPCR analysis in conjunction with the first. Nevertheless, the obstacle holding back results deduced by luciferase reporter assays is yet to be understood but predictions such as cell type may be the factor responsible.

Studying how ATF4 binds to the promoter element to upregulate the *ATF3* gene in response to amino acid deprivation within the integrated stress response pathway, we wanted to see if and how other transcription factors were potentially significant in this context. Collaborating with the lab of Dr. Juan Fuxman-Bass, enhanced yeast-one hybrid (eY1H) data revealed TFAP2A/2B to be a promising contributor as it showed strong DNA-protein interaction or binding of this factor to the *ATF3* promoter region. Having a binding motif encompassed within all promoter constructs, 2kb, ATG, exon, and Kilberg, as well as sequenced between both the CARE and ATF/CRE sites, TFAP2A/2B's role was proposed to be highly significant. However, statistical analysis of the activity produced from the *ATF3* gene through a luciferase reporter assay revealed the comparison between WT and mutant versions, not significant. This conclusion may be due to its dependence on other transcription factors or certain environmental effects. If time allowed, manipulating various experimental conditions and other elements to the response, may have provoked different results. Although TFAP2A/2B did not have significance in this context, perhaps TFAP2A/2B has other impacts or separate actions that we are unaware of at the current time. More research and additional experiments will assist with this conclusion.

Overall, through trial and error, we were able to recapitulate the results produced by previous literature using the exon promoter construct within a control environment. The results incorporating the critical amino acid response element (CARE) suggests that it is responsible for transcriptional activity within a stress induced environment such as being deprived of essential amino acids for survival. To mediate this, it is possible that the cell may upregulate autophagy to

breakdown proteins in order to resupply them if not available within the environment (Fulda et al., 2010). On the other hand, the ATF/CRE binding motif is suggested to be more responsible for basal levels of transcription due to its significance seen within controlled environment luciferase assay results. A remaining conclusion for any data discrepancies between my experiments and those of literature has come down to cell type. This was the only experimental parameter I did not change during my process of determining results. I predict that this could also likely be the cause of histidinol not functioning properly within the context of our reporter assay. Biologically, cell types are different therefore, it is highly probable that the effects of cellular stress and the ISR pathway between them are not the same. Examining published literature, different transcriptional gene activities were found between three various cell types and treatments including oxidative stress, endoplasmic reticulum stress, and DNA damage within breast cancer, HeLa cells, and keratinocytes, respectively (Murray et al., 2004). Due to the COVID-19 pandemic, time did not allow me to investigate and experiment with a new cell type (HepG2 in particular) however, this would be a question I would like to explore in the future. I would also like to look at the various other transcription factors detected by the yeast-one hybrid assay to determine the possibility of significance in regard to *ATF3* induction. It is ultimately reasonable to conclude that there are a multitude of factors and considerations that each play a role within the cell's response to stress through regulatory promoter interaction and activation of the *ATF3* gene to maintain homeostasis and initiate a response through the integrated stress response pathway.

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Appendix

Table 1
Primers ordered and used in various experiments[a]

Primer Name	Sequence[b,c]	Application
Exon	FWD: ctggcctaactggccggtacTTCCGCCTGTGGTCATTGC REV: ccaacagtaccggattgccaCTCACCTCCAGGCTCCGC	Gibson Assembly
First ATG	FWD: ctggcctaactggccggtacTTCCGCCTGTGGTCATTGCGTC REV: ccaacagtaccggattgccaTCCTCTCGGCGCGTGGGG	Gibson Assembly
Kilberg	FWD: ctggcctaactggccggtacAGCTATTAATAGCATTGCGGCAGCC REV: ccaacagtaccggattgccaGAGCTGTGCAGTGCGCGC	Gibson Assembly
ATF/CRE site	FWD: gcagCCTGGGACTGGCAACACG REV: cgcaaTGCTATTAATAGCTTCCCGGG	Site Directed Mutagenesis
CARE site	FWD: cctgcgtataGGGTGATGCAACGCTCTCC REV: ctcttcgcttGCGGCGCGGTCGTTTACT	Site Directed Mutagenesis
TFAP2A/2B site I	FWD: accGCTATAAAAGGGGTGATGCAACGCTC REV: caccCTGGCGGCGCGGTCGTTT	Site Directed Mutagenesis
ATF4 expression	FWD: CAGACGGTGAACCCAATTGG REV: CAACCTGGTCGGGTTTTGTT	qPCR
P21 expression	FWD: AGCGATGGAACTTCGACTTTG REV: CGAAGTCACCCTCCAGTGGT	qPCR
hASNS expression	FWD: GGTACATCCCGACAGTGATGATATT REV: spanningCCTGGACACTATGAAGTTTTGGATT	qPCR
hGAPDH expression	FWD: CCAGGTGGTCTCCTCTGACTTC REV: GTGGTCGTTGAGGGCAATG	qPCR

[a] All primers ordered from Integrated DNA Technologies (IDT)
[b] FWD: forward primer; REV: reverse primer
[c] All primer sequences run 5'- 3'

Table 2

Enhanced yeast-one hybrid data – ATF3 promoter [a, b]

Bait	TF	Strength 1	Strength 2	Sum
ATF promoter	TFAP2A	2	1	3
ATF promoter	TFAP2B	3	2	5

[a] Created and adapted from the full yeast-one hybrid data set

[b] Data was obtained from the lab of Juan Fuxmann-Bass of Boston University