MOLECULAR AND CHEMICAL MODULATION OF NRF2 FOR DISEASE INTERVENTION

By

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Dedication

This work is dedicated to the memory of
Wilbur F. 'Bill' Harder, who, at 89, past too soon

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List of Abbreviations

6-OHDA	6-hydroxydopamine
Aβ	Amyloid beta
AD	Alzheimer's disease
AhR	Aryl hydrocarbon receptor
AKR1C1	aldo-keto reductase family 1 member C1
ALS	Amyotrophic lateral sclerosis
AOM/DSS	Azoxymethane/dextran sodium sulfate
AP-1	activator protein-1
APP	amyloid precursor protein
ARE	antioxidant response element
ARNT	AhR nuclear translocator
ATF6	activating transcription factor 6
ATRA	all-trans retinoic acid
β-TrCP	β-transducin repeat containing family
BACH	BTB and CNC homology
BHA	butylated hydroxyanisole
BRCA1	breast cancer 1
CA	cinnamaldehyde
CBP; p300	CREB-binding protein
CDDO	Bardoxolone
CHD6	chromodomain helicase DNA-binding protein 6

List of Abbreviations- *Continued*

ChIP	chromatin immunoprecipitation
CNC-bZIP	cap'n'collar basic-region leucine zipper
CUL	Cullin
Cys	cysteine
DEPTOR	DEP domain containing MTOR-interacting protein
DMF	dimethylfumarate
DPP3	protein dipeptidyl peptidase 3
DUB	deubiquitylating enzyme
E2	Estrogen
ECH	erythroid cell-derived protein with CNC homology
EHR	Extended homology region
eIF2α	eukaryotic initiation factor 2 alpha
EPH1	Epoxide hydrolase
ER	endoplasmic reticulum
ERα	Estrogen receptor α
ERAD	Endoplasmic reticulum associated degradation
ERRβ	Estrogen-related receptor β
FDA	Food and Drug Administration
FH	Fumarate hydratase
GCN2	General control nonderepressible 2
GR	Glucocorticoid receptor
GSK-3	glycogen synthase kinase 3

List of Abbreviations- *Continued*

GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HD	Huntington's Disease
HRD1	HMG-CoA reductase degradation protein 1
IRE1	Inositol-requiring enzyme 1
KEAP1	Kelch-like ECH associated protein 1
LC3	Microtubule-associated proteins 1A/1B light chain 3A
LIR	LC3 interacting region
mTORC1	mammalian target of rapamycin complex 1
MLST8	mammalian lethal with SEC13 protein 8
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRP	Multidrug resistance protein
MS	multiple sclerosis
NCI	National Cancer Institute
Neh	NRF2-ECH homology
NF-κB	Nuclear factor-κB
NLS	Nuclear localization sequence
NRF2	Nuclear factor-erythroid 2 p45-related factor 2
NSCLC	Non-small cell lung cancer
NTD	N-terminal domain
NQO1	NAD(P)H quinone oxidoreductase

List of Abbreviations- *Continued*

$^{\cdot}O_2^-$	Superoxide anion
$^{\cdot}OH$	Hydroxyl radical
PAH	Polycyclic aromatic hydrocarbon
PALB2	Partner and localizer of BRCA2
PD	Parkinson’s disease
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
poly-Q	Polygultamine
PPARγ	Peroxisome proliferator-activated receptor gamma
ppb	parts per billion
PPP	Pentose phosphate pathway
PRCC	Papillary renal cell carcinoma
PRAS	Proline-rich AKT1 substrate
RAPTOR	Regulatory-associated protein of mTOR
RAR	Retinoid acid receptor
RBX1	RING-Box 1
RING	Really Interesting New Gene
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SKP1	S-phase kinase-associated protein 1
sMAF	small musculoaponeurotic fibrosarcoma
TRE	TPA-responsive element
TRIM21	Tripartite motif-containing protein 21

List of Abbreviations- *Continued*

UBA	ubiquitin-associated domain
UPS	ubiquitin proteasome system
VLDL	Very low density lipoprotein
WTX	Wilms Tumor Gene on X Chromosome
XRE	Xenobiotic response element

Abstract

Cells are frequently exposed to endogenous and environmental stressors that pose a threat to homeostatic cellular conditions. In response to such stress, the cell activates an adaptive antioxidant response, which is regulated by the transcription factor NRF2 to mitigate the harmful effects of electrophilic or oxidative species. Pharmacological activation of NRF2 has shown to be effective for preventing the initiation and promotion of cancer, neurodegenerative diseases, and other chronic illnesses, leading to the search for more specific molecules that activate this pathway. Intriguingly, because it is a pro-survival factor, NRF2 is frequently found to be deregulated in many cancer types that are resistant to chemotherapy, calling for the use of NRF2 inhibitors as an adjuvant therapy to enhance the effects of primary chemotherapeutic regimens. In this dissertation, detailed molecular studies have identified a new mechanism by which NRF2 can be aberrantly up-regulated in Type 1 endometrial carcinoma. Additionally, key mechanistic approaches were undertaken to understand the biological consequence of the first NRF2 inhibitor, brusatol. Furthermore, strategic approaches to identify novel chemical modulators of the NRF2 pathway were used, using natural products as a primary source. Assessment of the mechanism of action of biological activity for chemical modulators of the NRF2 pathway was of extreme interest, and rational approaches for the use of these compounds for disease intervention based on disease context will be discussed.

Chapter 1

Introduction

1.1 The antioxidant response to stress mediated by NF-E2 p45-related factor-2 (NRF2)

1.1.1 The cellular response to oxidative and electrophilic stress

The evolutionary origin of aerobic metabolism, dating back to approximately 2.9 billion years ago, was a momentous shift for the biochemical mechanisms of life [1]. In the presence of atmospheric oxygen, biochemical reactions became more energetically favorable, allowing eukaryotic organisms to evolve at a greater rate. With the continued use of oxygen in biochemical reactions, cells mounted a defensive response in order to protect themselves from the harmful byproducts of enzymatic reactions utilizing oxygen. These harmful byproducts, known as reactive oxygen species (ROS), include the superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) are detrimental to cells by means of altering native protein structure and function, which greatly impact cellular function [2]. While most ROS species cause harm, cells have made use of certain ROS, as some have been identified as key signaling molecules. Despite the importance of these signaling events caused by ROS, when the cell's capacity to keep ROS in check is overwhelmed, this leads to oxidative stress, a phenomenon that has been shown to be a pathological hallmark contributing to the ageing process, neurodegenerative diseases, and cancer.

ROS can be presented from either endogenous or exogenous sources. The major source of endogenous ROS occurs from organelles such as the mitochondria, endoplasmic reticulum (ER), or peroxisomes, but other enzymatic reactions catalyzed by cytochrome P450 enzymes, oxidases, or nitric oxide synthase also contribute to the basal production of ROS [3]. Moreover, exogenous ROS typically occurs from environmental factors such as UV exposure, ionizing radiation, and electrophilic xenobiotics greatly add to the growing burden of ROS levels in the cell, and it is with these, that the cell ultimately relies on the adapted defense response to mitigate the damaging effects caused by oxidative stress [4].

In addition to the formation of ROS, nature has provided a plethora of chemically diverse compounds that eukaryotes are exposed to that cause stress. Many of these molecules are electrophilc, in that they are electron deficient, and accept electrons from nucleophiles. In general, many biomolecules such as nucleotides and proteins contain nucleophilic centers and are susceptible to adduction by reactive electrophiles. Most notably, thiol groups (-SH) present in the amino acid cysteine, are strong nucleophiles and have since been regarded as key residues for sensing the oxidative environment [5]. While the majority of exposure to electrophiles comes from xenobiotics, such as toxins, pollutants, or plant-derived secondary metabolites, the cell also produces endogenous electrophiles in the form of reactive aldehydes and epoxides that occur from primary metabolism of xenobiotics, or products from amino acid, lipid, and carbohydrate oxidation. Indeed, these electrophilic species play a role in cellular

signaling due to the fact that they are capable of adduction to DNA and proteins. Since the realization that electrophiles can modulate defense response pathways, they have been used as mild stressors in order to prevent disease onset, providing the nomenclature of 'chemopreventive compounds' [6]. As long as chemopreventive compounds are administered at small doses, they can exert long-term benefit by promoting transcriptional responses that offers immediate neutralization of ROS and reactive species as well as the promotion of factors that can prevent cumulative damage.

1.1.2 The NRF2 transcription factor

NRF2 (Nuclear factor-erythroid 2 p45-related factor 2) was first identified by Moi *et.al.* in 1994 in their search for proteins that can bind to an extended activator protein-1 (AP-1) sequence [7]. It was established that the identified protein contained a cap'n'collar basic-region leucine zipper (CNC-bZIP) domain at its C-terminus and was thus recognized as the third member of the CNC-bZIP family of transcription factors, where it was then named NRF2. Additional members of the CNC-bZIP family include Nuclear factor-erythroid 2 p45 (NF-E2 p45), NRF1, NRF3, BTB and CNC homology 1 (BACH1), and BACH2. NF-E2 p45, the founding member of the family, is limited to hematopoietic cells, and has been shown to be a crucial factor for the generation of circulating platelets [8]. Because of this, NF-E2 -/- mice die early due to hemorrhage. Interestingly, NF-E2 p45 and NRF2 differ from NRF1 and NRF3 in that they are soluble factors. On the other hand, NRF1 and NRF3 contain an N-terminal domain (NTD) that

localizes them into the membrane of the ER [9,10]. When in the basal state, they are glycosylated and await activation in the form of a cleavage event from the ER. Once cleaved, they become soluble and can exert transcriptional function. NRF1 knockout mice are embryonic lethal, owing to the fact that its physiological role is related to development [11]. Additionally, NRF1 has been shown to regulate the basal and inducible expression of subunits that form the proteasome, implicating NRF1 as an important mediator of protein quality control [12]. Unlike NRF1, genetic ablation of NRF2 is tolerated in mice, although they are highly sensitive to xenobiotic stress and have a diminished antioxidant response causing them to be susceptible to chemical-induced carcinogenesis [13]. However, one interesting feature of NRF2 -/- mice is that they display decolorized teeth due to impaired iron transport [14]. Finally, NRF3 is a factor that is not ubiquitously expressed, but is present in B cells, monocytes, and the placenta. NRF3 knockout mice also develop normally and feature no obvious phenotypic changes to wildtype counterparts [15]. Despite the lack of knowledge on NRF3, the fact that its cleavage from the ER by certain stimuli suggests that there is potentially a stress-responsive role despite its ability to repress antioxidant gene expression [16].

The orthologous chicken NRF2 was first cloned by Masayuki Yamamoto and colleagues, where they described it as erythroid cell-derived protein with CNC homology (ECH) [17]. Seven separate regions of NRF2 are classified as NRF2-ECH homology (Neh) domains and all have individual functions.

The Neh1 domain is common to all other CNC-bZIP transcription factors and works to promote heterodimerization with other bZIP transcription factors, as well as to facilitate binding to DNA. Additionally, the Neh1 domain also contains a nuclear localization sequence (NLS), allowing NRF2 to be imported into the nucleus by karyopherin-mediated transport. The Neh2 domain is located at the N-terminus of NRF2 and plays an important role in its regulation. The Neh2 domain contains two critical binding motifs, 'DLG' and 'ETGE', that are required for interaction with Kelch-like ECH associated protein 1 (KEAP1), which functions as the negative regulator of NRF2 [18,19]. The Neh3 domain is located at the end of the C-terminus and allows the recruitment of a cofactor called chromodomain helicase DNA-binding protein 6 (CHD6), and also has been shown to take part in transactivation function [20]. Neh4 and Neh5 are two domains that are known as transactivation domains and enable binding to CREB-binding protein (CBP; p300) [21]. CBP has acetyltransferase activity and promotes acetylation of histones around regions of inactive transcription. Upon acetylation of key lysine and arginine residues in histones, the positive charge of the histones is neutralized, causing the surrounding negatively-charged DNA to lose its affinity for the histones, allowing for the recruitment of transcription factors, as well as RNA polymerase II. The Neh6 domain of NRF2 has been reported to contain additional 'redox-insensitive' degradation motifs, which contributes to NRF2 stability in times of cellular stress other than redox imbalance [22,23]. Finally, the Neh7 domain has been recently described to be a novel binding site for the retinoid X receptor alpha (RXRα), which negatively

regulates NRF2 activity [24]. This occurs by preventing the recruitment of coactivators to the Neh4 and Neh5 domains of NRF2. Due to this occurrence, the ligand for RXRα, bexarotene, has been shown to inhibit the expression of NRF2-regulated genes [25]. A schematic of the NRF2 protein with all 7 Neh domains is shown in Figure 1.1.

Based on sequence homology to other CNC transcription factors, NF-E2 p45, NRF1, and NRF3 contain Neh-like domains, similar to the ones described above. Interestingly, NRF1 contains to the two vital peptide sequences, 'DLG' and 'ETGE', however, NRF1 is not degraded in a KEAP1-dependent manner. KEAP1 can bind to the Neh2-like domain, but minor differences in domain structure likely contribute to its stability, such as the absence of 6 key lysine residues.

As previously mentioned, the Neh1 domain of NRF2 harbors a conserved CNC-bZIP region that is responsible for heterodimerization between other bZIP factors. In order for proper binding to DNA and subsequent activation, NRF2 must heterodimerize with small musculoaponeurotic fibrosarcoma (sMAF) proteins. sMAF proteins are bZIP transcription factors that lack a transactivation domain, disallowing facilitation of transcriptional activation on their own [26]. Importantly, NRF2 cannot bind to the DNA as a monomer, making sMAF proteins essential factors required for NRF2, and other CNC-bZIP transcription factors, to perform its function [27].

Three kinds of sMAF proteins, MAFF, MAFG, and MAFK, are part of the bZIP type of transcription factors. Their structure is simple, in that they contain an

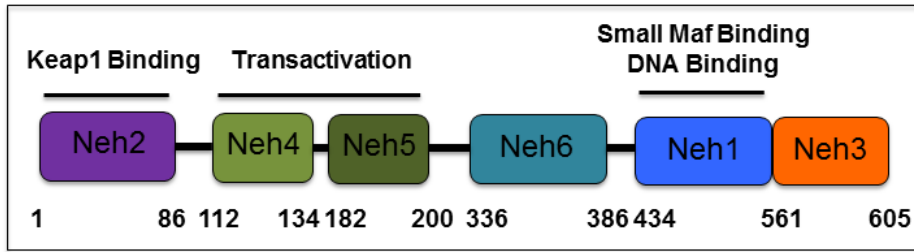

Figure 1.1 The domain architecture of NRF2. Known functions of imperative individual domains are represented above.

extended homology region (EHR), a basic region, and leucine zipper domain. The EHR is conserved between large and small MAF proteins, and functions to provide stable binding to DNA, once already bound. The basic region of sMAFs contains the DNA-binding motif as well as the NLS, in order for nuclear import, despite the fact that sMAFs are primarily located inside the nucleus. Lastly, the leucine zipper domain is required for dimer formation between other bZIP transcription factors. sMAFs have been shown to homodimerize with each other, but because they lack a transactivation domain, sMAF homodimers have been shown to repress NRF2-mediated gene transcription [28]. Because of the similarity between sMAF proteins, it has been assumed that any combination of sMAFs can form homodimers. sMAF proteins have also been shown to bind to BACH1 and BACH2, which, too, are incapable of binding to DNA and acting as monomers [29].

1.1.3 The antioxidant response element

In order for cells to protect themselves against harmful ROS and electrophilic species, they call upon an inducible defensive response that can sense, respond to, and combat the damaging effects before mutagenesis or cell death may occur. Once a cell senses an imbalance in the oxidative environment, the NRF2-mediated transcriptional program is the primary response to correct the imbalance. This response, collectively referred to as the antioxidant response, is accountable for the up-regulation of a battery of genes that help to restore redox homeostasis at the transcriptional level.

The bulk of the antioxidant response directed by NRF2 can be broken down into 3 subclasses of factors that help to restore redox homeostasis. These include (i) intracellular redox balancing enzymes, (ii) phase II detoxifying enzymes, and (iii) transporter proteins. Despite the integral role these proteins play in restoring redox balance, emerging evidence suggests NRF2 facilitates the regulation of additional genes involved in other survival-related processes such as the removal of damaged cellular components, DNA damage repair, and cell survival (anti-apoptosis and proliferation). On top of that, NRF2 has been shown to influence the metabolism of lipids, nucleotides, amino acids, and carbohydrates, which will be discussed in more detail in a later section. One particular feature of all of the genes regulated by NRF2 is the fact that they contain a *cis*-acting regulatory element: the antioxidant response element (ARE). Before the ARE was identified, common knowledge thought that the Aryl hydrocarbon receptor (AhR) was the principle regulator of a cell defense response system against planar hydrocarbons and environmental toxins because of the fact that the AhR could induce numerous cytochrome P450 enzymes. It wasn't until the observations by Talalay and colleagues that realized a chemoprotective response can be induced by electrophilic compounds by an AhR-independent mechanism [30]. Further work on this topic went on to identify an ARE in the promoter region of the rat glutathione-*S*-transferase Ya (GST-Ya) subunit gene, followed closely by the identification of an ARE in the promoter region of the rat and human NAD(P)H quinone oxidoreductase (NQO1) gene [31-33]. Characterization through mutation and deletion analysis revealed a

consensus sequence of 5'-GTGACNNNGC-3' was required for transcriptional activation by phenolic antioxidants and planar aromatic compounds such as β-naphthoflavone and 3-methylcholanthrene [34]. Moreover, it was found that the ARE was also responsive to H_2O_2 and other phenolic antioxidants that were capable of undergoing redox cycling, indicating that the ARE was a part of a signal transduction pathway that was responsive to oxidative stress. In 1997, Ken Itoh and colleagues published critical data indicating that the NRF2 transcription factor, along with a small MAF binding partner, recognized this ARE to activate gene transcription [35]. Further manipulation of the ARE uncovered an extended sequence, 5'-TMANNR**TGAYNNNGC**RWWWW-3', and suggested it was a better representation of a consensus ARE sequence [36]. Here, the 'core' sequence can be seen in the extended sequence as bold letters and '**Y'** indicates either C or T and 'W' indicates A or T. Six years later, the consensus sequence was changed once again upon identification that the newly identified AREs contained guanines at position 4 of the 'core' sequence, instead of cytosines or thymines [37]. Also, point mutations concluded that the 3' tetra-nucleotide motif (WWWW) was not required for gene induction. As a result, the consensus motif was reduced down to a 16 base pair sequence: 5'- TMANNR**TGABNNNGC**R-3', where **B** indicates either C, G, or T, and the 'core' sequence is highlighted in bold lettering. These amendments to the ARE sequence led to the finding of additional NRF2 target genes and showed that there is promiscuity associated with ARE sequence structure in the context of regulation by NRF2. Since then, over 200 genes have been found to be regulated by NRF2. Critically, *in silico* analysis of

identifying ARE-like sequences is an inadequate method to identify novel NRF2 target genes, as these sequences are quite common. Importantly, functional validation of the ARE must be performed in cell-based assays in order to confirm the identification of novel NRF2-regulated genes. In certain cases, it has been shown that the 'core' ARE sequence is insufficient to promote *in vitro* gene expression in cell-based assays, providing evidence that in certain contexts, the 16 base pair sequence may be required to drive gene expression. However, certain cell-based systems may be flawed in that some genes might be regulated by indirect effects because NRF2 has been shown to regulate other transcription factors.

1.2 Molecular mechanisms of NRF2 regulation

1.2.1 NRF2 is regulated by the ubiquitin proteasome system

Once proteins are synthesized, they must adopt their three-dimensional structure in order for the protein to have appropriate function. Occasionally, certain mistakes can occur that lead to the misfolding of a protein, which can cause stress to the cell. In order to avoid the stress associated with misfolded proteins, cells have put in place certain protein quality control mechanisms to preserve the functionality of the proteome. For the most part, these quality control mechanisms include chaperone systems in which chaperones can shuttle and facilitate the correct folding of the misfolded protein. If protein quality control mechanisms fail to maintain integrity, and misfolded proteins accumulate causing

disarray through protein aggregation or initiation of aberrant signaling, the cell is said to be under proteotoxic stress, which can profoundly affect cell viability. In order to defend against proteotoxic stress, the cell uses the ubiquitin proteasome system (UPS) as the primary cellular degradation pathway to dispose of unwanted protein products that have overwhelmed quality control components.

The key signal used by the cell to target proteins for degradation is ubiquitin. Ubiquitin is a small protein, containing 76 amino acids, that can be covalently attached to lysine residues on target proteins by E3 ubiquitin ligases present in the cell [38]. Because many proteins are targeted with ubiquitin for different functions, there are numerous E3 ubiquitin ligases that confer substrate specificity to their target proteins. Ubiquitin conjugation occurs via a series of thioesterification reactions, ultimately leading to the covalent addition of one ubiquitin molecule to a lysine residue to the target protein. This is known as mono-ubiquitylation has been postulated to govern signaling events such as membrane trafficking and endocytosis. More frequently, a subsequent ubiquitylation event will occur on a lysine residue within the single ubiquitin molecule, which is called poly-ubiquitylation. In this event, because ubiquitin contains 7 lysines, poly-ubiquitylation can have multiple downstream effects. For example, if a poly-ubiquitin chain is lysine-48 linked, the resulting chain will target the ubiquitylated protein for degradation by the 26S proteasome. Conversely, if a ubiquitin chain in lysine-63 linked, the resulting chain typically engages the protein in cell signaling events that mediate signal transduction or DNA-repair mechanisms.

In an unstressed state, the NRF2 cell defense response is not needed. Under basal conditions, cells mediate the constant degradation of NRF2 through the UPS, keeping NRF2 protein levels low and preventing transcription of unneeded genes. This regulation occurs through KEAP1, an adaptor protein of a Cullin3 (CUL3)-RING (Really Interesting New Gene) -Box 1 (RBX1) containing E3 ubiquitin ligase complex [39,40]. Dimeric KEAP1 is responsible for recognition of NRF2 through two key motifs in the Neh2 domain of NRF2 located in its amino terminus. The Kelch domain of each KEAP1 binds to the 'DLG' and 'ETGE' motifs, recognized as the low affinity and high affinity binding sites, respectively [19,41]. NRF2 is subsequently poly-ubiquitylated at seven key lysine residues within the Neh2 domain, condemning NRF2 to proteasomal destruction, depicted by red arrows in Figure 1.2 [40].

When the cell is exposed to electrophiles or ROS, the NRF2-mediated cytoprotective response is activated. Critical cysteine residues in KEAP1, especially Cys151, act as sensors of these cellular insults, and become covalently modified by electrophilic species or ROS [42]. Additional cysteine residues in KEAP1 may also be modified by electrophilic species [43,44]. Such modifications induce a conformational change in KEAP1, resulting in a disruption of the low affinity interaction between the Kelch domain and the DLG-motif, which leads to impaired ubiquitylation of NRF2, blocking UPS-mediated degradation and thus increasing NRF2 protein levels [45]. As newly synthesized NRF2 is continuously made, there are no longer sufficient KEAP1 molecules available to accommodate for the rising protein levels of NRF2. This cytosolic NRF2 is then

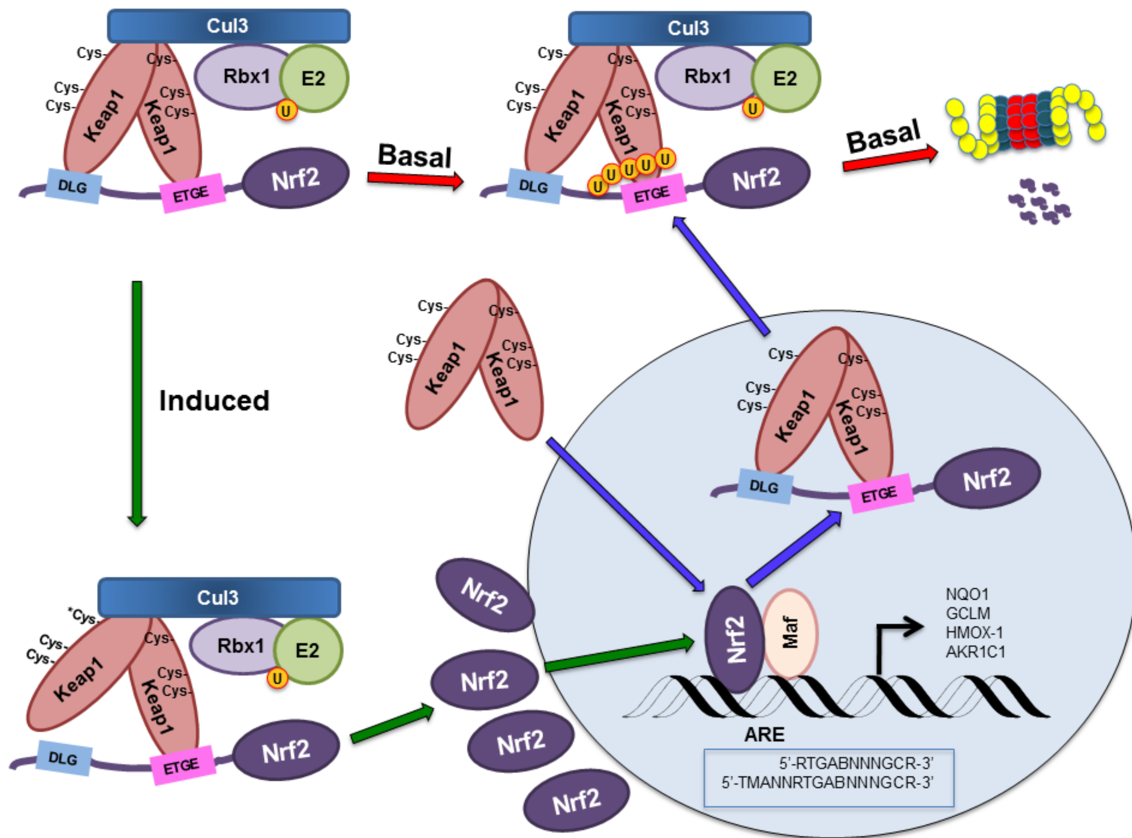

Figure 1.2 The canonical, KEAP1-CUL3-RBX1-mediated NRF2 regulatory pathway. Upon cellular insult, cysteine residues in KEAP1 are modified and the activity of the E3 ubiquitin ligase is suppressed. NRF2 levels rise and NRF2 enters the nucleus, where it dimerizes with MAF and transcriptionally up-regulates ARE-containing genes.

free to translocate into the nucleus and transcriptionally activate its target genes. After induction, when homeostasis is restored, KEAP1 translocates into the nucleus in a KPNA6-dependent manner to facilitate nuclear export of NRF2 and rejoin the KEAP1-mediated ubiquitylation and degradation machinery in the cytosol [46,47]. These events turn off the transcription of the NRF2 target genes and restore the low basal level of NRF2 until the next cellular insult is presented.

Although KEAP1 has been revealed as the primary redox-sensitive regulator of NRF2 through reactive cysteine residues (Figure 1.3A), a redox-insensitive degron within the Neh6 domain of NRF2 was reported in 2004 [22]. Subsequently, it was found that the Neh6 domain of mouse NRF2 contains a group of serine residues that can be phosphorylated by glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase. This phosphorylation event in the Neh6 domain creates a phosphorylated destruction motif (phosphodegron), which can then be recognized by the β-TrCP-SKP1-CUL1-RBX1 E3 ubiquitin ligase complex (Figure 1.3B) [48]. This E3 ligase complex ubiquitylates NRF2 and sends it to the proteasome for destruction. Further characterization of the Neh6 domain found two distinct motifs recognized by β-TrCP, 'DSAPGS' and 'DSGIS', the latter containing two serines which can be directly phosphorylated by GSK-3β [23]. Interestingly, the conditions that favor the GSK-3β/β-TrCP E3 ubiquitin ligase over the KEAP1-CUL3-RBX1 E3 ligase in controlling NRF2 remains to be determined.

More recently, our lab discovered that the NRF2-mediated protective response was suppressed during liver cirrhosis [49]. Liver cirrhosis is a

pathogenic state typically caused by chronic alcohol consumption or viral hepatitis infection, resulting in a profound scarring of the liver. Because hepatocytes are stressed during this disease state, the fact that NRF2 was down-regulated was a surprising result because KEAP1 was predicted to be inactivated by the high levels of ROS that occurs in cirrhotic livers, potentially leading to active NRF2 signaling by the canonical mechanism. In addition to ROS production, ER stress has been implicated during the pathogenesis of liver cirrhosis. ER stress occurs when misfolded proteins accumulate in the lumen of the ER. In order to prevent proteotoxic stress, a phenomenon that occurs when the UPS or other degradation pathways are inhibited, the cell initiates the UPR in order to reduce the burden of an accumulation of misfolded proteins, which can aggregate and prohibit the proper function of various cellular activities. Three sensors, IRE1, PERK, and ATF6, located on the ER membrane detect the accumulation of misfolded proteins and relays signaling cascades, resulting in induction of heat-shock proteins, autophagy factors, proteasomal subunits, and apoptotic factors, as well as a decrease of other secretory proteins until homeostasis is reached [50]. If the UPR system fails to correct the insult, apoptotic programs are the last to be activated to see that the cell undergoes coordinated cell death [51].

To understand the decrease of NRF2 in cirrhotic livers, our lab investigated the crosstalk between the ER stress pathways and the NRF2-mediated antioxidant stress pathway. It was found that decreased NRF2 levels correlated with activation of the IRE1 arm of the UPR. ER stress is known to

release the association between IRE1 and GRP78 (a chaperone also known as BiP, part of the HSP70 family), enabling free IRE1 to homodimerize and actively splice XBP1 mRNA into a mature mRNA encoding XBP1s, a transcription factor. HRD1 is a XBP1s target gene that is up-regulated upon activation of the IRE1-XBP1 signaling pathway. Based on the fact that the protein level of NRF2 was decreased in an HRD1 dependent manner whenever the IRE1 arm is activated, we identified HRD1 as a novel E3 ubiquitin ligase (Figure 1.3C) [49]. This discovery has important implications for the treatment and protection of cirrhotic livers.

1.2.2 '(E/S)TGE' containing proteins misregulate NRF2

In addition to regulation through the UPS, NRF2 is subjected to positive regulation by other proteins through disruption of the NRF2-KEAP1 interaction. A recent study identified numerous proteins with motifs identical (or similar) to the 'ETGE' motif of NRF2, which can compete with NRF2 for KEAP1 binding, thus stabilizing NRF2 [52]. Some examples of '(E/S)TGE' containing proteins include PALB2, WTX, and DPP3, but p62/SQSTM1 (henceforth p62) is perhaps the most recognized positive regulator of NRF2. p62, a protein containing the 'STGE' motif, is a scaffold protein recruited into forming autophagosomes to shuttle cargo proteins destined for degradation through the autophagy-lysosome pathway, the cell's bulk degradation process. Autophagy dysregulation leads to accumulation of autophagosomes, where p62 captures KEAP1 through binding with the 'STGE' motif, resulting in inactivation of KEAP1-mediated NRF2

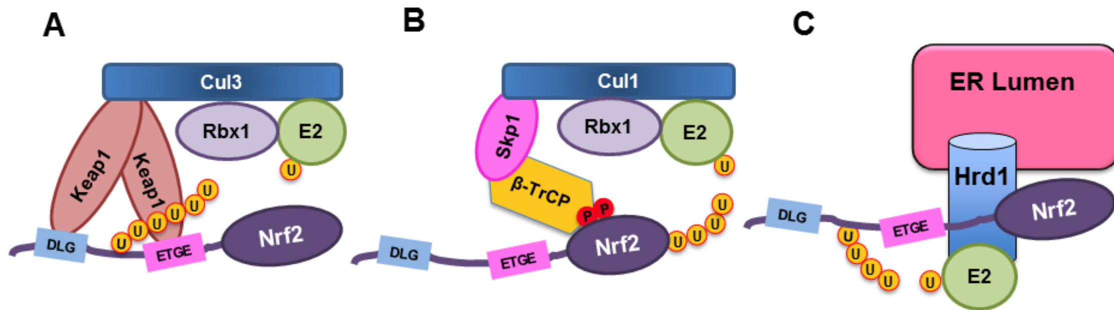

Figure 1.3 The three E3 ubiquitin ligases for NRF2. (A) KEAP1-CUL3-RBX1 E3 ubiquitin ligase binds to the 'DLG' and 'ETGE' motifs within the Neh2 domain of NRF2 and promotes polyubiquitylation on key lysine residues in between the two motifs. (B) β-TrCP-SKP1-CUL1-RBX1 E3 ubiquitin ligase complex recognizes redox-insensitive degrons within the Neh6 domain of NRF2. In certain cases, the 'DSGIS' motif can be phosphorylated by GSK-3β, creating a phosphodegron. (C) The HRD1 E3 ubiquitin ligase is anchored to the ER lumen and promotes polyubiquitylation of NRF2 during liver cirrhosis.

ubiquitylation and thus activation of NRF2 [53]. It is envisioned that activation of NRF2 by this p62-dependent, but KEAP1 cysteine-independent (non-canonical) mechanism takes a much longer time to attenuate, which leads to heightened cell survival and potential cellular transformation due not only because of the persistent expression of NRF2 target genes while NRF2 levels remain elevated, but also because of the metabolic changes the cell might undergo as a result of prolonged inhibition of autophagy, which deprives the cell of much-needed degradation products for biosynthetic pathways.

Recent advances regarding p62-mediated activation of NRF2 have identified phosphorylation of serine 351 (corresponding to serine 349 in humans) within the ‘STGE’ motif enhances the interaction between p62 and KEAP1 [54]. Interestingly, while autophagic dysfunction has been shown to amass p62 protein levels and trigger prolonged NRF2 activation, initiation of autophagy by mammalian target of rapamycin complex 1 (mTORC1) can also ultimately up-regulate NRF2 protein levels. mTORC1, a protein complex containing mammalian target of rapamycin (mTOR), regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (MLST8), and other non-core components PRAS40 and DEPTOR, functions to sense the cell’s nutrient, energy, and redox status [55]. mTORC1 is a master regulator of protein synthesis as well as the initiation of autophagy and recently has been described to phosphorylate serine 351 in p62, thereby enhancing the interaction between p62 and KEAP1. Indeed, when autophagy is turned on in an mTORC1-dependent manner, phosphorylated p62 can sequester KEAP1 into forming

autophagosomes and degrade KEAP1 when complete autophagosomes fuse with lysosomes to degrade the cargo. Additionally, p62 has been shown to be a direct target of NRF2, instigating a feed-forward mechanism of NRF2 up-regulation [56].

p62 is a stress responsive protein which can facilitate the shuttling of polyubiquitylated cargo destined for degradation by autophagy, if the proteasome becomes overwhelmed and damaged proteins and organelles accumulate. The UBA domain in p62 is required for binding to polyubiquitin chains on proteins to mediate the recruitment into autophagosomes [57]. Moreover, the LIR domain is responsible for binding to LC3 (ATG8), which is present on the outer membrane of the autophagosome [58]. While p62 acts as the intermediate between cargo and autophagy, p62 also contains the inherent ability to oligomerize to form large bulk products, allowing the recruitment of more cargo. Thus, the forming autophagosome can encapsulate the aggregates and degrade them once fusion with lysosomes occurs. Intriguingly, lysine 7 in p62 has been identified to be ubiquitylated by the E3 ligase TRIM21 [59]. This ubiquitylation event prohibits the oligomerization of p62 with itself, as well as the sequestration of KEAP1, indicating that TRIM21 can negatively regulate NRF2.

It was demonstrated that the human carcinogen arsenic, widely distributed in the ground water presenting a global health issue that affects approximately 200 million people, activates NRF2 through the non-canonical mechanism, resulting in prolonged NRF2 activation and possibly explaining the carcinogenic nature of arsenic [60]. While many reports aimed at determining the detrimental

consequences of arsenic exposure use micro Molar concentrations of arsenic, our group found that autophagic dysregulation by arsenic occurs at environmentally relevant concentrations in the parts per billion (ppb) range (about 500nM), potentially recognizing a specific attribute of arsenic toxicity and carcinogenicity.

Since the recognition that 'STGE'-containing p62 can augment NRF2-mediated signaling, albeit in a non-canonical manner, this prompted the search for additional proteins with 'E/STGE' motifs, which could potentially associate other signaling pathways with NRF2. Partner and localizer of BRCA2 (PALB2) was characterized to contain an 'ETGE' motif, and was subsequently found to bind to KEAP1 in such a manner [61]. Unlike autophagic dysregulation, which drastically increases p62 protein levels, PALB2 protein levels were not found to be altered, indicating that PALB2 expression in the cell contributes to the basal expression level of NRF2. Interestingly, because PALB2 in a nuclear protein, it was proven that PALB2 binds to KEAP1 in the nucleus, thereby preventing the post-induction repression of NRF2 and identifying a new mechanism of NRF2 regulation in the nucleus.

A second protein that was found to contain an 'ETGE' motif is Wilms Tumor Gene on X Chromosome (WTX). WTX is well documented as a tumor suppressor protein that is frequently lost or mutated in up to 30% of cases of Wilms Tumor, which is the most common pediatric kidney cancer. The established function of WTX promotes the ubiquitylation and degradation of β-catenin by interacting with the β-transducin repeat containing family (β-TrCP) of

E3 ubiquitin ligases. However, in this particular study, the authors identified WTX was able to bind to KEAP1 in an ‘ETGE’-dependent manner and thus prohibit the ubiquitylation of NRF2 and up-regulate NRF2-driven gene expression [62]. This observation implicates WTX in biological processes beyond regulation of β-catenin and suggests that WTX may play a role in diseases other than Wilms Tumor.

With the identification that these aforementioned ‘ETGE’ containing proteins can directly modulate NRF2 protein levels as well as NRF2-mediated transcription, researchers set out to identify even more KEAP1 interacting partners through proteomic analysis. Hast *et al* (2013) defined the KEAP1 protein interaction network through affinity purification and shotgun mass spectrometry [52]. Of the 42 high-confidence KEAP1 interacting partners identified, 17 of them harbored an ‘ETGE’ motif, ‘STGE’ motif, or both, supporting the notion that the presence of an ‘ETGE’ motif, or a similar one, provides competition for NRF2 biding to KEAP1. Of particular interest was the protein dipeptidyl peptidase 3 (DPP3), which was found to not only bind to KEAP1 in an ‘ETGE’-dependent manner, but also is overexpressed in squamous cell carcinoma. Moreover, augmented DPP3 positively correlated with high NRF2 expression.

1.2.3 NRF2 is induced at the transcriptional level

One pivotal feature of the NRF2 pathway regarding its molecular regulation is the fact that the gene encoding *NRF2* is constantly transcribed, translated, and destroyed, under ideal redox cellular conditions. This lends merit to the

importance of the NRF2 antioxidant pathway because the cell invests a large amount of energy to persistently transcribe and translate a protein that will succumb to degradation in a matter of minutes. However, in times of electrophilic and oxidative stress, the importance of timely activation of NRF2 has been well documented, therefore, the cell must continue to synthesize this critical transcription factor in order to be able to defend itself against harmful chemical species. Thus, basal transcription of *NRF2* has been shown to be regulated by other transcription factors to ensure that the NRF2 protein can accumulate if KEAP1 were to become modified by reactive species.

Early studies indicated that the NRF2 promoter region contains multiple xenobiotic response element (XRE)-like sequences, which allow for it to be regulated by the AhR [63]. The AhR, when activated, heterodimerizes with AhR nuclear translocator (ARNT) to induce expression of genes containing XREs. Therefore, polycyclic aromatic hydrocarbons (PAHs) can indirectly activate NRF2 target genes that otherwise do not harbor XREs in their promoter regions, through up-regulation of *NRF2* itself [64]. In addition to XREs, the NRF2 promoter region also contains 2 ARE-like sequences, which potentially allow for positive regulation by NRF2 in a feed-forward mechanism of activation, thereby prolonging activation of NRF2 target genes [65].

NRF2 has also been shown to be regulated by lipopolysaccharide because of the identification of a nuclear factor (NF)-κB binding element in its promoter region [66]. Due to this finding, constitutive NF-κB signaling in acute myeloid leukemia results in induced NRF2 [67]. One additional factor that helps

to facilitate the transcription of *NRF2* is breast cancer 1 (BRCA1) [68]. Binding of BRCA1 to the promoter of *NRF2* requires heterodimerization with ARNT, implying that the elements recognized by this pair may be similar to those recognized by the AhR/ARNT pair [69].

Lastly, chromatin immunoprecipitation (ChIP) experiments have revealed Jun and Myc binding sites near the transcription start site of *NRF2*. Murine *Nrf2* was recently reported to be up-regulated at the transcriptional level by oncogenic activation of K-RAS^{G12D}, B-RAF^{V619E} and MYC^{ERT2} [70].Our group demonstrated that activation of NRF2 by oncogenic K-RAS is facilitated through a TPA-responsive element (TRE) in the regulatory region of *NRF2* [71]. The precise mechanisms by which B-RAF^{V619E} and MYC^{ERT2} up-regulate the transcription of *NRF2* likely occur via similar mechanisms, however, definitive evidence is lacking.

1.2.4 Additional modes of NRF2 regulation

In addition to proteins capable of binding to KEAP1, other proteins have been shown to misregulate NRF2 by binding directly to it, rather than KEAP1. The first protein that was found to bind to NRF2 and direct an antioxidant response is the cyclin-dependent kinase inhibitor p21 [72]. A direct target of p53, p21 has been shown to regulate cellular differentiation and senescence, in addition to apoptosis depending on cell status. If the cell acquires damage to its DNA, p21 can mediate cell cycle arrest from the G1 to S phase, in order for the cell to repair the genome. This tumor suppressive role of p21 was further enhanced when it was

shown to activate the NRF2-mediated defense response, through direct interaction with NRF2.

Similarly, BRCA1 has been proven to directly interact with NRF2 and compete with KEAP1 for binding, thereby preventing the ubiquitylation and degradation of NRF2. As mentioned previously, BRCA1, along with ARNT, has also been identified to play a minor role in facilitating the transcription of *NRF2* through XRE-like sequences, further solidifying its tumor suppressive role by modulating NRF2 protein levels. Additionally, BRCA1 has been shown to positively regulate p53-dependent gene expression [73]. Accordingly, it is feasible to conceive that upstream BRCA1 activation can lead to p21 up-regulation through transcriptional activation of p53, however, this intricate feed-forward mechanism of NRF2 activation has not been fully delineated thus far.

Another significant study from our group identified that the stability of NRF2 can be modulated through USP15, which is a member of the ubiquitin-specific processing protease family of deubiquitylating enzymes (DUBs). The ability of KEAP1 to ubiquitylate NRF2 is well understood, however, KEAP1 itself can be ubiquitylated/deubiquitylated as well. Effectively removing ubiquitin from KEAP1 at lysine 39, USP15 was shown to stabilize the KEAP1-CUL3 complex, allowing the CNC-bZIP factor to be more efficiently degraded in a KEAP1-dependent manner [74]. In turn, overexpression of USP15 reduced NRF2 target gene expression and sensitized multiple cancer cell lines to chemotherapeutic agents.

While the mechanisms described above ultimately achieve up-regulation of NRF2, some emerging evidence has shown negative regulation of NRF2 through the activation of nuclear receptors. Still a growing body of research, the negative regulation of NRF2 by nuclear receptors will represent a significant contribution to the NRF2 field in the future, as nuclear receptors are greatly involved in a variety of physiological processes throughout the body. Moreover, the process by which nuclear receptors may exert their function can occur in a ligand-dependent and/or –independent manner, which could potentially shape the drug discovery process given that certain cancers are driven by hormones that activate nuclear receptors.

In 2007, it was reported that the induction of ARE-driven luciferase expression was inhibited by all-trans retinoic acid (ATRA) [75]. ATRA belongs to a class of compounds called retinoids, which are structurally related to vitamin A. Retinoids exert their effects through the interaction of either retinoid acid receptors (RARs) or retinoid X receptors (RXRs), which can heterodimerize with each other or with other nuclear receptors, and research on retinoids suggests that they have significant anti-cancer activity by means of regulating redox status as well as apoptosis. ATRA was shown to inhibit the mRNA and protein expression of aldo-keto reductase family 1 member C1 (AKR1C1) and AKR1C2, which are both NRF2 target genes. Substantially, ATRA was not shown to affect the half-life of NRF2 or its accumulation/nuclear translocation. More specifically, RARα directly inhibited the luciferase activity of ARE-driven gene expression in a follow-up experiment. Years later, further work on this topic revealed that

heterodimerization with RXRα was not required for inhibition of ARE-driven luciferase expression [24]. It was found that RXRα was able to bind to a region in NRF2 adjacent to the Neh5 domain. Because of this, a new domain, the Neh7 domain, was reported (introduced in section *1.1.2*) and is now known for its ability to bind RXRα.

In addition to the retinoic acid receptors, other nuclear receptors can negatively regulate NRF2 activity in various cell culture models. PPARγ, which is also capable of heterodimerization with RXRs under normal circumstances, can repress the transactivation of NRF2 through direct interaction. Further, NRF2 signaling is negatively correlated with estrogen treatment. The first description of this identified that upon stimulation with estrogen (E2), phase II gene expression was blunted *in vitro* and *in vivo*, suggesting that E2 treatment may lead to oxidative DNA damage. Additional experiment showed that activation of estrogen receptor α (ERα) with E2 directly represses NRF2-mediated transcription [76]. In accordance with this data, inhibition of estrogen signaling causes activation of the NRF2 pathway. The estrogen-related receptor β (ERRβ) has also been implicated in the negative regulation of NRF2 [77]. Yet another nuclear receptor that obstructs NRF2 signaling is the glucocorticoid receptor (GR). The GR is activated by steroid hormones called glucocorticoids and plays a major role in intermediary metabolism, cardiovascular function, and immunity. Dexamethasone, a synthetic glucocorticoid that activates the GR, was found suppress GSTA2 gene expression that was induced with multiple small molecule inducers of NRF2 [78]. Though the relationship between NRF2 and nuclear

receptors has only recently come to light, additional work must be completed in order to determine the mechanisms by which ligand activated nuclear receptors can negatively regulate the stress-responsive NRF2. The insights gained from these studies will help to elucidate more specific mechanisms of NRF2 regulation and clarify transcriptional responses to various compounds that trigger nuclear receptor activation or inhibition.

1.3 Chemoprevention and NRF2

1.3.1 Phytochemicals activate NRF2

Despite vast research efforts, cancer incidence is still on an upward trend, ultimately leading to approximately 600,000 deaths in the United States in 2017. While our understanding of the molecular events that are associated with cancer initiation, progression, and promotion have greatly improved; lifestyle alterations, environmental factors, and increasing age still critically contribute to an increase in cancer rates. Of particular interest is the notion that many cancers are attributed to environmental exposure to toxicants or pollutants that contain carcinogens. Quite simply, in order to reduce the risk of acquiring these cancers caused by exposure, avoidance of carcinogenic species remains the most feasible strategy. While this is easier said than done, alternate approaches have been put in place in order to minimize exposure to substances that cause cancer.

It has been previously described that consumption of certain dietary constituents help to minimize the incidence of cancer [79,80]. These dietary

constituents have since been recognized as ‘chemopreventive’ agents, which are typically identified as non-nutritive components from consumable plants, because of their ability to slow or block the promotion stage of carcinogenesis. Though poor diet has been heavily implicated in cancer progression, established chemopreventive agents which suppress carcinogenesis are typically phytochemicals from fruits and vegetables and can have a positive health outcome, arguing that diet has a large role to play in cancer progression.

The salubrious benefits of many foods and herbal medicines have been known for thousands of years. In the mid-1970s, Michael Sporn coined the term ‘chemoprevention’, which described the process of blocking or slowing the onset of premalignant tumors with chemical compounds that are relatively nontoxic. Around this time, The National Cancer Institute (NCI) began to evaluate the safety profile of many phytochemicals found in plants and vegetables for their ability to suppress carcinogenesis. The mechanisms by which these agents offered beneficial effects remained without explanation, but extensive research has revealed that alteration to key cell survival pathways is responsible for the beneficial effects. Most notably, research has shown that activation of NRF2 by many foods and traditional medicines as an explanation of benefit. As discussed earlier, KEAP1 is the primary negative regulator of NRF2, and is subject to oxidation and electrophilc modification. Many foods (i.e. broccoli, grapes, cinnamon, etc.) contain natural electrophiles that react with Cys151 in KEAP1 and increase NRF2 protein levels. Up-regulation of NRF2 then targets phase II metabolism enzymes, which mediate the cell’s ability to detoxify carcinogens and

to combat mutagenic ROS. Typically, canonical activation of NRF2 is turned off once the chemopreventive agent is metabolized and removed from the cell, and NRF2 protein levels are reduced to basal levels. Therefore, additional applications of chemically diverse chemopreventive compounds (from different kinds of fruits and vegetables) are needed in order to intermittently activate NRF2 for cellular protection against malignant transformation.

1.3.2 Tumor suppression by NRF2

Early chemoprevention studies identified that up-regulation of phase II enzymes during the progression phase of carcinogenesis greatly reduced tumor burden in mice that were challenged with a carcinogen. These studies employed the use of newly identified NRF2 inducers, such as oltipraz or sulforphane, to enhance the expression of phase II enzymes to suppress carcinogenesis [81-83]. Despite their beneficial effects in the preclinical setting, the electrophilicity of NRF2 inducers meant that there was a strong potential for modulation of other signaling pathways, as chemopreventive compounds have been shown to affect the degradation mechanisms of additional cell-survival pathways such as NF-κB and β-catenin [79]. To address this matter, the *Nrf2*-knockout mouse was generated by Yamamoto and colleagues [84]. Imperatively, it was observed that the chemopreventive action of certain NRF2 inducers was abolished in *Nrf2*-knockout mice, because of the fact that phase II metabolism enzymes were no longer responsive [85]. This critical finding confirmed that NRF2 is an indispensable factor for biochemical targeting as a strategy for chemoprevention.

Early pharmacological targeting of NRF2 used synthetic antioxidant compounds such as butylated hydroxyanisole (BHA) and oltipraz, which, from a safety perspective, had been thoroughly investigated because of their original use as a food preservative and an anti-schistosomal drug, respectively [86,87]. Because it was deemed safe, BHA was used as a chemopreventive agent, as it showed an ability to induce both the mRNA levels as well as the activity of hepatic microsomal epoxide hydrolase (EPH1), glutathione-S-transferase (GST), and NAD(P)H:quinone oxidoreductase 1 (NQO1), against carcinogenic aromatic amines [88-90]. Oltipraz was also shown to have significant efficacy as a chemopreventive agent. Intermittent dosing of oltipraz, a dithiolethione, was able to sustain NRF2-target protein levels and inhibit tumorigenesis in rats that were treated daily with the potent hepatocarcinogen aflatoxin B_1 [91]. Indeed, detoxication of aflatoxin B_1 was mediated primarily by the coordinated induction of cytochrome P450 (CYP) enzymes, but most notably by GSTs. Interestingly, BHA and oltipraz were identified to modulate NRF2 signaling by producing ROS. While they were shown to be effective in blunting carcinogenesis via NRF2 induction in numerous studies, the reliance on these compounds became less favorable because of the toxicological implications associated with sulfhydryl reactivity and lack of specificity with prolonged use and high doses.

An additional class of chemopreventive agents capable of activating the NRF2 pathway is the isothiocyanates and the glucosinolates (isothiocyanate precursors), which are found in cruciferous vegetables (i.e. broccoli). Because previous agents induced NRF2 through ROS production, there was a need to

develop a more specified category of NRF2 inducers. With the recognition that various inducers were highly reactive to thiols, an effort was put forth to use a quinone reductase bioassay to identify individual isothiocyanate-containing analogs from a series of extracts from broccoli. This experiment identified sulforaphane, which to this day, is still the most commonly used plant-derived NRF2 inducer due to its potency and availability/affordability. Extensive biochemical studies showed that sulforaphane activates NRF2 by covalently modifying C151 in KEAP1, thereby preventing the ubiquitylation and degradation of NRF2. Chemopreventive studies using sulforaphane or sulforaphane-rich broccoli extracts have proven to protect against tumor progression in organ sites such as skin, bladder, colon breast, lung, and stomach using rodent models of carcinogenesis after initiation by carcinogenic species [92].

The success of sulforaphane in preventing tumorigenesis in animal models has led to its pursuit in the clinic. Because sulforaphane is a phytochemical from broccoli and is thus widely consumed all over the world, chemoprevention studies in humans took place in Qidong, People's Republic of China [93]. As an endpoint, the disposition of aflatoxin metabolites was measured because exposure aflatoxin B_1 to is common and is known to cause hepatocellular carcinoma (HCC). Between the placebo and treatment group (glucosinolate-rich beverage every night for 2 weeks), analysis revealed an inverse relationship between sulforaphane metabolites excreted and aflatoxin-DNA adducts [94]. Additionally, further analysis also revealed that the metabolism of polycyclic aromatic hydrocarbons could be modulated.

Importantly, the results of this study showed that administering a glucosinolate-rich consumable over a brief period of time was sufficient enough to alter aflatoxin disposition, which is a logical and cost-effective way to minimize the incidence of HCC in a region where exposure to aflatoxins are common.

An additional dietary NRF2 inducer that has recently emerged is the cinnamon-derived factor cinnamaldehyde (CA) [95]. Cinnamon is spice that is widely used all over the world and is easy to obtain. CA contains a Michael acceptor, which is responsible for its reactivity with thiol groups (especially C151) in KEAP1. After early research efforts proved that CA could up-regulate NRF2 target genes in a canonical manner, chemopreventive studies then focused on CAs ability to suppress the formation of chronic diseases such as cancer. Specifically, CA was shown to suppress tumorigenesis in the colon after an initial exposure to AOM/DSS in mice that were fed a CA-rich diet [96]. Notably, the mice which did not receive the CA-rich diet developed a large tumor burden, implying that ingesting CA, like sulforaphane, is a feasible strategy to slow or potentially reverse the process of carcinogenesis upon exposure to harmful insults that we might not even be aware of. Taken together, daily consumption of fruits, vegetables, spices, herbs, etc. is an easy way to minimize the risk of cancer due to the beneficial consequences of NRF2 induction.

In addition to phytochemicals from foods and traditional medicines, two NRF2 inducers; Bardoxolone-methyl (CDDO-Me) and dimethylfumarate (DMF) have advanced to clinical trials and the clinic, respectively. Bardoxolone, a natural product derived triterpenoid, was reported to interact with the BTB

domain of KEAP1, disrupting the BTB-CUL3 interface, leading to NRF2 activation [97]. Bardoxolone was entered into phase II clinical trials for the treatment of diabetic nephropathy and other complications. In this trial, an increased glomerular filtration rate in patients with chronic kidney disease was attributed to long-term bardoxolone treatment. However, upon matriculation to phase III, it was retracted due to safety issues [98]. Conversely, another compound capable of KEAP1 adduction, dimethyl fumarate (DMF, also known as BG-12 and Tecfidera) was recently awarded Food and Drug Administration (FDA) approval for the treatment of multiple sclerosis (MS). Pre-clinically, DMF was shown to have significant neuroprotective effects in transgenic murine models of Huntington's disease as well as experimental models of demyelination and neurodegeneration [99,100]. In addition to these findings, DMF was shown to exhibit significant protection against the neuroinflammatory effects caused by MS, ultimately leading to its FDA approval.

1.3.3 NRF2 and neurodegeneration

Neurodegeneration is a term that is used to describe the progressive loss of neurons in a patient, resulting in a dramatic inability to control movements or mental function. Because neurodegenerative diseases result in the death of certain neurons, the outcome is permanent and quality of life plummets as the disease progresses further. Typically, signs of neurodegeneration do not usually appear until too much damage has been sustained, and the patient must rely on drugs to manage the disease. Unfortunately, treatment options are limited, and

aside from genetic screening, there is no way to know if one is at major risk for acquiring a neurodegenerative disease, as they are, in most cases, idiopathic. Though specific causes are unknown, different neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's Disease (HD), and Amyotrophic lateral sclerosis (ALS), share similar mechanisms of pathology on both macro- and microscopic levels. For instance, it is well accepted that overproduction of ROS is a key feature in all neurodegenerative diseases. There is also mounting evidence for the role of protein degradation pathways, as these diseases often feature the accumulation of misfolded protein species which aggregate and initiate cell death. Certain genetic factors contribute to a fraction of these diseases, and common mutations that are found usually occur in proteins that affect cell functions responsible for controlling ROS and protein degradation, but for the most part, causes are still unknown. Because ROS is implicated in the pathogenesis of these disorders, NRF2 has emerged as a potential factor to target to decelerate progression [101].

A possible explanation of cell death due to ROS accumulation could be defective mitochondria [102]. During cellular respiration, the mitochondria contribute to the majority of the ROS the cell faces, but because of the antioxidant response by NRF2, the cell is able to cope with this mild stress. However, various familial mutations that have been associated with PD, occur in genes that code for mitochondrial proteins. Therefore, defective mitochondria become increasingly problematic overtime, as they enhance ROS production.

Although the antioxidant function of NRF2 is well established, recent research has begun to show that NRF2 induction can protect against mitochondrial toxins, as well as influence mitochondrial biogenesis and mitophagy (mitochondrial degradation through the autophagy pathway).

Similar in execution to the cancer chemoprevention studies discussed above, phytochemical activators of NRF2 have been used to delay the onset of neurodegenerative diseases in mouse models of PD, AD, HD, and ALS. Early studies using NRF2 $^{-/-}$ mice identified an increased sensitivity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (dopaminergic toxins used in chemically-induced models of PD), and reports from multiple labs showed that the protective effects of the compounds given were in fact due to NRF2 induction [103,104]. Two of these compounds that were used were sulforaphane and CDDO-ethyl amide (CDDO-EA). Additional synthetic triterpenoids (i.e. CDDO-trifluoroethyl amide (CDDO-TFEA)) have been used in models of ALS, and were shown to increase lifespan in the hSOD^{G93A} mouse model when fed CDDO-TFEA in the diet [105]. CDDO-methylamide (CDDO-MA) also showed a positive effect in Tg19959 mice, a transgenic mouse model for AD that expresses the mutant amyloid protein precursor bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations [106]. In this study, CDDO-MA was able to improve the memory of these mice, as well as reduce plaques, Amyloid beta (Aβ)1-42, and other markers of oxidative stress. However, one limitation to the research regarding NRF2 and AD is the fact that NRF2 $^{-/-}$ mice have not been utilized to confirm that NRF2 is required for the

protective effects offered by the compounds tested, such as CDDO-MA or sulforaphane. Unlike the complex mechanisms that drive the pathogenesis of PD, AD, and ALS, HD is well understood, and as a result, there is limited research on the effects of NRF2 induction and HD progression. HD is an autosomal dominant neurodegenerative disease, where affected patients have an expansion of ‘CAG’ repeats in exon 1 of the Huntington (Htt) gene. These ‘CAG’ repeats code for a stretch of glutamine residues, and when the protein is translated, the aggregation potential is directly proportional to the amount of glutamine repeats. Since this disease is driven by genetic predisposition, pharmacological induction of NRF2 only provides mild protection, as aggregation of polygultamine (poly-Q)-containing proteins can enhance proteotoxic/oxidative stress. In this particular case, clearance (degradation) of this toxic protein species through the autophagy/lysosome pathway has been conceived as an ideal strategy to minimize cell death induced by proteins with poly-Q expansions. Because the influence of NRF2 on autophagy is still emerging, future work will aim to see if NRF2-based therapeutics protects against poly-Q toxicity, or if autophagy inducers will act as stand-alone drugs to delay onset of HD symptoms.

An added element that contributes to AD pathogenesis is the hyperphosphorylation of the tau protein. In addition to altered splicing of amyloid precursor protein (APP), which leads to Aβ(1-42) and other similar species, tau is a cytoskeletal protein that, when phosphorylated, displays an enhanced propensity to aggregate, contributing to the plaques which are found in AD

patients post-mortem. Because phospho-tau is a pathological hallmark of AD, efforts have focused on preventing the phosphorylation of tau. The primary kinase that governs this modification is GSK-3. As mentioned previously, GSK-3 also contributes to the stability of NRF2. Therefore, the beneficial outcome of inhibiting GSK-3 is two-fold; decreasing tau phosphorylation and inducing NRF2, so the pursuit of better drugs to treat AD should focus on inhibition of GSK-3 over canonical NRF2 inducers. However, because GSK-3 is not implicated in the pathogenesis of PD, ALS, or HD, the use of NRF2 inducers should still be used for preventive or concurrent means.

1.4 NRF2 and disease progression

1.4.1 Chemoresistance and the 'dark side' of NRF2

NRF2 undeniably plays a prominent role in cancer prevention; however, reports from our lab have revealed the "dark-side" of NRF2 [107]. Because NRF2 improves cellular survival under cytotoxic challenges, cells heavily rely on NRF2 activation to circumvent cell death. Unfortunately, this is also the case with cancer cells that are exposed to cytotoxic chemotherapeutic agents, and it was predicted early on that NRF2 could be up-regulated to provide a cellular environment conducive for survival against constant battering by chemotherapeutics with diverse mechanisms of action.

Non-small cell lung cancer (NSCLC) contributes to the majority of lung cancer cases and is frequently refractory to chemotherapy. Analysis of NSCLC

tumors revealed overexpression of key xenobiotic metabolism genes such as GSTs and multidrug resistance proteins (MRPs) [108]. Acting in concert, the expression of all of these detoxication gene products provide a robust resistance to drug regimens, making the treatment of NSCLC very difficult. With this antioxidant phenotype in mind, Biswal's group sequenced *KEAP1* in multiple NSCLC cell lines and tumor samples. Their results revealed that *KEAP1* is frequently mutated within conserved amino acid residues. More specifically, these residues were located in the Kelch or Intervening region domains, which would likely eliminate the repressor activity of KEAP1. Soon after this finding, it was described that NRF2 specifically enhances the resistance of NSCLC cells to chemotherapeutic drugs. Detailed experiments showed that knockdown of NRF2 sensitized A549 (NSCLC) cells to cisplatin, doxorubicin, and etoposide [107,109]. Furthermore, overexpression of NRF2 in other cancer cell lines such as MDA-MB-231 and SH-SY5Y cells demonstrated that the NRF2-dependent survival response to chemotherapeutic challenge was not limited to NSCLC or a particular anticancer drug because an increased resistance to multiple drugs was also observed. This important finding conveyed that inhibition of NRF2 could be a generalized strategy for cancer treatment. In conjunction with these findings, Yamamoto's group identified a somatic mutation and a gene variation, also in the Kelch domain of KEAP1, in lung cancer cells [110]. These mutations induced a conformational change in KEAP1 and reduced the affinity for NRF2 binding and provided key mechanistic insights into NRF2s gain of function in lung cancer.

1.4.2 Oncogenic role of NRF2

Upon malignant transformation, certain cancers, in addition to NSCLC, can achieve constitutively high levels of NRF2 resulting in uncontrolled NRF2 activation, compared to normal cells (Figure 1.4). Mounting evidence has indicated that elevated NRF2 levels due to loss of function mutations in *KEAP1* are associated with resistance to chemotherapeutic agents and thus a poor prognosis in many cancer types including NSCLC, endometrial carcinoma, ovarian carcinoma, breast cancer, and gallbladder cancer [111-114]. In addition to *KEAP1*, mutations found in *NFE2L2* (the gene encoding NRF2) are also common in esophageal, lung, and head and neck cancers [115,116]. Lastly, mutations in *CUL3* also confer constitutive activation of NRF2 in sporadic type 2 papillary renal cell carcinoma (PRCC) [117]. It is certain that many somatic mutations in other genes encoding NRF2-regulatory proteins will be identified in addition to these. For instance, NRF2 has been found to be up-regulated as a result of *PTEN* mutations [118]. PTEN inactivation consequently activates the PI3K/AKT pathway, where uncontrolled AKT can suppress GSK-3s negative regulation of NRF2 through the phosphodegron. Another mechanism for NRF2 overexpression in cancer cells, which is consistent in brain, lung, and prostate cancers, is the epigenetic silencing of the *KEAP1* gene, leading to deregulated NRF2 protein levels [119-121]. In these cases, the *KEAP1* promoter region was found to be hypermethylated, preventing the expression of KEAP1 mRNA and conferring an oncogenic gain of function for NRF2. Oncometabolite adduction of KEAP1 has also been reported as a mechanism of NRF2 activation in PRCC.

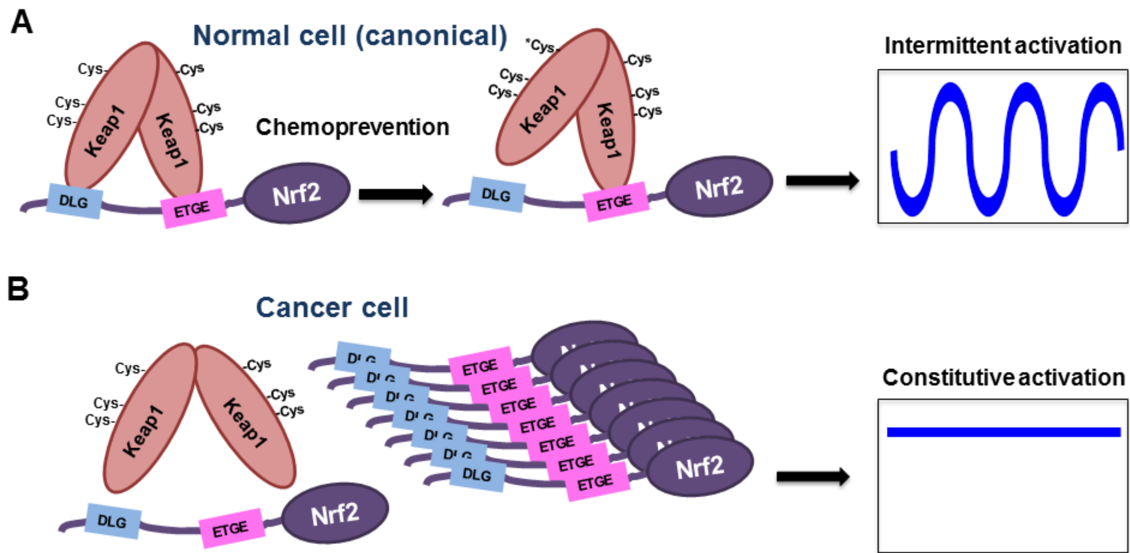

Figure 1.4 Modes of NRF2 activation during disease progression. (A) The canonical mechanism of NRF2 regulation in a normal cell exposed to chemopreventive compounds that target KEAP1. (B) Misregulated NRF2 signaling in a cancer cell that leads to constitutive activation of NRF2. The molecular events underlying this misregulation are discussed in the text.

Fumarate hydratase (FH)-inactivating mutations lead to the accumulation of fumarate and the subsequent activation of NRF2 target genes through modification of cysteines in KEAP1 [122]. Lastly, as mentioned previously, NRF2 has been shown to be controlled at the transcriptional level by the oncogenes K-Ras^{G12D}, B-Raf^{V619E} and Myc^{ERT2}. This oncogene-directed induction of *NRF2* was identified to promote tumorigenesis through suppression of ROS *in vitro* and an *in vivo* model of K-Ras^{G12D}-driven pancreatic cancer [70]. As a follow up to this landmark study, our lab focused on identifying the molecular mechanisms responsible for the induction of NRF2, as this point was not addressed. Ultimately, KRAS was found to drive the transcription of NRF2 through a TRE enhancer located within the proximal promoter of *NRF2* [71].

Since the emergence of the 'dark side' of NRF2, detailed reports of the oncogenic role of NRF2 are on the rise. Initially, it was recognized that enhanced NRF2 expression could provide a reduced environment for cancer cells to thrive and eliminate cytotoxic chemotherapies, but the manner in which NRF2 regulates these processes was overgeneralized. In an effort to answer how NRF2 contributes to aggressive proliferation, microarray analysis of A549 cells treated with control or *NRF2* siRNA was performed to identify potentially new targets of a constitutively active NRF2 [123]. Interestingly, in addition to the prototypic phase II enzymes and antioxidant proteins, gene products involved in the pentose phosphate pathway (PPP), *de novo* nucleotide synthesis, and NADPH generation were diminished in the *NRF2* siRNA treated group. These data illustrate the importance of NRF2 is proliferating cells versus cells that are

quiescent. Cancer cells are highly proliferative and thus have a great metabolic demand, providing explanation for NRF2s ability to control purine nucleotide synthesis, as well as glucose and glutamine metabolism to favor anabolic pathways. In a similar fashion to this study, another group reported the activation of a different subset of genes that are involved in a related pathway that also controls cancer cell growth and proliferation. To fuel these processes, the serine biosynthetic pathway has been recently proven to provide the cancer cell with a source of metabolic intermediates that feed into glycine production and the folate cycle to stimulate nucleotide production. Through gene set enrichment analysis, NRF2 was predicted to regulate *PHGHD*, *PSAT1*, and *SHMT2*, which are key genes within the serine biosynthetic pathway [124]. In cells treated with ^{13}C-glucose, it was found that cells that had a high expression of NRF2 yielded a strong enrichment for ^{13}C-labeled serine. It was subsequently shown that silencing of PHGDH was detrimental for the growth of a serine-high cell line and thus contributed to a decrease in tumorigenesis. Human tumors with high NRF2 also showed a strong positive correlation with *PHGDH*, *PSAT1*, and *SHMT2*, and conferred a poor prognosis.

NRF2 has also been identified to contribute to carcinogenesis through regulation of mRNA translation in pancreatic cancer [125]. In this study, global cysteine proteomics revealed that translational machinery is highly susceptible to oxidative modification of cysteines, and that NRF2 is heavily involved in maintaining the effectiveness of translational machinery, via protecting it from oxidative damage. Interestingly, not only was global translation impaired in

NRF2-deficient cells, but NRF2 also was shown to enhance the cap-dependent translation of mRNA transcripts, many of which code for pro-survival proteins. In this sense, NRF2 is able to sustain tumors by not only providing an advantage against chemotherapeutics, but also preventing damage to key cellular translational machinery allowing cancer cells to properly synthesize cell-survival proteins, thereby contributing to aggressiveness.

1.4.3 Inhibition of NRF2

In order to combat this oncogenic function of NRF2, our lab has focused on the discovery and development of inhibitors of the NRF2 pathway. Our first success in this vain, brusatol, potently decreases NRF2 protein levels and target gene expression at nanomolar concentrations in cancer cells with constitutively high NRF2 expression, enhancing the cytotoxic effect of cisplatin and other chemotherapeutics [126]. As mentioned above, oncogenic K-RAS up-regulates NRF2 mRNA levels, which offers a possible explanation for why oncogenic K-RAS mutations lead to chemoresistant tumors. In line with this interpretation, we demonstrated that co-administration of brusatol enhanced the efficacy of cisplatin and improved survival of mice with lung cancer using a K-RAS^{G12D} mouse model. As a result, brusatol has illuminated the value of NRF2 inhibitors as adjuvants to chemotherapeutic drugs that are typical first line regimens for cancers, especially for those that express uncontrolled activation of NRF2.

The success of brusatol prompted a large effort to identify additional inhibitors of the NRF2 pathway. In this regard, certain compounds such as

ochratoxin A, luteolin, and AEM1 have emerged from high-throughput screens as inhibitors of NRF2-mediated transcription [127-129]. Unfortunately, the mechanisms of action of these compounds are not well defined, as they have not undergone further characterization (AEM1) or have conflicting data regarding the mechanism of action (ochratoxin A and luteolin). Discovery of how these compounds inhibit NRF2 protein levels or activity would enhance our understanding of the mechanisms that govern the negative regulation of the NRF2 pathway. Despite the importance of this venture, NRF2 is a protein with a short half-life, which can be easily reduced when the cell undergoes cell death, leading researchers to identify a number of compounds with supposed inhibitory action on NRF2 without providing key mechanistic studies aimed at identifying specificity towards the NRF2 pathway. Nevertheless, multiple compounds are still being pursued to identify a molecule that can specifically deregulate the transcriptional activity of NRF2, which would provide extreme benefit for the treatment of cancers that over express NRF2, while eliminating off-target effects that the current arsenal of NRF2 inhibitors display.

One interesting approach to the discovery of an inhibitor of de-regulated NRF2 signaling came from Komatsu's lab in 2016 [130]. NRF2 is frequently up-regulated in HCC, due to an overexpression of p62. Phosphorylation of serine 349 in p62 is frequently observed in cases of HCC, and results in persistent activation of NRF2 due to an enhanced interaction with KEAP1. Through a fluorescence polarization screening method using recombinant KEAP1 and a 6-carboxyfluorescein-labelled phospho-S349 peptide of p62, a compound was

identified to inhibit the interaction between phospho-p62 (S349) and KEAP1. Prevention of the association between phospho-p62 (S349) and KEAP1 leaves KEAP1 to negatively regulate NRF2 protein levels, ultimately restoring the cells ability to canonically regulate NRF2 despite the status of p62.

Additionally, other small molecules have recently been identified as novel NRF2 inhibitors. Biswal's group identified a molecule they named ML385, which inhibits NRF2-mediated transcription through direct interaction with the Neh1 domain of NRF2 [131]. ML385 was also shown to be effective in enhancing the cytotoxic effects of chemotherapy *in vivo*. Moreover, Yamamoto's group recently identified a molecule called halofuginone that was capable of inhibiting protein levels of NRF2 [132]. Interestingly, halofuginone exerted it inhibitory action on NRF2 through starving the cells of amino acids, resulting in repressed global protein synthesis, which had a drastic effect on NRF2 protein levels in a short period of time. The GCN2 kinase, which is one of the four kinases that comprise the integrated stress response, was rapidly phosphorylated, leading to the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), which controls cap-dependent translation. Despite the effect on global protein synthesis, halofuginone was shown to be effect for the treatment of cancers with high expression of NRF2 both *in vitro* and *in vivo*.

It is now widely accepted that pharmacological inhibition of NRF2 is an appealing strategy to sensitize tumor cells to chemotherapeutics and thus has sparked a major movement to characterize new NRF2 inhibitors with heightened specificity. However, in many other contexts, NRF2 remains an indispensable

cytoprotective factor that protects against the formation of many chronic diseases, so modulation of NRF2 should be met with precise attention. There is an overabundance of research on the beneficial effects of NRF2 activation with countless NRF2 activators; most of them coming from natural products, and there is still mounting evidence to support this case, especially for the prevention of cancer. Consequently, additional research into the molecular mechanisms that modulate NRF2 expression and activity warrants further study in order to fully understand the precise contexts in which NRF2 should be activated or inhibited to intervene with disease progression. The following chapters in this dissertation aim to describe additional modes of NRF2 regulation as well as other signaling pathways that may provide crosstalk with NRF2 and interfere with its activity. Research into the mechanism of action of natural products that modulate NRF2 has also been pursued in order to characterize and provide the framework for the next generation of compounds that either activate or inhibit NRF2. Together, the following studies provide new insights into the molecular mechanisms that govern NRF2 and provide further evidence for the fact that NRF2 is a factor that should be targeted depending on disease context.

Chapter 2

Interaction between TSC22D4 and KEAP1 promotes the NRF2-mediated cell defense response

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2.1 Abstract

The redox-sensitive transcription factor Nuclear factor-erythroid factor 2-like 2 (NRF2) is the master regulator of a critical transcriptional program that defends the cell against harmful oxidative and xenobiotic insult. Although this program can be upregulated to prevent the initiation of cancer, a growing number of studies have associated constitutively active NRF2 signaling with resistance to chemo- and radiotherapy, indicating that NRF2 contributes to the survival of cancer cells. Here, a newly identified interaction between KEAP1, the negative regulator of NRF2, and TGFβ-stimulated clone 22 Domain Family Member 4 (TSC22D4) results in activation of the NRF2 pathway in Ishikawa cells (a human Type 1 endometrial endometrioid adenocarcinoma cell line). TSC22D4, a putative transcriptional regulator with little known function, competes with NRF2 for KEAP1 binding in an 'ETGE' dependent manner, thereby preventing the ubiquitylation/degradation of NRF2. Additionally, TSC22D4 was found to be responsive to the synthetic progestin Medroxyprogesterone acetate (MPA), via a functional hormone response element in its promoter region, showing that the protein levels of TSC22D4 can be modulated. These findings potentially link the complex signaling networks between the progesterone receptor (PR) and NRF2,

arguing for inhibition of the NRF2 pathway during treatment with MPA, which is re-emerging as an attractive therapy for hormone-driven malignancies. Collectively, this anti-oxidant phenotype induced by TSC22D4 could provide framework for additional studies that attempt to understand the role of TSC22D4 in cancer progression.

2.2 Introduction

Endometrial cancer is the most common gynecological malignancy in the world and according to the American Cancer Society, it is estimated that approximately 60,000 new cases will arise in 2017, resulting in over 10,000 deaths. Depending on histological classification, endometrial cancer can be divided into two groups, Type 1 and Type 2. Type 1 endometrial endometrioid carcinoma (EEC) is more prevalent and is of endometrioid origin, which is generally associated with a favorable prognostic outcome. In contrast, Type 2 endometrial serous carcinoma (ESC) are nonendometrioid, arising from either serous or clear cell types, and are associated with much poorer prognosis [133]. One clinically relevant feature that distinguishes Type 1 from Type 2 is the responsiveness to hormonal therapy. In most cases, Type 1 endometrioid tumors express the progesterone receptor (PR), whereas Type 2 serous or clear cell tumors do not [134]. As a result, certain contraindications to surgery or the unwillingness to use more aggressive chemotherapy regimens may allow patients with Type 1 EEC to receive the hormonal therapy medroxyprogesterone acetate (MPA). Beyond PR

expression, recent studies have provided evidence for the expression of NRF2 as a potential prognostic marker [135,136].

The redox-regulated transcription factor NRF2 has been well established as the key regulator of the antioxidant response inside all cell types. Critically, NRF2 is modulated at the protein level by the Kelch-like ECH associated protein 1 (KEAP1)- Ring-Box1 (RBX1)- Cullin3 (CUL3) E3 ubiquitin ligase complex [39,40]. One molecule of NRF2 is bound by two molecules of KEAP1, through the association of the 'DLG' and 'ETGE' motifs (low and high affinity binding, respectively) located at the N-terminal end of NRF2 [137]. Once bound, NRF2 is polyubiquitylated at key lysine residues and sent for proteasomal degradation [40]. However, when cells are exposed to stressors such as reactive oxygen species (ROS), electrophilic compounds, or environmental toxicants, a critical cysteine residue (Cys 151) on KEAP1 is covalently modified inducing a conformational change in KEAP1 [42]. This alteration prohibits the proper interaction between NRF2 and KEAP1, and NRF2 is not polyubiquitylated or degraded. Thus, newly synthesized NRF2 then accumulates and translocates into the nucleus, where it heterodimerizes with a small MAF protein and locates the antioxidant response element (ARE) enhancer sequence found in its target genes and promotes transcription of a battery of genes that together encompass the antioxidant response [35].

While NRF2 plays a massive role in orchestrating cellular survival in response to stress, certain cancers have taken advantage of this protective response. Over time, cancers acquire chemoresistance through achieving

constitutively active NRF2 and many studies have identified that high levels of NRF2 are associated with a decreased efficacy of chemotherapeutics, a phenomenon that has been termed 'the dark side of NRF2' [107,126]. In an effort to further understand how NRF2 is regulated, key proteomic studies have revealed a variety of proteins capable of binding to KEAP1, which can aberrantly induce the NRF2-mediated antioxidant response [52]. Not surprisingly, the majority of KEAP1 interacting proteins contained 'ETGE' or 'ETGE'-like motifs, which is the motif responsible for the high-affinity binding between NRF2 and KEAP1. One particular protein that was found to associate with KEAP1 was TGFβ-Stimulated Clone 22 Domain Family 4 (TSC22D4), which contains an 'ETGE' motif [52].

TSC22D4 is a leucine zipper protein that has putative transcriptional regulatory activity, but a clear regulatory function has yet to be explicated. Early studies identified that TSC22D4 can homodimerize or heterodimerize with its TSC22 family member TSC22D1 [138]. Later studies strongly argue for the role of TSC22D4 as a gene involved in neurodevelopment, and identify that TSC22D4 is responsive during cerebellar granule neuron apoptosis [139-141]. Additionally, TSC22D4 was shown to be an important regulator of VLDL secretion in a cachexia phenotype, potentially arguing further for its role as a stress-responsive gene [142].

In 1997, differential display identified TSC22D1 (then known as TSC22) to be a progestin-responsive gene in T-47D cells [143]. Here, we have identified TSC22D4 as progestin-responsive gene through the identification of a hormone

response element (HRE) 6kb upstream of its transcription start site. To date, there is little known concerning the relationship between the PR and NRF2, so we set out to elucidate how TSC22D4 could potentially provide a link between these two transcriptional programs. We have shown that TSC22D4 can up-regulate the NRF2-dependent antioxidant response in Ishikawa cells and argue that a more in-depth understanding of the molecular mechanisms that drive misregulated NRF2 activation is needed to develop better treatment strategies for the treatment of Type 1 EEC.

2.3 Materials and Methods

2.3.1 Cell culture

Ishikawa cells are a Type 1 endometrial adenocarcinoma cell line isolated from the uterus of a middle-aged female patient. They were purchased from the American Type Culture Collection (ATCC) and were maintained in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS). T-47D and HEK293 cells were also purchased from the ATCC and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. All cell lines were grown at 37°C with 5% CO_2.

2.3.2 Construction of plasmids and transfection of siRNA

The TSC22D4 gene was amplified from HEK293 cDNA and was sub-cloned into a pCMV-FLAG tag expression vector (Clonetech) using the HindIII and Sal1 fast digest restriction enzymes (ThermoFisher Scientific).

The HRE upstream of the TSC22D4 transcription start site was amplified from HEK293 genomic DNA and sub-cloned into the pGL4.22 Luciferase expression vector using the BglII and Kpn1 fast digest restriction enzymes.

Site-directed mutagenesis using PCR and the Dpn1 method has been previously described in order to generate the presented mutations [42]. All constructed plasmids were verified by sequencing.

The siRNA against TSC22D4 was purchased from Qiagen and was transfected using the HiPerFect transfection reagent according to the manufacturer's instructions. All transfections occurred for 72 h.

2.3.3 Dual luciferase assay

T-47D cells were seeded at 80% confluence and left to adhere overnight. Transfection of Firefly luciferase expression constructs (pGL4.22, Promega) containing the wildtype or mutant HREs along with *Renilla* luciferase was performed using the Lipofectamine 3000 reagent according to the manufacturer's instructions. After an 8 h transfection, cells were treated with the indicated doses of MPA for 16 h. Cells were then lysed in 1x passive lysis buffer (Promega) and a dual luciferase assay was performed using a luminometer (Turner BioSystems).

2.3.4 Antibodies and western blot

Antibodies for NRF2, AKR1C1, GCLM, and GAPDH were purchased from Santa Cruz Biotechnology. The antibodies used to detect TSC22D4 and FLAG were purchased from Sigma-Aldrich. For experiments using western blotting, Ishikawa

cells were seeded at 80% confluence and left to adhere overnight. After treatment, the cells were harvested using 1x NuPage LDS Sample Buffer (Invitrogen), boiled for 5 minutes, and were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.5 Immunoprecipitation/ubiquitylation assay

HEK293 cells were seeded at 80% confluence and left to adhere overnight. Cells were then transfected with FLAG-tagged constructs with or without expression of a KEAP1 construct tagged with a chitin-binding domain (CBD) using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. 24 h later, cells were harvested in Radio immunoprecipitation assay (RIPA) buffer containing 10mM sodium phosphate (pH 8.0), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail (PIC) (Sigma) were added to the RIPA buffer. To blot for total lysate, 10% of each sample was taken and mixed with 2x NuPage LDS Sample Buffer (Invitrogen) and boiled for 5 min. Cell lysates for immunoprecipitation were incubated with chitin beads (New England Biolabs) at 4°C overnight with rotation. The next day, the immunoprecipitated complexes were washed with RIPA buffer (plus DTT, PMSF, and PIC) three times and eluted with the addition of sample buffer followed by boiling for 5 min. All samples were resolved by SDS-PAGE and FLAG-tagged constructs were detected with the anti-FLAG antibody.

To detect ubiquitylation of NRF2, HEK293 cells were seeded at 80% confluence and left to adhere overnight. The next day, cells were transfected with HA-tagged NRF2 and with the appropriate FLAG-tagged constructs using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. 24 h later, the cells were treated with 10μM MG132 for 4 h and then harvested in a buffer containing 2% SDS, 150mM NaCl, 10mM Tris-HCl, and 1mM DTT. Cell lysates were boiled immediately for 10 minutes, sonicated, and 10% of each sample was taken and mixed with 2x NuPage LDS Sample Buffer (Invitrogen) for a total lysate blot. Samples were diluted with a Tris-buffered salt (TBS) solution containing 1% Triton-X-100 and immunoprecipitated with HA beads (Sigma) overnight at 4°C with rotation. The following day, immunoprecipitated proteins were subjected to immunoblot analysis using antibodies against FLAG, HA, and ubiquitin.

2.3.6 Real-time quantitative PCR

Total RNA was harvested using the TRIzol reagent (Invitrogen). 1μg of purified RNA was reverse transcribed using the M-MLV Reverse Transcriptase (Promega). Once cDNA was obtained, a PCR reaction was prepared using CYBR Green Mastermix (Fisher Scientific) and the reaction was run on the LightCycler 480 instrument (Roche). The primers for NRF2, AKR1C1, GCLM, NQO1, and GAPDH have been previously reported [53]. The primers designed to detect the TSC22D4 transcript are as follows: F- GACCCCTTCGGAGCAGTAG

and R- GGCTTCCGGAGCCACTAT. Data was expressed as relative mRNA levels and were normalized to GAPDH.

2.3.7 Live cell imaging

Ishikawa cells were seeded at 80% confluence in glass bottom D35 dishes and left to adhere for 24 h. Either a single or dual transfection with the indicated plasmids was performed with the Lipofectamine 3000 reagent according to the manufacturer's instructions. 24 h later, live images were acquired using the Zeiss Observer.Z1 microscope with the Slidebook 4.2.0.11 imaging program (Intelligent Imaging Innovations, Inc.).

2.3.8 MTT/cell viability assay

Ishikawa cells were seeded at approximately 50% confluence in a 96 well plate and left to adhere overnight. After treatment with indicated treatments and doses for 48 h, cell viability was determined via the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. A 2mg/ml stock solution of MTT in 1x phosphate buffered saline (PBS) was prepared fresh, and 20μl was added to each well. The plates were then incubated for 2 h at 37°C. After 5-minute centrifugation, the media was removed and 100μl of isopropanol/HCl was added and the plates were shaken at room temperature for 5 minutes to dissolve the crystals. Absorbance was measured at 570nm using a plate reader (Synergy 2, BioTek). The experiment was conducted with triplicate wells to obtain error bars.

2.4 Results

2.4.1 TSC22D4 up-regulates the NRF2 pathway

Previous proteomic analysis of KEAP1 interacting partners showed that the KEAP1 interaction network is enriched for proteins containing an ETGE motif. In this study, TSC22D4 was shown to bind to KEAP1, but no further analysis was performed. In order to substantiate a relationship between TSC22D4 and NRF2, FLAG-tagged TSC22D4 was exogenously added to Ishikawa cells, and protein levels for NRF2, and its target genes GCLM and AKR1C1 were measured. Overexpression of Wildtype (WT) TSC22D4 led to an increase in NRF2, GCLM, and AKR1C1 protein levels, but not the Mutant (Mut) version of TSC22D4 ('ETGE' to 'AAAA' mutation) (Figure 2.1A). Furthermore, overexpression of TSC22D4 was able to induce the mRNA transcript levels of GCLM and AKR1C1, but not NRF2, indicating that TSC22D4 is not transcriptionally activating NRF2, and that instead amplified NRF2 protein levels induce NRF2-mediated transcription (Figure 2.1B). In an attempt to verify if TSC22D4 binds to KEAP1 in an 'ETGE' dependent manner, an immunoprecipitation against KEAP1 was performed. A KEAP1 construct with a Chitin binding domain (CBD) attached was able to pull down WT TSC22D4, but not Mut TSC22D4, confirming the interaction between KEAP1 and TSC22D4 is ETGE-dependent (Figure 2.1C). Additionally, WT TSC22D4 was found to decrease the endogenous ubiquitylation of HA-tagged NRF2 using an *in vivo* ubiquitylation assay. Moreover, Mut TSC22D4 was

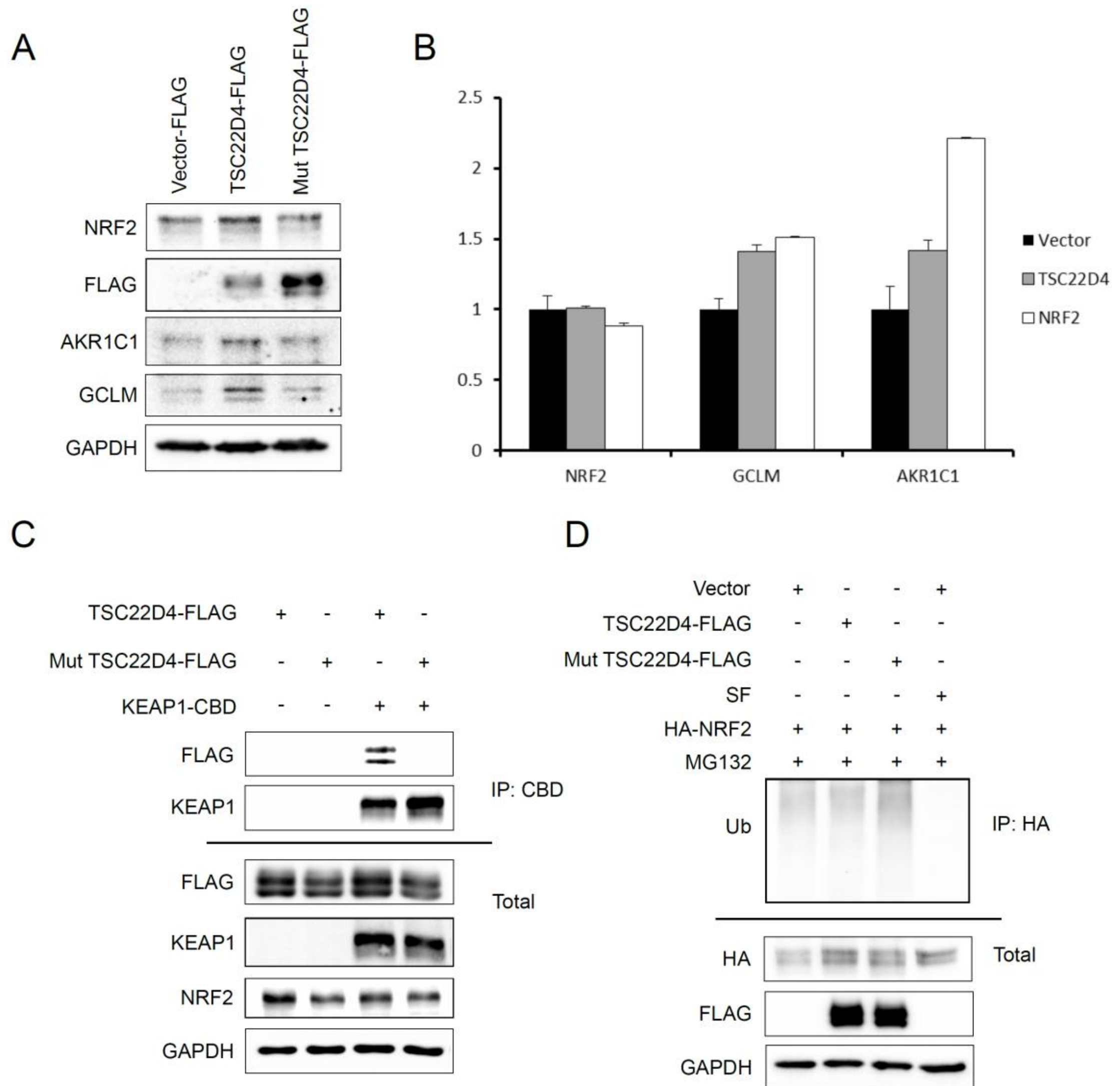

Figure 2.1 TSC22D4 up-regulates the NRF2 pathway. (A) Overexpression of Wildtype (WT), but not the Mutant (Mut), version of FLAG-tagged TSC22D4 up-regulates NRF2 protein levels, as well as the protein levels of GCLM and AKR1C1 in Ishikawa cells 24 h after transfection. (B) Overexpression of FLAG-tagged TSC22D4 for 16 h induces the mRNA transcript levels of GCLM and AKR1C1, but not NRF2. Overexpression of untagged NRF2 was used as a positive control. (C) Immunoprecipitation analysis reveals TSC22D4 can bind to KEAP1 in an ETGE-dependent manner. HEK293 cells were transfected with WT and Mut versions of FLAG-tagged TSC22D4 with or without KEAP1-CBD. KEAP1 was pulled down with chitin beads and the presence of TSC22D4 was determined using an anti-FLAG antibody. (D) WT, but not Mut, TSC22D4 decreases the ubiquitylation of HA-tagged NRF2. HEK293 cells were transfected with the WT and Mut versions of FLAG-tagged TSC22D4 and HA-tagged NRF2. HA-conjugated beads were used to pull down HA-tagged NRF2 and a ubiquitin antibody was used to determine the endogenous ubiquitylation of HA-NRF2.

not able to reduce the ubiquitylation of HA-NRF2. A sulforaphane (SF) treatment (5μM) was used as a positive control (Figure 2.1D).

These results were further confirmed using the WT and Mut versions of TSC22D4 tagged with Green fluorescent protein (GFP). These constructs were overexpressed in Ishikawa cells alone, or co-expressed with Red fluorescent protein (RFP)-tagged versions of either KEAP1 or NRF2. Again, the WT-TSC22D4-GFP, but not Mut-TSC22D4-GFP, was found to co-localize with KEAP1-RFP (Figure 2.2A). Moreover, neither WT-TSC22D4-GFP nor Mut-TSC22D4-GFP was found to co-localize with NRF2-RFP (Figure 2.2A).

2.4.2 TSC22D4 is a Progesterone Receptor responsive gene

TSC22D4 has been identified as a gene that is quickly responsive to certain stressors, but the key regulatory mechanisms that govern this response remain elusive. Previous reports have shown TSC22D1 to be a progestin responsive gene in T-47D cells, but there have been no reports on TCS22D4. *In silico* analysis of the promoter region of TSC22D4 revealed a canonical hormone response element (HRE) 6kb upstream of the transcription start site (Figure 2.3A). Because the PR can recognize this canonical inverted repeat HRE, we treated Ishikawa cells with MPA for 4 h and measured TSC22D4 mRNA and protein levels. Accordingly, MPA treatment increased both the mRNA and protein levels of TSC22D4 in a dose-dependent manner (Figure 2.3B and 2.3C). In order to validate the functionality of the HRE, we sub-cloned a 400bp region containing the HRE upstream of a luciferase expression vector. Transfection of this

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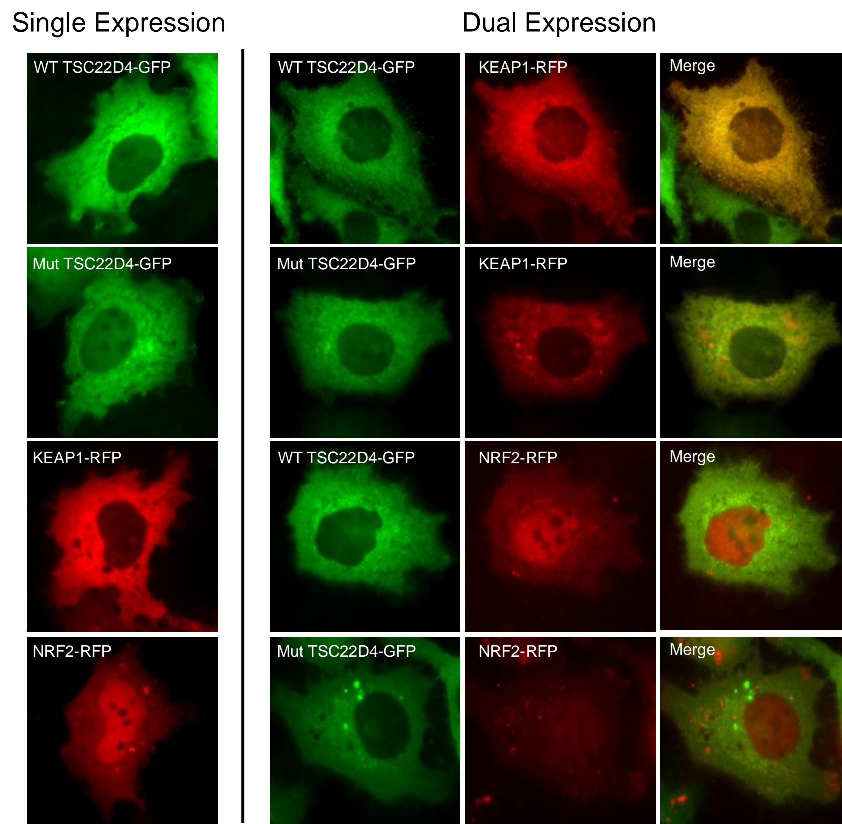

Figure 2.2 WT TSC22D4-GFP, but not Mut TSC22D4-GFP, co-localizes with KEAP1-RFP in Ishikawa cells. WT and Mut TSC22D4 constructs tagged with GFP were overexpressed alone (single expression), or co-expressed with KEAP1-RFP or NRF2-RFP (dual expression). 24 h after transfection, live cell images were acquired with a Zeiss Observer.Z1 microscope. Co-localization was determined by merged images, which give the appearance of a yellow color.

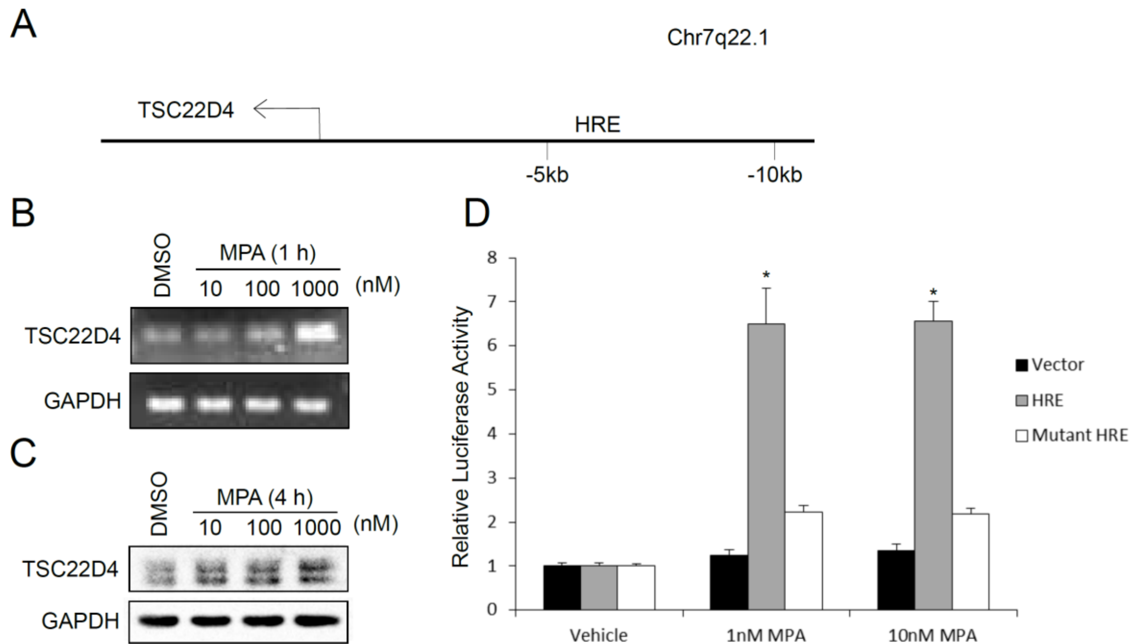

Figure 2.3 TSC22D4 is a Progesterone Receptor responsive gene. (A) *In silico* analysis of the promoter region of TSC22D4 revealed a canonical inverted repeat hormone response element (HRE) approximately 6kb upstream of the transcription start site. (B) Ishikawa cells were treated with 3 doses of MPA for 1 h. PCR of the resulting cDNA product revealed a dose-dependent increase in TSC22D4 transcript in response to MPA treatment. (C) Ishikawa cells were treated with the same three doses from (B) and samples were harvested for western analysis. Similarly, a dose-dependent increase in the protein levels of TSC22D4 was observed within 4 h. (D) To validate the functionality of the identified enhancer sequence, a 400bp region containing the HRE was sub-cloned into a luciferase expression vector (pGL4.22). T-47D cells were transfected with the WT or the mutated version of the HRE and treated with 1 and 10nM MPA for 16 h. The WT HRE was able to significantly induce relative luciferase activity (RLA); however, this effect was abolished with the expression of the Mutant HRE construct.

construct into T-47D cells and subsequent treatment with 1 and 10nM MPA for 16 h showed a 6-fold increase in relative luciferase activity (RLA), which seemed to peak at 1nM MPA, for the construct containing the HRE, but not for the construct containing the mutated version of the HRE (Figure 2.3D).

2.4.3 MPA induces the NRF2-mediated antioxidant response

Given that TSC22D4 was identified as a KEAP1 interacting partner, we predicted MPA, through up-regulation of TSC22D4, could positively regulate the NRF2 pathway. Indeed, MPA (100nM) was found to induce NRF2 protein levels in 4 h, but not at 8 or 12 h (Figure 2.4A). Interestingly, 100nM Dexamethasone (Dex) did not have an effect on NRF2 or TSC22D4 protein levels (Figure 2.4B). To verify that MPA induced NRF2 is TSC22D4-dependent, TSC22D4 was knocked down with siRNA (72 h) and treated with DMSO or 100nM MPA for 4 h. As expected, in the absence of TSC22D4, MPA could no longer induce NRF2 protein levels, indicating TSC22D4 is required for the MPA-dependent induction of NRF2 (Figure 2.4C). In order to address the role of TSC22D4 on the transcriptional activity of NRF2, Ishikawa cells were treated with either non-targeted (NT) siRNA or TSC22D4 siRNA for 72 h. Subsequently, the cells were treated with 10, 100, and 1000nM MPA for 16 h. Accordingly, MPA was able to induce the expression of AKR1C1 and GCLM in a dose-dependent manner in the cells treated with NT siRNA, but MPA was not able to induce the expression of these two NRF2 target genes in the cells treated with TSC22D4 siRNA (Figure 2.4D). Finally, because MPA is a known transcriptional activator, we tested whether or not MPA was

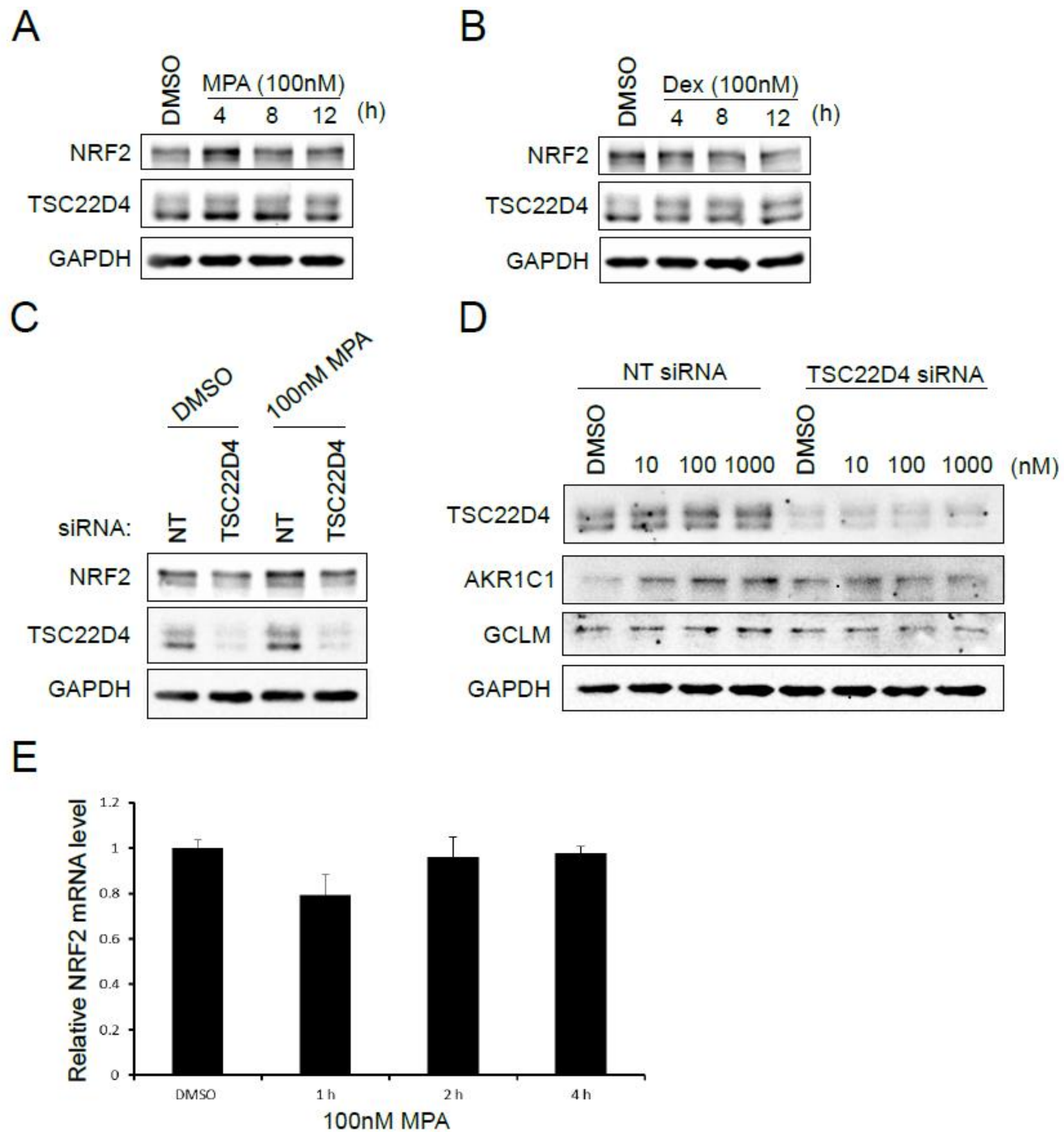

Figure 2.4 MPA induces the NRF2-mediated antioxidant response. (A) Ishikawa cells were treated with 100nM MPA for 4, 8, and 12 h to evaluate the effect on NRF2. 100nM MPA was able to induce NRF2 protein levels at 4 h, but not 8 or 12 h. Similarly, the most robust induction of TSC22D4 protein levels was also found to be at 4 h. (B) 100nM Dexamethasone (Dex) treatment did not induce NRF2 or TSC22D4 protein levels at any time point shown. (C) Ishikawa cells were transfected with non-targeted (NT) or TSC22D4 siRNA (5nM, 72 h) and treated with either DMSO or 100nM MPA for 4 h. Upon knockdown of TSC22D4, MPA was no longer able to induce NRF2 protein levels, indicating that the induction of TSC22D4 by MPA is required to enhance NRF2 protein levels. (D) Ishikawa cells were transfected with either NT or TSC22D4 siRNA (5nM, 72 h) and treated with 10, 100, and 1000nM MPA for 16 h. MPA was able to induce the expression of AKR1C1 and GCLM in a dose-dependent manner in the cells

transfected with NT siRNA, but not in the cells transfected with TSC22D4 siRNA, confirming that the ability of MPA to induce NRF2 target genes is dependent upon the modulation of TSC22D4 protein levels. (E) Ishikawa cells were treated with MPA (100nM) for 1, 2, and 4 h. Quantitative real-time PCR analysis showed no induction of NRF2 mRNA transcript levels, confirming that the effect of MPA on NRF2 protein levels is not through transcriptional up-regulation of *NRF2*.

capable of directly inducing the transcription of NRF2 mRNA. Treatment with MPA (100nM) at 1, 2, and 4 h showed no induction of NRF2 transcript levels, confirming that *NRF2* is not being transcribed and is altered at the protein level (Figure 2.4E).

2.4.4 Knockdown of TSC22D4 inhibits the NRF2 pathway

To further validate the relationship between TSC22D4 and NRF2, we measured NRF2 protein levels following siRNA knockdown of TSC22D4 (72 h). Knockdown of TSC22D4 protein levels produced an observable decline in NRF2 protein levels, as well as the protein levels of GCLM and AKR1C1 (Figure 2.5A). This result was confirmed at the mRNA level using quantitative real-time PCR, which revealed a reduction in NQO1, GCLM, and AKR1C1 mRNA transcript levels, confirming that TSC22D4 is able to regulate NRF2-mediated transcription (Figure 2.5B).

2.4.5 MPA rescues Ishikawa cells from cisplatin toxicity

It has been well established that compounds capable of positively regulating the NRF2 pathway can increase cell survival and protect cells from the detrimental effects of certain toxins. Because MPA was shown to up-regulate the NRF2 pathway, we predicted that MPA treatment could affect Ishikawa cell viability either alone, or in the presence of cisplatin, one of the more aggressive chemotherapies given to patients with Type 1 EEC. Interestingly, when treated with MPA alone, the cell viability of Ishikawa cells was diminished slightly at

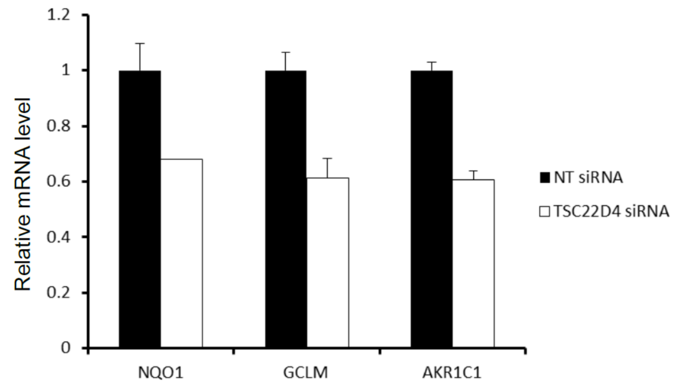

Figure 2.5 Knockdown of TSC22D4 inhibits the NRF2 pathway. (A) Ishikawa cells were transfected with NT or TSC22D4 siRNA (5nM, 72 h) and lysate was collected for western analysis. Depletion of TSC22D4 protein levels caused a decline in NRF2, AKR1C1, and GCLM protein levels. (B) Quantitative real-time PCR also revealed a decline in the NRF2 target genes NQO1, GCLM, and AKR1C1 when Ishikawa cells were transfected with TSC22D4 siRNA (5nM, 72 h).

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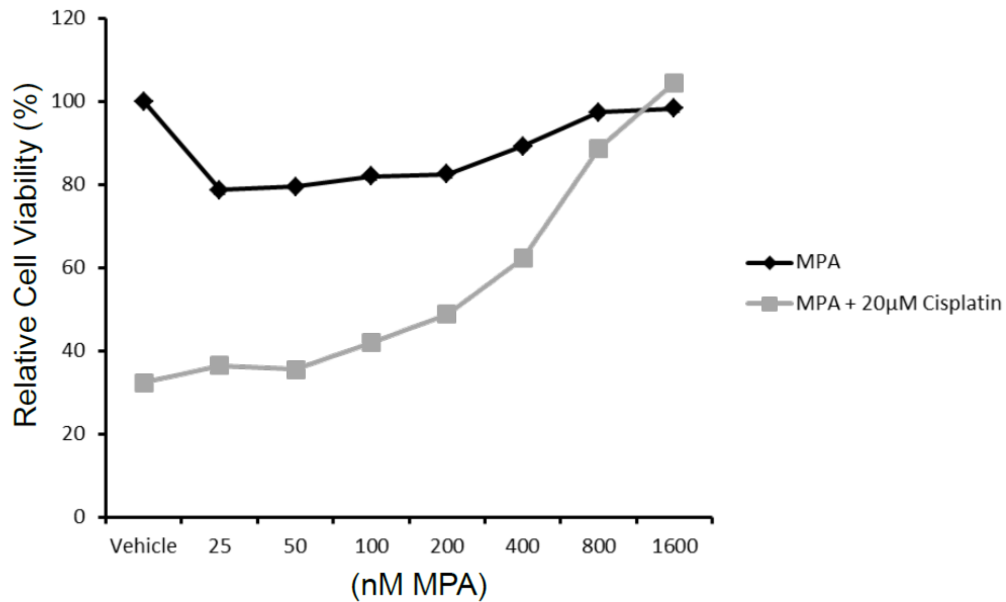

Figure 2.6 MPA rescues Ishikawa cells from cisplatin toxicity. (A) Ishikawa cells were treated with a dose range of MPA alone, or with 20µM cisplatin. The lower doses of MPA (25-200nM) caused a slight decline in cell viability, but the 400-1600nM dose range did not appear to affect viability. 20µM cisplatin alone or co-treatment in the low MPA dose range produced a large decline in cell viability, but as the MPA dose was increased, there was a rescue of cell viability.

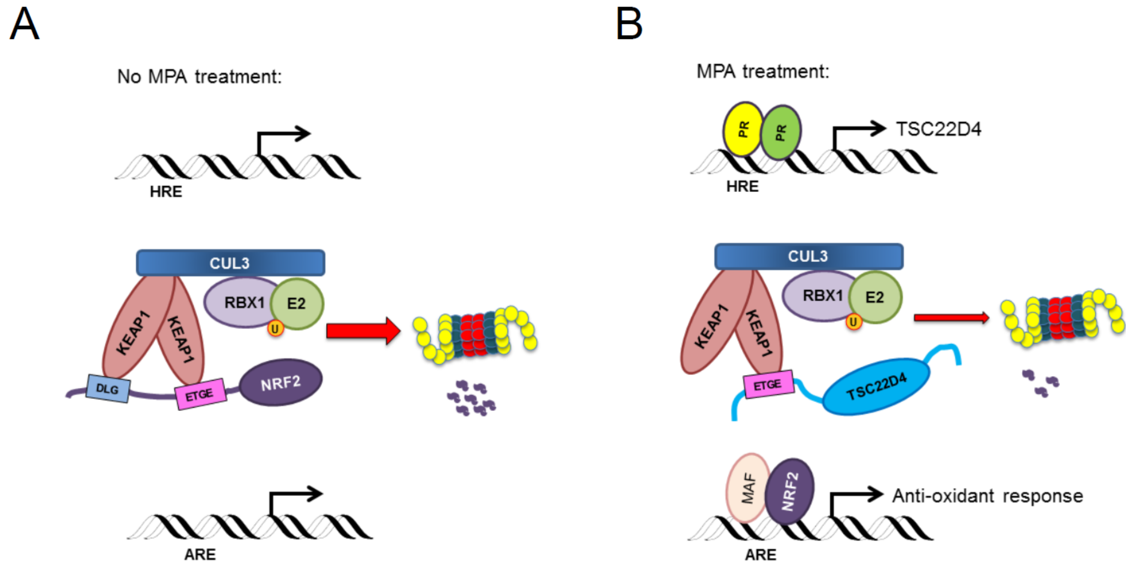

Figure 2.7 Schematic of the proposed mechanism of NRF2 induction by MPA/TSC22D4. (A) Under basal conditions, without the presence of MPA, NRF2 is degraded by the KEAP1-RBX1-CUL3 E3 ubiquitin ligase complex, and NRF2 protein levels remain low in the cell. However, in (B), when cells are exposed to MPA, this activates the transcription and translation of TSC22D4. The presence of an ETGE motif in TSC22D4 forces competition between TSC22D4 and NRF2 for KEAP1 binding. In turn, NRF2 protein levels increase and activate the NRF2-mediated antioxidant response by transcriptionally up-regulating genes containing AREs.

lower doses (25-200nM), but not at higher doses (400-1600nM) (Figure 2.6A). Additionally, MPA treatment was able to dose-dependently rescue Ishikawa cells from the cytotoxic effects of 20μM cisplatin within the higher dose range (Figure 2.6A).

2.5 Discussion

A growing number of reports are continuously revealing a strong role for NRF2 activation in cancer progression. While NRF2 is still regarded as a critical cell-survival mechanism in response to stress, the emergence of 'the dark side of NRF2' has lately earned more interest. Determining the molecular events that lead to aberrant NRF2 activation have proven vital in order to overcome deregulated NRF2 signaling and rescue cells from malignant transformation. Most commonly, somatic mutations occur in either *NRF2* or *KEAP1* coding regions, which ultimately leads to constitutively high NRF2 protein levels. DNA sequencing of endometrioid endometrial adenocarcinoma tumors from patients has uncovered a common single nucleotide polymorphism in KEAP1 that correlates to an impaired KEAP1-NRF2 interaction [144]. Additionally, high NRF2 expression is a key feature in Type 2 ESC that confers chemoresistance to therapy in comparison to Type 1 EEC, which has a lower expression of NRF2, but the reason for this is not well understood [135]. Likewise, AKR1C1 was recently identified as a potential mediator of progestin resistance in Type 1 EEC; however, these results are confounding, as both the PR and NRF2 can direct AKR1C1 transcription [136]. Because NRF2 activation is associated with tumor

resistance to chemotherapy, genetic screening for mutations or an understanding of the molecular mechanisms of NRF2 activation may offer key prognostic insights for the treatment strategies concerning endometrial cancer.

In the present study, we have identified a novel mechanism of NRF2 regulation in Type 1 EEC. Because there is mounting evidence to support the fact that 'ETGE'-containing proteins are capable of up-regulating NRF2, Hast *et. al.* performed proteomic analysis of KEAP1 and revealed that the KEAP1 interaction network is enriched for proteins containing 'ETGE' or 'ETGE'-like motifs. Among those associated with KEAP1 was TSC22D4. Here, TSC22D4 was found to induce protein levels of NRF2, as well as the NRF2 target genes AKR1C1 and GCLM. Furthermore, mutation of the 'ETGE' motif to 'AAAA' was able to abolish the interaction between TSC22D4 and KEAP1, strongly supporting that TSC22D4 may have a role in cancer progression because of its ability to deregulate the NRF2 transcriptional program. Our group has previously reported a similar 'non-canonical' mechanism of NRF2 regulation whereby p62, a cargo adaptor protein, can also bind to KEAP1 in a similar manner. p62 shuttles KEAP1 into forming autophagosomes, removing it from the cytosol, inducing a robust increase in NRF2 protein levels [60]. Here, we observe a similar, albeit less robust increase in NRF2 protein levels potentially due to the retention of KEAP1 in the cytosol.

Interestingly, previous reports on TSC22D4 have mainly focused on its ability to repress transcription when bound to itself or TSC22D1 [138]. Here, we show evidence that overexpression of TSC22D4 can indirectly activate ARE-

mediated transcription. Currently, the role of TSC22D4 in the cell has focused on certain stressors such as neuronal apoptotic conditions or osmotic stress, in addition to TGFβ stimulation, however, there have not been any reports indicating that TSC22D4 may respond to oxidative stress or contribute to the progression of cancer [140,145]. Despite this, an early report illustrated that TSC22 (now TSC22D1) was a novel progestin-responsive gene in T-47D cells [143]. Because of the relationship between TSC22D1 and TSC22D4, we suspected that TSC22D4 could also be regulated by progestins. Indeed, TSC22D4 mRNA was responsive to MPA in 1 h in a dose-dependent manner and TSC22D4 protein levels were induced by MPA in as little as 4 h, also in a dose-dependent manner. This induction of TSC22D4 also coincided with an increase in NRF2 protein levels. We also show that the ability of MPA to induce NRF2 protein levels and NRF2 target gene expression is diminished when TSC22D4 protein levels are significantly reduced.

With the identification of a functional HRE 6kb upstream of its transcription start site, TSC22D4 was identified to be a direct PR target gene. Yet, the identification of the HRE which, to our knowledge has not been reported on, is of particular interest given that there is potential for TSC22D4 to be regulated not only by the PR, but also the Glucocorticoid Receptor (GR), Androgen Receptor (AR), or the Mineralocorticoid Receptor (MR), depending on cellular context, because all of these hormone receptors share the same canonical HRE inverted repeat sequence. In the present report, we did not observe an activation of TSC22D4 by the GR agonist Dex, however, GR expression or activity was not

established in Ishikawa cells. Further work on this topic will be needed in order to establish relevant cell culture models to study steroid hormone-dependent regulation of TSC22D4. Moreover, the insights gained from future studies will reveal further relationships between steroid hormone signaling and NRF2 activation.

Currently, there is little known regarding the relationship between PR activation and NRF2 signaling, however, there are select reports showing that NRF2 is repressed by other nuclear receptors such as the GR and the Estrogen receptor (ER) [146]. Our report that activation of the PR through MPA treatment up-regulates NRF2 activity provides evidence of a molecular mechanism of NRF2 regulation by a standard hormonal therapy used for the treatment of EEC. In the future, additional studies should assess the long-term effects of MPA and NRF2 activation and determine if misregulated NRF2 is a causative factor in malignant transformation over time. If so, therapeutic intervention with NRF2 inhibitors should potentially be used to suppress this effect.

In conclusion, we report a novel mechanism of NRF2 regulation in Type 1 EEC with the identification of TSC22D4 as a KEAP1 binding partner. The TSC22D4-KEAP1 interaction prevents KEAP1-mediated degradation of NRF2, thereby inducing the antioxidant response in Ishikawa cells. Additionally, we show that TSC22D4 is a novel PR target gene with the identification of a functional HRE 6kb upstream of its transcription start site. There are therapeutic implications associated with these findings because of the fact that patients with Type 1 EEC can receive MPA as a hormonal therapy in lieu of more aggressive

chemotherapeutic regimens. The fact that MPA treatment has been shown to increase NRF2 protein levels and activity should be considered because NRF2 activation is associated with chemo- and radioresistance and cancer progression.

2.6 Acknowledgements

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Chapter 3

Brusatol overcomes chemoresistance through inhibition of protein translation

Text and figures derived from Harder B. *et al.* 2017 *Mol Carcinog.* 56(5):1493-1500

Bryan Harder, Wang Tian, James J. La Clair, Aik-Choon Tan, Aikseng Ooi, Eli Chapman, and Donna D. Zhang

3.1 Abstract

The NRF2 pathway activates a cell survival response when cells are exposed to xenobiotics or are under oxidative stress. Therapeutic activation of NRF2 can also be used prior to insult as a means of disease prevention. However, prolonged expression of NRF2 has been shown to protect cancer cells by inducing the metabolism and efflux of chemotherapeutics, leading to both intrinsic and acquired chemoresistance to cancer drugs. This effect has been termed the "dark side" of NRF2. In an effort to combat this chemoresistance, our group discovered the first NRF2 inhibitor, the natural product brusatol, however the mechanism of inhibition was previously unknown. In this report, we show that brusatol's mode of action is not through direct inhibition of the NRF2 pathway, but through the inhibition of both cap-dependent and cap-independent protein translation, which has an impact on many short-lived proteins, including NRF2.

Therefore, there is still a need to develop a new generation of specific NRF2 inhibitors with limited toxicity and off-target effects that could be used as adjuvant therapies to sensitize cancers with high expression of NRF2.

3.2 Introduction

Chemoresistance to current drug regimens for the treatment of cancer has become a major health concern leading to the usage of highly cytotoxic chemicals with many unwanted side effects. The molecular mechanisms that lead to chemoresistance are not well understood, resulting in the continued reliance on broad-spectrum, highly toxic chemotherapeutics. There are a number of potential avenues to combat chemoresistance. One is through personalized medicine, which would foster the development of highly-specific targeted therapies based on the molecular profile of each individual patient's cancer. This means that the treatment would be more effective in a shorter amount of time due to the decreased likelihood that chemoresistance may occur. A second approach would be to develop a better molecular understanding of the mechanisms underlying chemoresistance and develop adjuvant therapies to increase the efficacy of chemotherapeutics [147]. In order to address this second option for targeted therapy, we developed the first inhibitor of the nuclear factor-erythroid factor 2-related 2 (NRF2) pathway based on the discovery that high levels of NRF2 are associated with resistance to chemotherapeutics [107].

NRF2 is a redox-sensitive transcription factor that maintains the crucial intracellular reductive/oxidative (redox) balance of the cell. To do so, it up-

regulates genes that are involved in phase I and II drug metabolism, glutathione synthesis, and xenobiotic drug transport [148]. Because of the critical role played by NRF2 in cellular protection, timely activation by NRF2 is needed. As a result, NRF2 is constantly translated by the cell; however, under stress-free conditions, when NRF2 activation is not needed, its negative regulator KEAP1 constantly targets NRF2 for degradation [39,40]. KEAP1 is a substrate adaptor protein that is part of an E3 ubiquitin ligase complex that polyubiquitylates NRF2 and targets it for degradation by the 26S proteasome [39,40]. A critical cysteine residue in KEAP1, cysteine 151 (Cys151), can become oxidized or covalently modified by reactive oxygen species (ROS) or electrophiles, causing a conformational change in KEAP1, preventing polyubiquitylation and subsequent degradation of NRF2 [42]. Newly synthesized NRF2 then accumulates in the cytosol and translocates into the nucleus, where it forms a heterodimer with small MAF proteins, binds to an enhancer sequence, the antioxidant response element (ARE), in the promoter region of its target genes, and promotes the antioxidant response.

Early investigations determined that up-regulation of NRF2 with dietary phytochemicals (sulforaphane, cinnamaldehyde, etc.) could protect against cancer and other diseases, prompting the search for NRF2-inducing compounds [79,149]. However, the recognition that certain cancers overexpress NRF2, either through somatic mutations or epigenetic silencing of key negative regulators, and that chronic upregulation of NRF2 can lead to tissue damage and cancer progression, led to the concept of the “dark side” of NRF2 [107,148,150]. To

overcome this “dark side”, our lab identified the first NRF2 inhibitor, brusatol, and proved that inhibition of NRF2 sensitized non-small cell lung cancer (NSCLC) cells to cisplatin treatment, making combination therapy with an NRF2 inhibitor a promising new initiative for the treatment of cancer with high levels of NRF2 [126]. While treatment with brusatol, a quassinoid compound extracted from *Brucea javanica*, was effective in the nanomolar range at inhibiting NRF2 signaling, the mode of action was unknown [126]. Despite this, brusatol was shown to be effective in enhancing the antitumor action of cisplatin in a mutant KRAS G12D-induced lung cancer model [71]. In the present study, the mode of action of brusatol as an NRF2 inhibitor was determined. Brusatol was found to be a general translation inhibitor, which causes a decline in the protein levels of short-lived proteins, including NRF2. While these findings do not negate previous studies showing inhibition of NRF2 by brusatol as an effective strategy to sensitize cancers to chemotherapies, they do argue for the development of new inhibitors with heightened specificity for inhibition of the NRF2 pathway.

3.3 Materials and Methods

3.3.1 Cell culture

A549 non-small cell lung carcinoma (NSCLC) cells were purchased from the American Type Culture Collection (ATCC) and were maintained with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2.

3.3.2 RNA-seq profiling

A549 cells were plated at 80% confluence in D35 dishes and left to adhere overnight. Cells were treated (in duplicate) with 40 nM brusatol for 16 h and total RNA was extracted as previously described [72]. RNA-sequencing analysis was performed as previously described by The Genomics and Microarray Shared Resource at the University of Colorado [117]. A 5% false discovery rate (FDR) cut off was applied.

3.3.3 Fluorescent brusatol probe

The Immunoaffinity (IAF) tag was synthesized as previously described [151]. Briefly, a) brusatol (1) was treated with *t*-butyl-bromoacetate in basic dimethylformamide (DMF) and then b) deprotected in tri-fluoroacetic acid (TFA) to yield (2). c) Coupling with IAF tag (3) was carried out by treatment with HATU in $EtN^{i}Pr_2$. A549 cells were treated with the brusatol-IAF probe (1 μM) for 4 h, which was followed by the addition of staining markers for the lysosome (LysoTracker red, Life Science Technology), endoplasmic reticulum (ER tracker, ThermoFisher Scientific), and Golgi (Golgi tracker, ThermoFisher Scientific) for 15 min. Live cell images were acquired with a deconvolution microscope.

3.3.4 Antibodies and western blot

Antibodies for NRF2, p53, p21, p97 (VCP), and GAPDH were purchased from Santa Cruz Biotechnology. The antibody used to detect GFP was purchased from GeneTex. For western blot experiments, A549 cells were seeded at 80%

confluence and left to adhere overnight. After the indicated treatments, cells were harvested in 1x NuPAGE LDS Sample Buffer (Invitrogen) and subjected to SDS-polyacrylamide gel electrophoresis.

3.3.5 Dual reporters for translation inhibition

The FF-Ren and FF-EMCV IRES-Ren plasmids were a kind gift from Dr. Jerry Pelletier (McGill University). The mRFP-IRES-GFP plasmid was constructed from the pIRES-EGFP-Puro construct acquired from Addgene (Plasmid #45567). Briefly, mRFP from a previous construct was sub-cloned within the multiple cloning site using the Xho1 and Nhe1 digestion enzymes.

For dual luciferase experiments, A549 cells were seeded at 80% confluence and left to adhere overnight. Plasmid transfection for FF-Ren and FF-EMCV IRES-Ren was performed using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. After a 4 h transfection, the media/transfection reagent was removed and the cells were treated with indicated compounds in normal media for 16 h. Cells were lysed in a 1X passive lysis buffer (Promega) and a dual luciferase assay was performed with a luminometer (Turner BioSystems). The experiment was repeated 3 times, with triplicate wells, for acquisition of statistical significance as reported by the standard error of the mean.

For the dual fluorescence reporter (mRFP-IRES-GFP), A549 cells were seeded at 80% confluence in glass bottom D35 dishes and left to adhere overnight. Transfection of the mRFP-IRES-GFP plasmid was performed as

mentioned previously for 4 h and treatment occurred for 16 h. After treatment, live cell images were acquired using the Zeiss Observer.Z1 microscope with the Slidebook 4.2.0.11 imaging program (Intelligent Imaging Innovations, Inc.).

3.4 Results

3.4.1 RNA-seq profiling reveals similar gene set enrichment patterns between brusatol and other translation inhibitors

The effects of brusatol on gene expression in A549 cells, a NSCLC cell line that has high, constitutive NRF2 expression, were analyzed by RNA-seq. These results were compared to publicly available gene enrichment data sets for a variety of different treatments, such as chemotherapeutics, hormones, cytokines, and other signaling factors, to try to infer a mechanism of action for brusatol. Brusatol treatment in A549 cells up-regulated the expression of 2,914 genes and down-regulated the expression of 2,991 genes. The top 10 up- and down-regulated genes are shown in Table 3.1. Interestingly, when comparing the gene expression modulation of brusatol with other compounds, brusatol shared a similar gene-set enrichment pattern with the translation inhibitor cycloheximide (Figure 3.1A). A more in-depth analysis revealed that brusatol and cycloheximide shared 1,339 up-regulated and 1,320 down-regulated genes (Figure 3.1B). As a follow-up, brusatol was compared to ricin and puromycin, two other translation inhibitors with distinct mechanisms of action. Brusatol also showed a similar gene-set enrichment pattern to both inhibitors (Figure 3.1C). Gene-sets that were shared between brusatol and ricin (176 genes up-regulated and 175 down-

TABLE 1 Top 10 gene expression changes associated with brusatol treatment in A549 cells

	ID	Base mean	Fold change	log2 Fold change	P value	P adjusted
Up-regulated	DUSP27	126.1137	506.8652	8.985458	0.002388	0.010455
	ATP10B	78.41707	314.7887	8.29824	0.005938	0.022985
	CYP1A1	75.48413	302.9777	8.243068	2.58E-14	6.45E-13
	MAL2	259.7108	270.6116	8.08008	0.001155	0.005488
	SEMA5A	54.76455	219.5391	7.778334	1.65E-10	2.67E-09
	ASCL2	42.4191	169.8235	7.407892	9.95E-08	1.08E-06
	SPINK1	233.5567	155.7571	7.283154	0.000276	0.001527
	PPP1R1B	449.7914	143.133	7.161213	4.15E-05	0.000276
	SLC5A1	85.09216	113.2232	6.823026	8.87E-05	0.000549
	ACE2	51.0301	101.7502	6.668887	4.41E-09	5.84E-08
Down-regulated	NR2E3	29.39972	0.0068	−7.20022	3.75E-06	3.08E-05
	GSTA2	158.6975	0.010111	−6.62789	1.68E-05	0.000122
	SNORD116-17	15.97673	0.015506	−6.011	0.001941	0.008685
	SNORD116-19	15.97673	0.015506	-6.011	0.001941	0.008685
	LOC93432	77.42563	0.031753	−4.97695	0.002499	0.010873
	SERPING1	15.76144	0.033605	−4.89518	0.002523	0.010955
	LDLRAD1	15.76144	0.03928	−4.67005	0.009018	0.033027
	PLCB2	18.31799	0.041617	−4.5867	0.002526	0.010963
	SEC14L3	36.5122	0.04227	−4.56423	0.001689	0.007693
	GSTM5	104.4568	0.046495	−4.42679	1.24E-12	2.59E-11

Table 3.1 Top 10 gene expression changes associated with brusatol treatment in A549 cells.

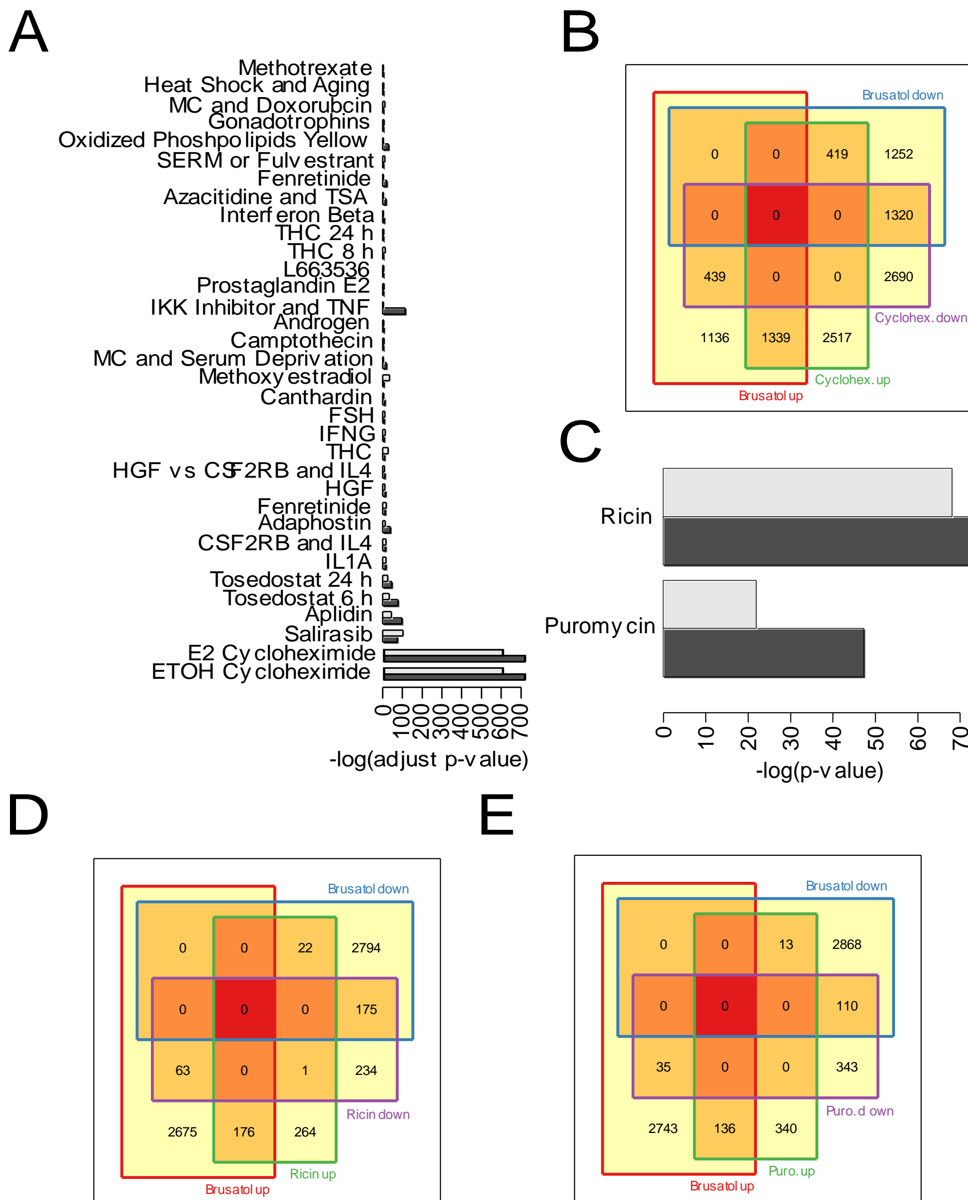

Figure 3.1 RNA-seq profiling reveals similar gene set enrichment patterns between brusatol and other protein translation inhibitors. (A) Gene sets that were up-regulated (light gray) and down-regulated (dark gray) were acquired and compared to gene sets altered by brusatol treatment. The –log of the fold change of each treatment was plotted and compared to brusatol. (B) A Venn diagram representing the up- and down-regulated genes shared by both brusatol and cycloheximide. (C) Brusatol-modulated gene expression was compared to that of the translation inhibitors, ricin and puromycin, and the –log of the fold change caused by these treatments was also plotted and compared to brusatol. (D and E) Venn diagrams show the up- and down-regulated genes shared by the treatment of brusatol and ricin (D) and brusatol and puromycin (E).

regulated) (Figure 3.1D) or brusatol and puromycin (136 up-regulated and 110 down-regulated) (Figure 3.1E) treatment were found to be equally significant, considering that ricin and puromycin regulated a smaller subset of genes compared to cycloheximide. These results indicated that brusatol might function as a protein translation inhibitor. Additionally, RNA-seq profiling after brusatol treatment revealed a 0.6 fold increase in NRF2 mRNA transcript levels. This phenomenon likely occurs as a compensation for the rapid loss of the NRF2 protein, and has been previously reported [152].

3.4.2 Brusatol localizes to the endoplasmic reticulum

Given that the effects of brusatol treatment resembled that of other established protein translation inhibitors, we attempted to determine if brusatol co-localizes in the cell to a site of active translation. In order to do this, brusatol was chemically modified with a fluorescent immunoaffinity (IAF) tag to track its subcellular location using live cell fluorescent microscopy (Figure 3.2A). After a mixture of two distinct probes (henceforth brusatol-IAF) was successfully synthesized, A549 cells were treated with increasing concentrations of the brusatol-IAF mixture for 4 h to test if the tag affected the drug's potency. As shown in Figure 3.2B, addition of the tag did not alter the native action of brusatol, since the inhibitory effect of 40nM brusatol-IAF on NRF2 was similar to that of 40 nM brusatol. Moreover, there was a dose-dependent reduction of NRF2 protein levels by brusatol-IAF. Next, the localization of brusatol-IAF was tested. A549 cells were treated for 4 h with brusatol or brusatol-IAF (1μM), followed by the addition of fluorescent

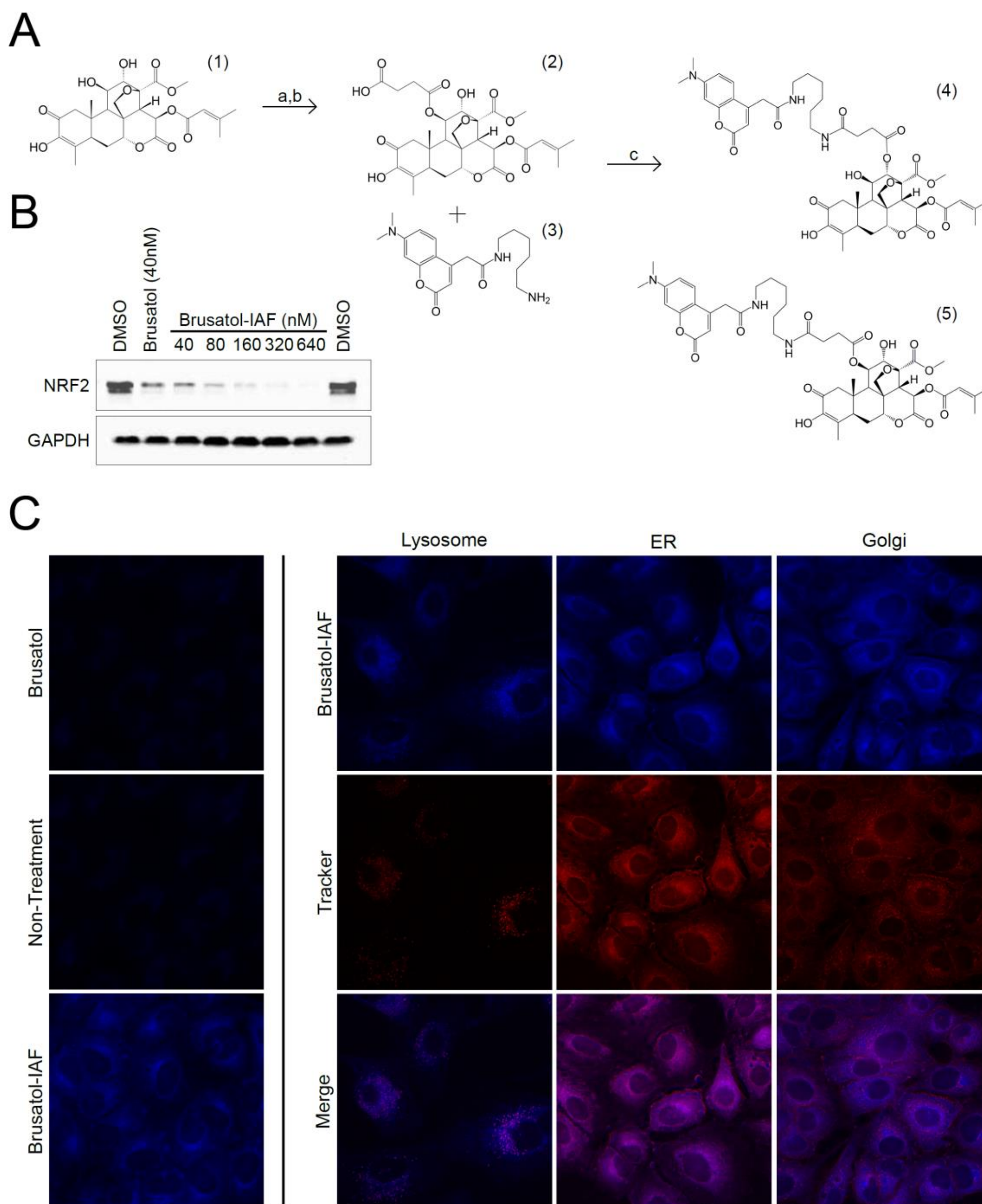

Figure 3.2 Brusatol localizes to the endoplasmic reticulum. (A) Synthesis of the brusatol-IAF probe. Reagents and conditions: a) *t*-butyl-bromoacetate, K_2CO_3, DMF, rt, 65%; b) then TFA, CH_2Cl_2 to cleave t-butyl ester; c) tag (3) HATU, EtN^iPr_2, DMF, rt, 53%. Overall yield 34%. (B) A549 cells were treated with the indicated doses of brusatol-IAF for 4 h to determine if the probe maintained the native activity of brusatol, inhibition of NRF2 protein levels. (C) Images of live A549 cells obtained using deconvolution microscopy show brusatol-IAF localizes to the ER over the Golgi apparatus or lysosomes.

probes that selectively stain the lysosome, ER, or Golgi. Live cell images were acquired via deconvolution microscopy and the merged images between brusatol-IAF and the different organelle tracker dyes indicated that brusatol-IAF localized to the endoplasmic reticulum (ER) (Figure 3.2C). Treatment with non-tagged brusatol was used as a negative control, and no autofluorescence was observed.

3.4.3 Inhibition of cap-dependent and cap-independent translation by brusatol

Since brusatol mimics the effects of other translation inhibitors and concentrates to the ER, the ability of brusatol to inhibit cap-dependent or cap-independent translation was measured. A549 cells were transfected with a plasmid that expresses mRFP under the control of a cytomegalovirus promoter (CMV, cap-dependent translation) and GFP under the control of an internal ribosomal entry site (IRES, cap-independent) (Figure 3.3A) and then treated with brusatol, cycloheximide (CHX, both cap-dependent and cap-independent inhibitor), and rapamycin (cap-dependent inhibitor). Brusatol and CHX abolished the expression of mRFP, and significantly reduced the GFP signal, as analyzed by live cell fluorescent microscopy (Figure 3.3B) and Western blot (Figure 3.3C). However, rapamycin only inhibited the expression of mRFP without affecting GFP expression (Figure 3.3B and 3.3C). In addition to GFP, the levels of the cap-dependent proteins NRF2 and p53, were shown to decrease with brusatol or CHX treatment (Figure 3.3C). Furthermore, the inhibitory effects of brusatol were quantified using the Firefly (FF) and *Renilla* (Ren) dual reporter constructs in

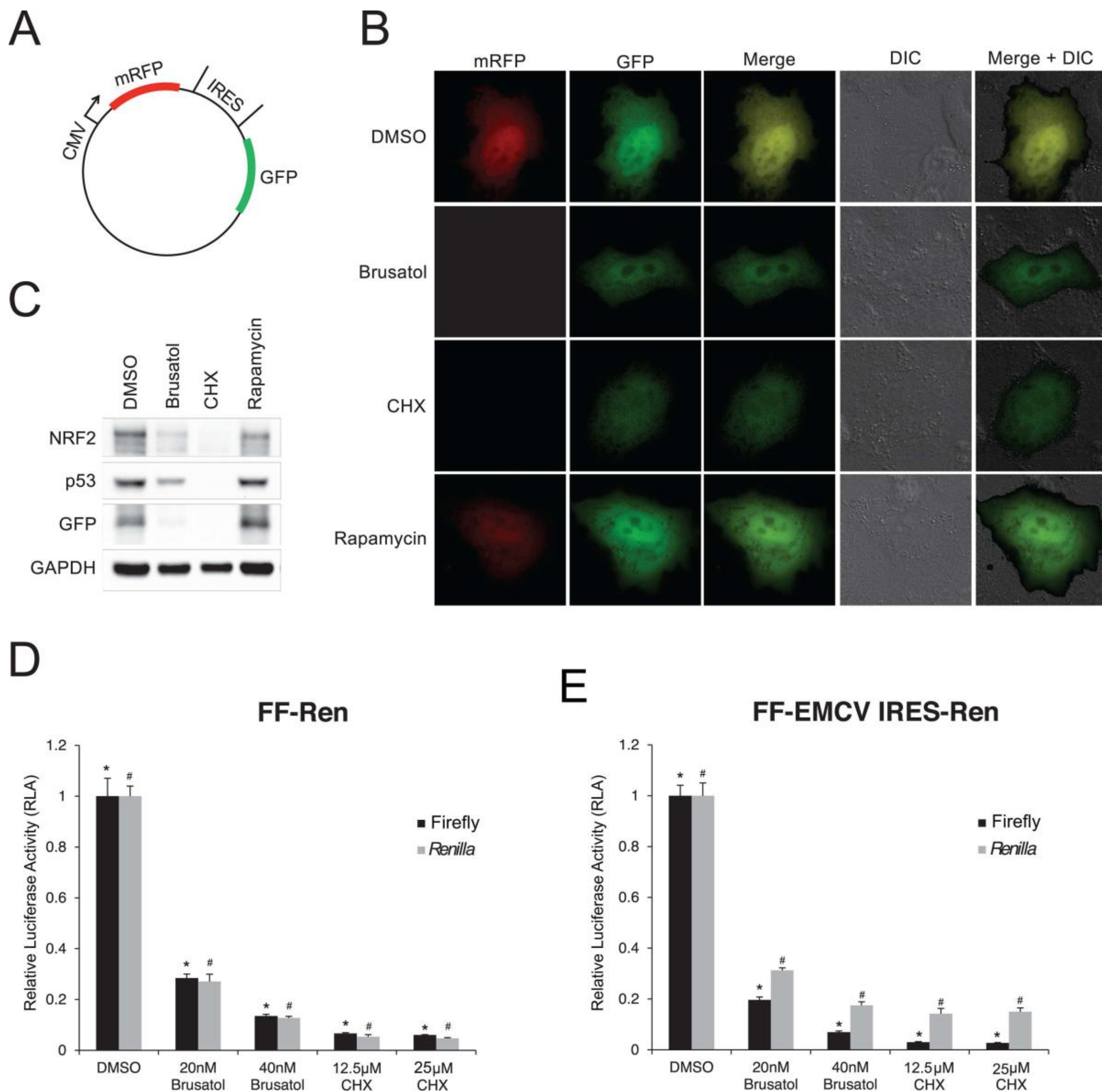

Figure 3.3 Inhibition of cap-dependent and cap-independent translation by brusatol. (A) Plasmid map of the dual fluorescent reporter constructed for live cell visualization (mRFP, cap-dependent translation; GFP, cap-independent translation). (B) Live-cell images of A549 cells after 16 h of indicated treatments. Differential interference contrast (DIC) images were also taken in conjunction with fluorescent images to define the cell body. (C) The experiment from (B) was performed under identical conditions, except cells were plated in D35 dishes and subjected to western blotting. (D and E) Brusatol and cycloheximide (CHX) inhibited the expression of both Firefly (FF) and *Renilla* (Ren) in a dose-dependent manner in both of the FF-Ren and FF-EMCV IRES-Ren constructs, indicating that brusatol inhibits cap-dependent and cap-independent translation. Data are shown as the mean ± SEM (n=3 independent groups) and the * (for Firefly) and # (for *Renilla*) symbols indicate p<0.05 vs control.

A549 cells. In the FF-Ren construct, a T3 RNA polymerase promoter drives the expression of a FF-Ren fusion transcript that is translated in a cap-dependent manner. Conversely, in the FF-EMCV IRES-Ren construct, FF is translated in a cap-dependent manner (T3 promoter), but Ren is translated in a cap-independent manner due to the EMCV (Encephalomyocarditis virus) IRES upstream of it [153]. In accordance with the fluorescence reporter data, brusatol indeed inhibited the expression of both FF and Ren, as indicated by Relative Luciferase Activity (RLA), in both constructs in a dose-dependent manner, while the maximum inhibitory effect of CHX seemed to be reached at 12.5μM, since 25μM CHX did not further decrease FF and Ren expression (Figure 3.3D and 3.3E). Together, these results indicated that brusatol inhibits both cap-dependent and cap-independent translation.

3.4.4 Brusatol inhibits the expression of short-lived proteins

Having identified that brusatol localized to the ER and inhibited both cap-dependent and cap-independent protein translation, it was predicted that brusatol would affect short-lived endogenous proteins or other endogenous proteins that are constantly translated. Therefore, the dose-dependent inhibitory effects of brusatol on NRF2, other short-lived proteins (p53 and p21), or long-lived proteins (the AAA+ chaperone p97 and GAPDH) were tested [126,154,155]. Bruceantin, another translation inhibitor with a chemical structure similar to that of brusatol was also tested. After a 4 h treatment, both brusatol and bruceantin reduced the protein levels of NRF2, p53, and p21 in a dose-dependent manner, while the

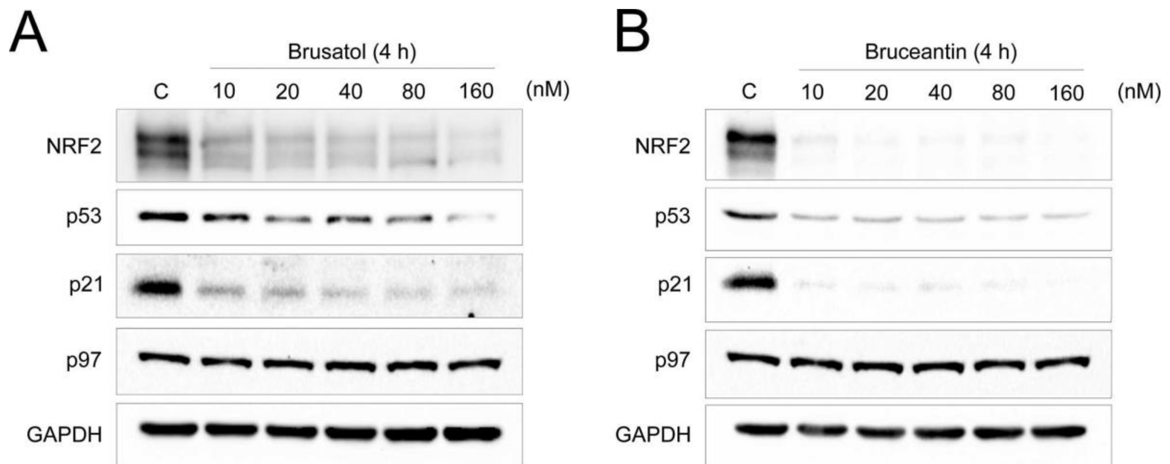

Figure 3.4 Brusatol inhibits the expression of short-lived proteins. (A and B) Western blot analysis of short- (NRF2, p53, and p21) and long-lived (p97 and GAPDH) proteins in A549 cells after treatment with brusatol (A) and bruceantin (B) for 4 h.

expression of p97 and GAPDH were not affected (Figure 3.4A and 3.3B). Taken together, these results indicate that brusatol inhibits NRF2 and other short-lived, constantly translated proteins through inhibition of protein translation.

3.5 Discussion

There is substantial evidence indicating that NRF2 up-regulation prevents cancer initiation, which has encouraged the development of NRF2 inducers, such as those found in fruits, vegetables, and other sources of natural products [79]. However, high constitutive levels of NRF2 can confer chemoresistance to cancer cells, the dark-side of NRF2, which argues for the development of NRF2 inhibitors. After successfully identifying the first inhibitor, brusatol, we provided experimental evidence demonstrating that suppressing the NRF2 pathway leads to chemosensitization in a variety of cancer cell lines, and enhances the efficacy of cisplatin using the KRAS-G12D induced murine lung tumor model *in vivo* [71]. Given that it was the first of its class, we set out to elucidate the mechanism of action of brusatol in regulating NRF2 levels using A549 cells which have constitutively high NRF2 due to a mutation in KEAP1 [108].

In this study, we identified brusatol as a potent inhibitor of protein translation. RNA-seq profiling was initially used to assess changes to the transcriptome following brusatol treatment. Brusatol was shown to induce gene expression changes similar to those of cycloheximide, a known inhibitor of the translational elongation step during protein synthesis. Brusatol also showed a similar gene set enrichment pattern to ricin and puromycin, two other translation

inhibitors, suggesting that brusatol may have a greater effect on the proteins with short half-lives.

In an effort to identify the target of brusatol, we modified brusatol with an immunoaffinity fluorescent tag (IAF), a method that has been previously reported to be effective for real time visualization of the subcellular localization of the tagged compound, and for identification of target proteins by immunoprecipitation using an antibody targeting the fluorescent probe [151]. Localization of brusatol-IAF to the ER, coupled with the gene set enrichment patterns shared between brusatol and other translation inhibitors, suggested that brusatol could be concentrating to ribosomes, since the majority of translation occurs at the ER. Consistent with the notion that brusatol may be an inhibitor of protein translation, early reports on brusatol and bruceantin claimed that these drugs inhibit the peptidyl transferase reaction in biochemical assays [156-159]. Furthermore, a crystal structure of a partial ribosome bound to bruceantin was reported in 2009, and molecular footprinting data suggested that bruceantin binds to specific nucleotides within the A-site of the ribosome, some of which are conserved between eukaryotes, prokaryotes, and archaea [160]. Most recently, using a mass spectrometry profiling approach, it was reported that brusatol is an inhibitor of proteins with short half-lives [161].

An interesting feature of brusatol is that its EC_{50}, the effective concentration in reducing NRF2 protein levels to 50%, is 40nM in most cancer cell lines tested. However, previous studies performed in rabbit reticulocyte lysate utilized brusatol in micromolar concentrations [156-159]. In order to

address this finding, we also performed an *in vitro* transcription and translation assay and determined that brusatol inhibited translation *in vitro* with an EC_{50} of 1 µM, consistent with previous reports (data not shown). The large discrepancy between effective doses *in vitro* may be due to the fact that brusatol concentrates to the ER following cellular uptake (Figure 3.2C). Moreover, a previous structure-activity relationship study indicates that the hydrophobic side chain of related quassinoids is responsible for their cellular uptake and retention [156], supporting the hypothesis that brusatol and bruceantin concentrate to the ER. This allows for a lower effective concentration in live cells versus cellular lysate or biochemical assays, which are more dilute systems with a variable concentration of translational machinery compared to live cells. Additionally, brusatol blocks both cap-dependent and cap-independent translation, arguing against inhibition of PKR-like endoplasmic reticulum kinase (PERK), a membrane bound kinase in the ER that can inhibit cap-dependent translation through phosphorylation of eIF2α. It is now clear that brusatol is a global translation inhibitor that selectively targets short-lived proteins, including NRF2.

The unwanted side-effect of global protein translation inhibition makes the development of brusatol into a commonly used adjuvant for chemosensitization less desirable. Thus, there is once again a need to develop next generation inhibitors that specifically target the NRF2 pathway. There have been a growing number of reports of NRF2 inhibitors, however, many of these target upstream or unknown factors that may result in other off-target effects [75,129,162]. Encouragingly, a recently reported compound called ML385 was found to bind to

the Neh1 domain of NRF2 and inhibit NRF2-MAFG heterodimerization, selectively interfering with NRF2 target gene expression and enhancing cytotoxicity in KEAP1 deficient NSCLC cells, compared to single agent treatment [131]. However, a reduction in NRF2 mRNA and protein levels was also reported using doses of 5 and 10μM, potentially indicating that there is either toxicity, or an effect on global protein translation associated with this compound, since inhibiting the NRF2-MAFG interaction should not affect NRF2 expression [131]. In summary, specific NRF2 inhibitors will be highly effective for overcoming chemoresistance in tumors with high levels of NRF2, which is a major obstacle for cancer therapy, and these drugs will have a substantial impact on the future treatment of NSCLC and other cancers with high levels of NRF2.

3.6 Acknowledgments

The authors are grateful to Dr. Jerry Pelletier for kindly providing the dual Firefly/*Renilla* reporter constructs and to Dr. Matthew Dodson and Montserrat Rojo de la Vega for their critical review of this manuscript. This work was funded by the National Cancer Institute (RO1CA154377).

Chapter 4

An analog of withaferin A induces autophagy and the NRF2 transcriptional program in differentiated SH-SY5Y cells

Bryan Harder, Tao Jiang, A. A. Leslie Gunatilaka, Lalitha Madhavan, Eli Chapman, and Donna D. Zhang

4.1 Abstract

Identifying the molecular mechanisms that lead to Parkinson's Disease (PD) pathogenesis is vital in order to find ways to either slow the progression or completely prevent the disease form occurring. Because the etiology of PD is largely unknown, and the cases in which familial mutations in key genes only contribute to a small fraction of all diagnoses, there is a growing need for early therapeutic intervention applicable for all cases. Here, differentiated SH-SY5Y neuroblastoma cells have been used to characterize a structural analog of withaferin A (A6) that is capable of inducing both the NRF2 transcriptional program as well as autophagy. Differentiated SH-SY5Y cells appear to have higher basal autophagy and increased protein levels of the small neuronal protein α-synuclein, making these cells a suitable cell culture model to study the potential neuroprotective effects of A6. A6 was found to activate autophagy independent of endoplasmic reticulum (ER) stress induction or mTORC1 activity. Furthermore, we show that A6 is capable of inducing the clearance of α-synuclein via induction of autophagy, possibly illuminating a new therapeutic

opportunity to prevent neurotoxicity associated with α-synuclein oligomerization/aggregation and subsequently decrease Lewy body formation. The dual neuroprotective properties of A6 may well be used not only for chemoprevention against PD, but also other neurodegenerative diseases where NRF2 activation provides protection as well as aggregate prone proteins that largely contribute to disease progression can be degraded by autophagy.

4.2 Introduction

Neurodegenerative diseases encompass a multifarious group of degenerative disorders that cause deterioration of the central or peripheral nervous system. These typically affect the elderly, which contribute to a large burden on patients, but also to care givers and health systems because patients with these devastating diseases can live for years while showing symptoms. In most cases, symptoms of neurodegenerative diseases affect motor function, causing involuntary or uncontrollable movements, but additional clinical features also show mental degeneration, eventually leading to dementia. Unfortunately, there are limited treatment options available to those affected by these diseases. At the time of diagnosis, the disease has progressed too far, and the only option is to manage the symptoms and maintain quality of life.

One common feature of neurodegenerative diseases, especially in Parkinson's disease (PD), is the accumulation of intracellular protein aggregates consisting of mostly α-synuclein [163]. Post-mortem brain slices from the substantia nigra pars compacta from patients who had PD reveal large protein

aggregate structures containing α-synuclein, in addition to other proteins such as ubiquitin and neurofilaments [164]. Because these aggregates largely contribute to the toxicity of affected neurons, induction of autophagy has emerged as a feasible strategy to divest the cell of protein aggregates, or aggregate-prone proteins that have the potential to form cytotoxic species [165]. Autophagy is the cell's bulk degradation process where cellular components such as misfolded proteins, damaged organelles, and invading pathogens, among others, are encapsulated by an autophagosome; a vesicular double membrane structure that is the delivery site of cargo that is to be degraded. Once fully formed, the autophagosome fuses with a lysosome, forming an autolysosome, which, at low pH, is an environment conducive for degradation to occur. It has been recently shown that pharmacological activation of autophagy can induce the clearance of proteins associated with PD, and provide significant neuroprotection against both WT α-synuclein as well as other mutant versions that cause familial-early onset PD such as the A53T or A30P mutations [166].

In addition to autophagic activation, the induction of the Nuclear factor-erythroid 2-related factor 2 (NRF2) transcription program has also shown significant neuroprotective effects against the progression of multiple neurodegenerative diseases including PD [167]. NRF2, the primary regulator of the antioxidant response, is kept at low basal levels through constant degradation mediated by the 26S proteasome. To accomplish this, the negative regulator of NRF2, Kelch-like ECH-associated protein 1 (KEAP1), constantly polyubiquitylates NRF2, marking it for destruction [40]. However, when the cell

undergoes challenge via oxidative or electrophilic stress, key cysteine residues in KEAP1 are covalently modified and the stability of NRF2 increases due to KEAP1 inactivation [42,43]. Newly synthesized NRF2 then translocates into the nucleus and directly regulates the expression of genes bearing an antioxidant response element (ARE) in the regulatory genomic regions of its target genes. The genes that are expressed act to restore an ideal reductive/oxidative (redox) environment in the cell. Interestingly, oxidative stress has been identified as a key pathogenic hallmark in the progression of various neurodegenerative diseases [168]. Many of the toxins used to initiate neuronal toxicity in cell culture or rodent models and recapitulate neurodegenerative phenotypes often act by severely enhancing oxidative stress to eliminate neurons. Therefore, genetic and pharmacological up-regulation of NRF2 has also proven to be a feasible strategy to combat further deterioration of the nervous system to delay the progression of the disease [169].

Here, we describe an analog of withaferin A (A6) that shows an ability to induce both autophagy and the NRF2 pathway. Because many non-nutritive phytochemicals from plants, fruits, and vegetables have been identified as NRF2 inducers (chemopreventive agents), significant efforts have described their ability to delay the progression of neurodegenerative diseases. Conversely, the number of well characterized autophagy inducers for the treatment of PD or other neurodegenerative diseases are severely limiting, mainly due to a lack of a reliable high-throughput assay to screen for target-based inducers of the autophagy pathway. In the present report, A6 is described to potentially offer dual

neuroprotective effects primarily due to the induction of autophagy, in addition to activation of the antioxidant response pathway mediated by NRF2.

4.3 Materials and Methods

4.3.1 Cell culture

SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH3T3 cells were also obtained from the ATCC and were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum. iBMK cells stably expressing GFP-LC3 cells have been previously described [60]. In all cases, cells were cultured at 37°C with 5% CO_2.

4.3.2 Differentiation of SH-SY5Y cells

In order to achieve a more neuronal phenotype, SH-SY5Y cells were differentiated with all-trans retinoic acid (AT-RA). SH-SY5Y cells were seeded at approximately 30% confluence. After 24 h, the cells were treated with 10μM AT-RA in normal media (day 0). 72 h later (day 3), 10μM AT-RA was re-applied in DMEM containing 1% FBS for an additional 48 h. On day 5, the desired neuronal phenotype was achieved and for experiments using differentiated SH-SY5Y cells, treatment times began at day 5. All experiments using differentiated SH-SY5Y cells were treated in DMEM containing 1% FBS, but AT-RA was not reapplied. Our unpublished observations demonstrate that removing AT-RA from fully

differentiated cells (day 5) for 24 h did not have any effect on the differentiation status, nor did the cells begin to revert back to an undifferentiated phenotype. Any measurement of differentiation status beyond 24 h was not investigated. Images of differentiated SH-SY5Y cells were acquired with the IncuCyte Zoom S3 Live-Cell Analysis System (Essen Bioscience).

4.3.3 Antibodies and Western blotting

Antibodies used to detect the following proteins in this study: NRF2, NQO1, and GAPDH were purchased from Santa Cruz Biotechnology, LC3 and α-synuclein were purchased from Sigma Aldrich, p-eIF2α (ser 52), eIF2α, XBP1S, p-Akt (ser 473), Akt, p-S6RP (ser 235/236), S6RP were purchased from Cell signaling Technologies, p62 was purchased from Abnova, and Neurofilament-M (NF-M) was purchased from Covance. For experiments involving western blotting, non-differentiated cells were seeded at 80% confluence and left to adhere overnight. For differentiated SH-SY5Y cells, cells were treated on beginning on day 5 in DMEM media containing 1% FBS. Cells were then treated with the indicated compounds, and were harvested in 1x NuPAGE LDS Sample Buffer (Invitrogen) at the indicated times. Protein abundance from total cellular lysate was determined by SDS-PAGE.

4.3.4 Live cell fluorescent microscopy

Assessment of autophagic status via fluorescent microscopy has been previously described [60]. Briefly, NIH3T3 cells were seeded at 80% confluence in glass

bottom D35 dishes and left to adhere overnight. Transfection of the mRFP-GFP-LC3 plasmid was performed using the Lipofectamine 3000 reagent according to the manufacturer's instructions. 24 h-post transfection, cells were treated at the given indications, followed by imaging of live cells. Multiple images for each treatment group were acquired using the Zeiss Observer.Z1 microscope with the Slidebook 4.2.0.11 imaging program (Intelligent Imaging Innovations, Inc.) and a single representative image is shown.

4.4 Results

4.4.1 Differentiated SH-SY5Y cells display enhanced expression of autophagy markers and α-synuclein

The SH-SY5Y neuroblastoma cell line is commonly used as a cell culture model to study molecular mechanisms of toxicity that are associated with PD [170]. Despite being of neuronal origin, SH-SY5Y cells are highly proliferative and display misregulated signaling pathways, thus contributing to their malignancy, which may convolute data from cellular assays aimed at identifying neuroprotective compounds. As a result, previous reports have described various protocols to differentiate SH-SY5Y cells to a more neuronal phenotype with either all-trans-retinoic acid (AT-RA), 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), or Brain-derived neurotrophic factor (BDNF) [170-172]. SH-SY5Y cells were treated with either DMSO or 10μM AT-RA for 5 days (see differentiation protocol). Around day 3, the SH-SY5Y cells exposed to AT-RA begin to display an elongated cellular morphology, consistent with alterations to the cytoskeleton

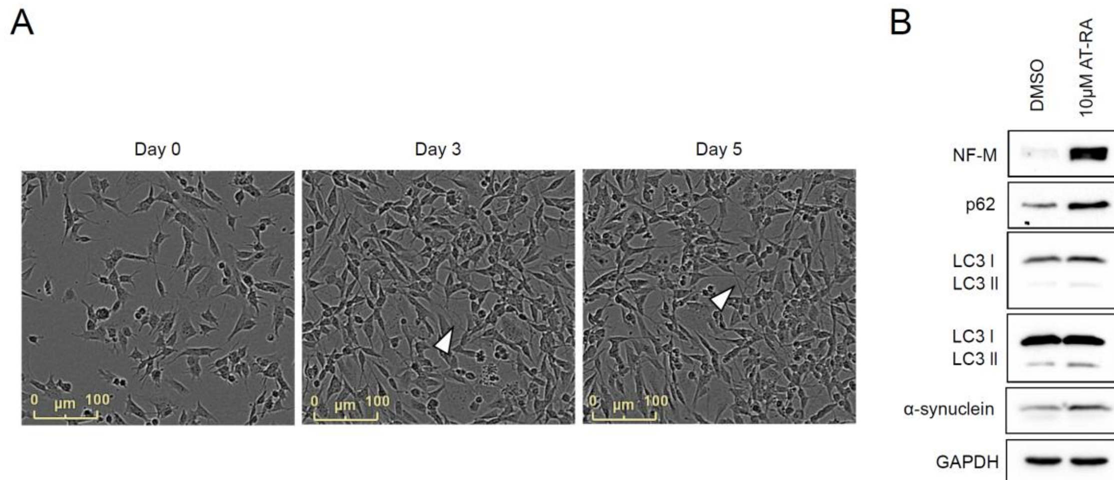

Figure 4.1 Differentiated SH-SY5Y cells display enhanced expression of autophagy markers and α-synuclein. (A) SH-SY5Y cells were differentiated over 5 days using 10μM AT-RA. Pictures were taken at day 0, day 3, and day 5, and the presence of neurite outgrowth is seen as early as day 3 and are fully extended at day 5 (white arrows indicate neurites). (B) Western blot analysis of SH-SY5Y cells exposed to DMSO (vehicle) or 10μM AT-RA for 5 days. Expression of NF-M in the cells exposed to AT-RA indicates cytoskeletal changes associated with neurite outgrowth, confirming differentiation. Additionally, basal levels of LC3 I and LC3 II, as well as p62 were enhanced in differentiated cells versus the non-differentiated counterparts. Moreover, the expression of α-synuclein was also enhanced in differentiated cells.

(Figure 4.1A, second panel). On day 5, cells appear to have fully developed neurites and a smaller, soma-like, body (Figure 4.1A, third panel), indicating fully differentiated SH-SY5Y cells. Because differentiated SH-SY5Y cells are no longer proliferative, previous reports have provided evidence for major changes associated with the bioenergetics of differentiated versus non-differentiated SH-SY5Y cells [173]. One major contributing factor to cellular energetics is the autophagy pathway, due to its ability to recycle cellular components. In order to compare undifferentiated versus differentiated cells, alterations in key autophagy markers were assessed after 5 days of DMSO, or 5 days of 10μM AT-RA, according to the differentiation protocol. To confirm differentiation, NF-M protein levels were substantially enhanced in accordance with neurite outgrowth (Figure 4.1B). Protein levels of p62 and both LC3 I and LC3 II were increased in differentiated cells, suggesting increased basal autophagic flux due to the demanding energetic needs of post-mitotic cells (Figure 4.1B). Additionally, enhanced protein levels of α-synuclein were also observed in differentiated cells compared to their undifferentiated counterparts (Figure 4.1B). This finding potentially enables the use of differentiated SH-SY5Y cells as a cellular model to study biologically relevant protein levels of WT α-synuclein as opposed to other models that induce the expression, causing abnormal accumulation of the protein.

4.4.2 A6 induces the NRF2 transcriptional program in differentiated SH-SY5Y cells

An analog of withaferin A (A6) was found from a screen to identify new inducers of the NRF2 pathway (data not shown) and the structure of A6 is provided in Figure 4.2A. A6 was found to induce NRF2 protein levels within 4 h in a dose-dependent manner, while not altering KEAP1 or GAPDH protein levels in differentiated SH-SY5Y cells (Figure 4.2B). In order to determine that the elevated NRF2 protein levels resulted in downstream transcriptional activation, differentiated SH-SY5Y cells were treated with A6 for 24 h using the same doses as the 4 h time point. Indeed, protein levels of NQO1, a prototypical NRF2 target gene, increased dramatically in a dose-dependent manner (Figure 4.2C). Interestingly, protein levels of NRF2 remained elevated in a dose-dependent manner at 24 h, potentially indicating that multiple mechanisms of activation may be involved.

4.4.3 A6 induces autophagy

Because NRF2 is stress-responsive transcription factor, there are multiple mechanisms by which protein levels of NRF2 can be induced. Our lab has previously shown that inhibition of autophagic flux can ultimately lead to a steady increase in NRF2 protein levels, followed by prolonged expression of NRF2 target genes, such as NQO1 [60]. In an attempt to understand how A6 induces NRF2, iBMK cells stably expressing LC3-GFP were treated with either DMSO or A6 for 4 h. Interestingly, A6 induced puncta formation within 4 h, indicative of autophagic inhibition instead of canonical induction of NRF2 by modification of KEAP1 which to not promote puncta formation (Figure 4.3A). The presence of

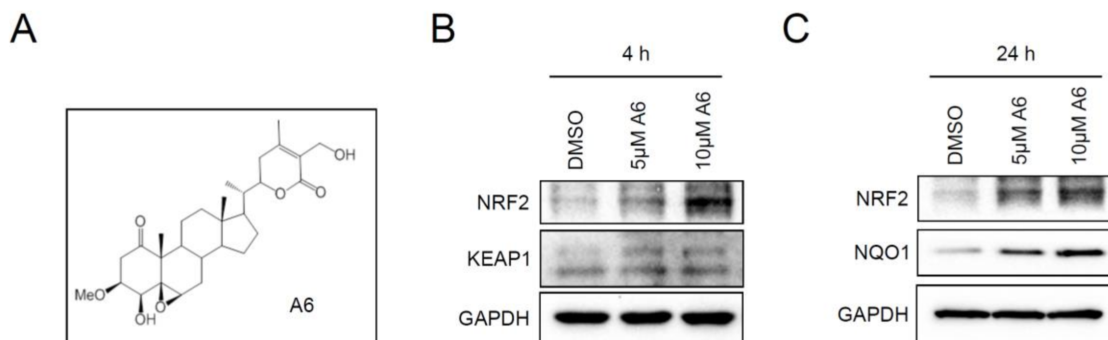

Figure 4.2 A6 induces the NRF2 transcriptional program in differentiated SH-SY5Y cells. (A) The chemical structure of A6, an analog of withaferin A. (B) Protein levels of NRF2 increase when exposed to 5 and 10μM A6 for 4 h in differentiated SH-SY5Y cells, but the protein levels of KEAP1 were not altered. (C) Expression of NQO1, an NRF2 target gene, increases in a dose-dependent manner when exposed to 5 and 10μM A6 for 24 h in differentiated SH-SY5Y cells. Additionally, protein levels of NRF2 remain elevated at 24 h.

LC3-GFP puncta does not yield enough information to determine if autophagy is inhibited or induced, so the LC3 flux assay was performed in differentiated SH-SY5Y cells. Cells were treated with DMSO, 100nM Bafilomycin (Baf), 10μM A6, and 10μM A6 plus 100nM Baf, and autophahic flux was determined via comparison of the LC3 II bands between Baf alone and the A6/Baf co-treatment. LC3 II protein levels were slightly enhanced in the A6/Baf co-treatment compared to Baf alone, indicating that A6 initiated autophagy rather than blocked flux (Figure 4.3B). Moreover, A6 treatment alone slightly induced LC3 II protein levels compared to the DMSO control. To further confirm that A6 can induce autophagy, NIH3T3 cells were transfected with the tandem mRFP-GFP-LC3 dual fluorescent reporter and subsequently treated with A6 and 500nM rapamycin as a positive control to induce autophagy. As expected, rapamycin induced the formation of mostly red puncta, indicting the presence of autolysosomes because of the fact that the GFP signal is quenched in acidic environments. Additionally, A6 also produced red puncta, confirming its role as an autophagy inducer (Figure 4.3C).

4.4.4 Induction of autophagy by A6 is independent of mTORC1 activity and ER stress and minimizes α-synuclein protein levels in differentiated SH-SY5Y cells

There are many mechanisms that govern the initiation of autophagy, however, most of which typically are in response to deprivation to nutrients. One master regulator of autophagic initiation is the mechanistic target of rapamycin (mTOR) [174]. In an attempt to discovery how A6 promotes autophagic flux, differentiated

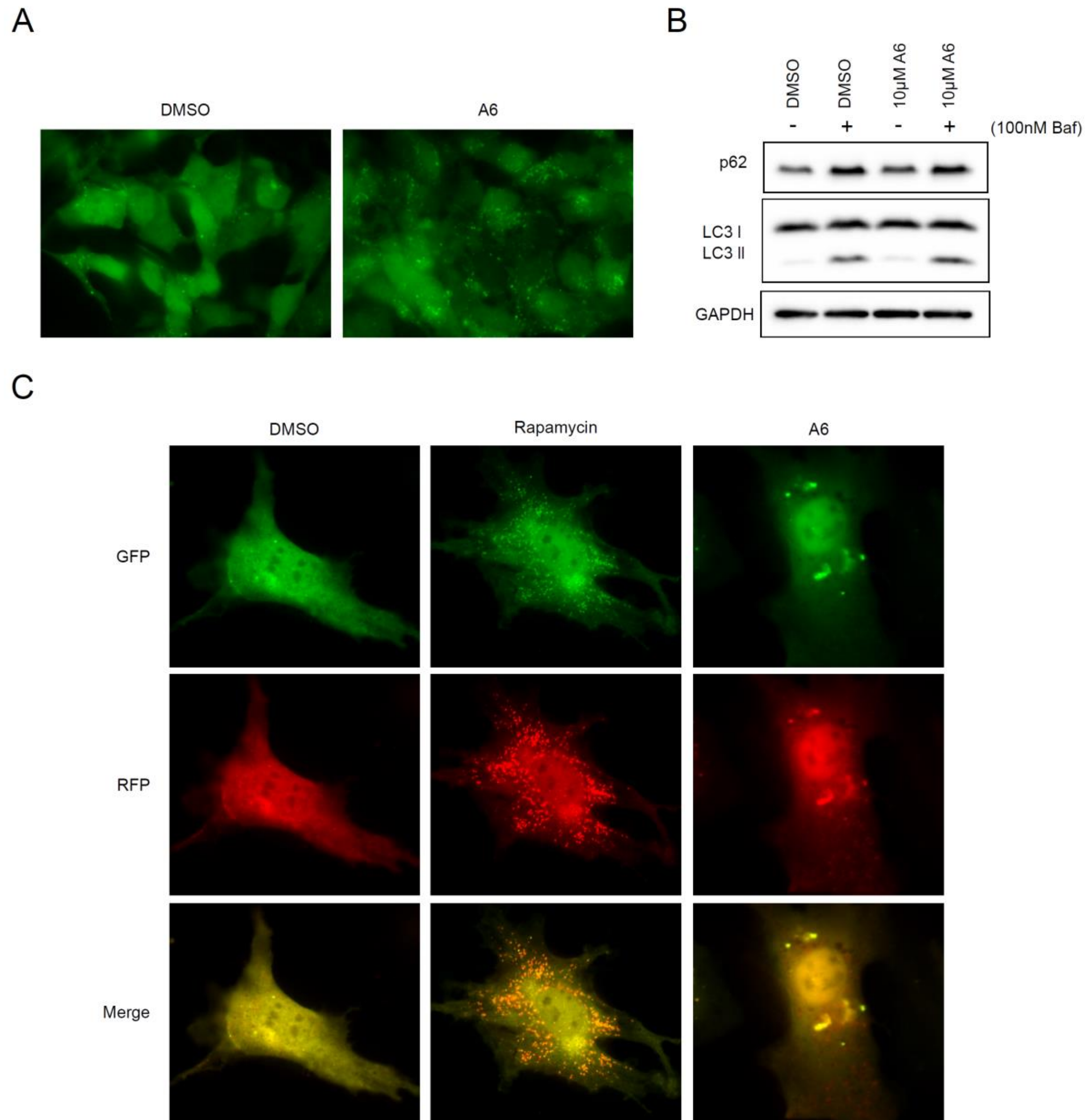

Figure 4.3 A6 induces autophagy. (A) A6 (10μM) induced puncta formation in iBMK cells stably expressing GFP-LC3, but the DMSO vehicle control did not, indicating that A6 may affect the autophagy pathway. (B) A6 (10μM) induced autophagic flux in differentiated SH-SY5Y cells using the LC3 turnover assay. The LC3 II band in the lane treated with both A6 and Baf (100nM) was increased in comparison to the LC3 II band in the lane treated with DMSO and Baf (100nM), signifying that autophagy is stimulated upstream, leading to the production of more LC3 II. (C) NIH3T3 cells were transfected with the mRFP-GFP-LC3 construct and treated with DMSO, rapamycin (500nM), and A6 (10μM) for 4 h. The DMSO treatment did not form any puncta, however, the formation of red puncta by rapamycin and A6 demonstrates the presence of functional autolysosomes.

SH-SY5Y cells were treated for 4 h and 2 markers of mTOR signaling were surveyed. A6 did not alter the phosphorylation S6 ribosomal protein (S6RP) (ser 235/236) or Akt (ser 473) or within 4 h, which are downstream targets of mTORC1 and mTORC2 complex activity, respectively (Figure 4.4A). Moreover, endoplasmic reticulum (ER) stress has been shown to activate autophagy. A6 did not enhance the expression of XBP1S or the phosphorylation status of eIF2α (ser 52), indicating that ER stress did not play a role in the induction of autophagy within the 4 h time point, while Tunicamycin (10μM) was used as a positive control (Figure 4.4B). Due to the fact that α-synuclein is a substrate of autophagy, differentiated SH-SY5Y cells were treated with 10μM A6 for 24 h in addition to 100mM Trehalose as a positive control. Surprisingly, A6 was able to diminish the protein levels of monomeric α-synuclein more so than Trehalose within 24 h (Figure 4.4C). Despite evidence that α-synuclein can be cleared by autophagy induction; Bafilomycin was not able to enhance the expression of monomeric α-synuclein protein levels. Differentiated SH-SY5Y cells were treated with A6 (10μM) alone, Bafilomycin (10nM) alone, or a combination of the two compounds. As previously shown, A6 can reduce the levels of monomeric α-synuclein, but Bafilomycin or A6 plus Baf had no effect on the expression of monomeric α-synuclein compared to the vehicle treatment (DMSO) (Figure 4.4D).

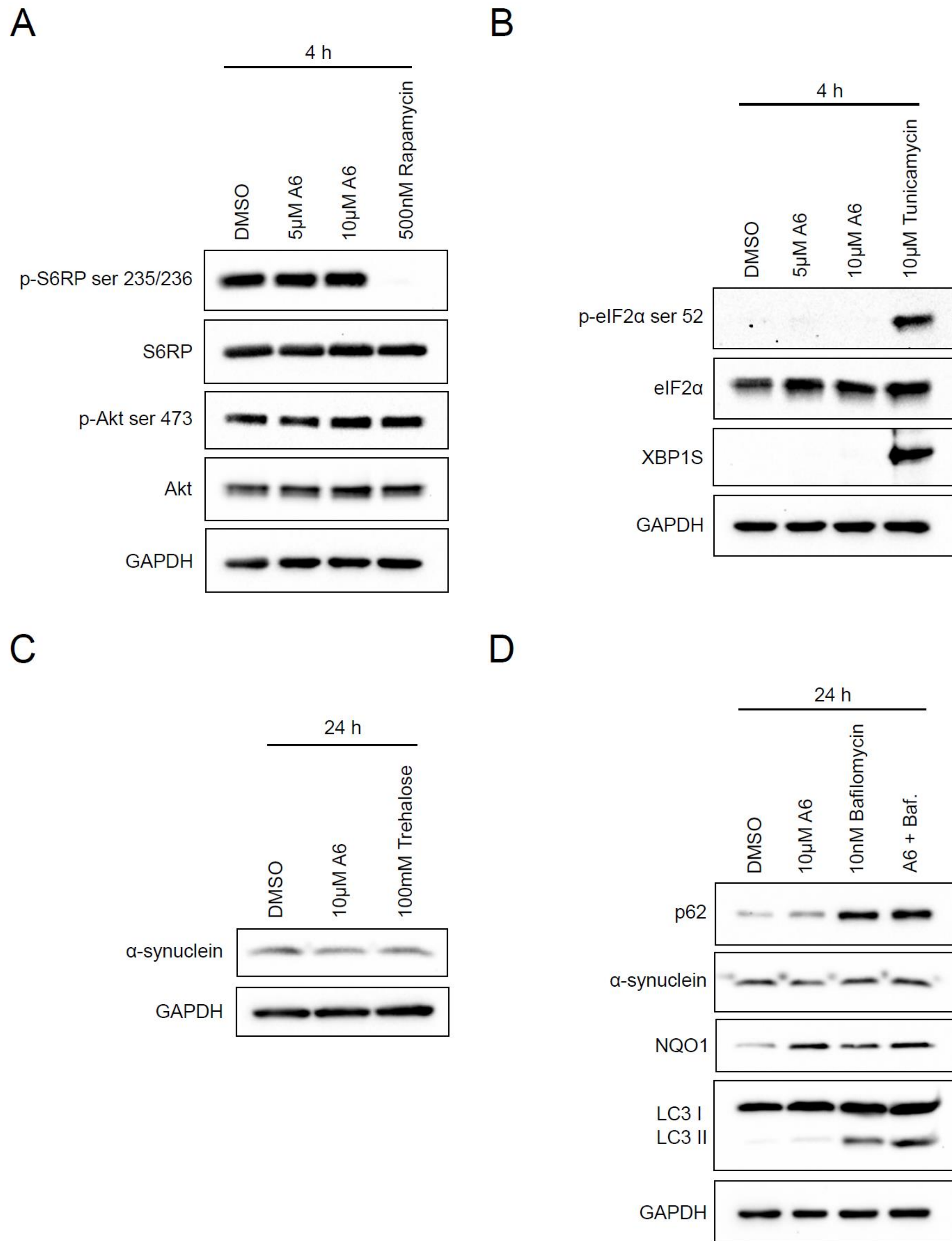

Figure 4.4 Induction of autophagy by A6 is independent of mTORC1 activity and ER stress and minimizes α-synuclein protein levels in differentiated SH-SY5Y cells. (A) A6 did not alter the phosphorylation of S6RP at ser 235/236 or affect the phosphorylation of Akt at ser 473 in differentiated SH-SY5Y cells, indicating that A6 does not inhibit mTORC1 or mTORC2 activity. 500nM Rapamycin was

able to inhibit the phosphorylation of S6RP at ser 235/236, but did not affect the phosphorylation of Akt at ser 473, confirming that Rapamycin is able to inhibit mTORC1 activity. (B) Differentiated SH-SY5Y cells treated with 5 and 10μM A6 did not induce the phosphorylation of eIF2α at ser 52 or the expression of XBP1S in 4 h, indicating that A6 does not induce ER stress pathways. 10μM Tunicamycin was used as a positive control, which was able to induce the phosphorylation of eIF2α at ser 52 and the expression of XBP1S within 4 h. (C) A6 (10μM) induced the clearance of monomeric α-synuclein after 24 h in differentiated SH-SY5Y cells. Trehalose (100mM) was used as a positive control, however, A6 showed to be more effective in decreasing protein levels of α-synuclein. (D) Autophagy inhibition does not alter monomeric α-synuclein levels in differentiated SH-SY5Y cells. The LC3 turnover assay was performed for 24 h, but Bafilomycin (10nM) did not alter the expression of monomeric α-synuclein. The expression of p62 was slightly enhanced by A6 (10μM), but drastically enhanced in the presence of Bafilomycin (10nM). Combination of A6 and Bafilomycin showed no further enhancement of p62 protein levels. Additionally, NQO1 expression was considerably up-regulated by A6 treatment alone and moderately altered by Bafilomycin due to the non-canonical mechanism of NRF2 activation by blockage of autophagy, however, co-treatment of A6 and Bafilomycin did not show a further increase.

4.5 Discussion

Here, we have identified an analog of withaferin A that is capable of inducing the NRF2 transcriptional response as well as autophagy. Induction of each of these pathways has shown to be extremely effective for the treatment of multiple neurodegenerative disease in both cell culture and *in vivo* models, but the pharmacological activation of both pathways conjointly has not been pursued.

Differentiated SH-SY5Y cells are a useful cell culture model to study the early molecular mechanisms that lead to PD pathogenesis. As a result of differentiation, tyrosine hydroxylase expression is significantly enhanced, thus contributing to a more dopaminergic phenotype. Because dopaminergic neurons are lost in the substantia nigra pars compacta during the progression of PD, identifying compounds that provide neuroprotection is of extreme interest. Despite the relevance of this useful model, little is known regarding the mechanisms of proteostasis or bioenergetics. Differentiated SH-SY5Y cells displayed elevated markers for autophagy, as well as the small neuronal protein α-synuclein (Figure 4.1B). Because neurons are not mitotic, they must rely more heavily on oxidative phosphorylation for more efficient energy production. The autophagy pathway is a bulk degradation process that provides the cell with various recycled cellular components for energy production used for anabolic pathways. To accommodate with the increase in oxidative phosphorylation, neuronal cells enhance basal autophagy in order to ensure quick turnover of the oxidized proteins/organelles that are modified as a result from high metabolic activity. Additionally, α-synuclein is a protein whose function is largely unknown,

however, it is speculated to play a role in vesicle trafficking, helping to facilitate neurotransmitter release. Accordingly, because differentiated SH-SY5Y cells have a more neuronal phenotype, they presumably would require more α-synuclein for vesicle movement in the extended neurites that were produced, shown in Figure 4.1A.

In an attempt to identify new NRF2 inducers, an analog of withaferin A (A6) was found to activate both NRF2 protein levels as well as transcriptional activity in differentiated SH-SY5Y cells. Further characterization led to the finding that A6 could also induce autophagy in addition to the NRF2 pathway. While there are numerous NRF2 inducers, mostly cysteine 151-dependent phytochemicals, the number of well-established autophagy inducers is limiting. Interestingly, withaferin A was recently reported to be an NRF2 activator by Kensler's group, however, it's mechanism of action remained questionable as it appeared to be acting independent of cysteine modification on KEAP1 [175]. This is corroborated by our data due to the fact that A6 displays sustained NRF2 protein levels at 24 h, atypical of a compound capable of cysteine modification. Because A6 was identified as an autophagy inducer as well, this may shed light on the mechanism of action of NRF2 induction because these two pathways are intimately related.

Autophagy was initially described as mechanism of cell death, though currently it is generally accepted to be a stress-responsive cell-survival pathway. Autophagy stimulation is usually a result of caloric restriction, though; there are numerous other stressors that can activate this critical process. Because

autophagy is a bulk degradation process, it is heavily involved in maintaining proteostasis. When misfolded proteins accumulate, three sensors within the lumen of the ER can initiate the unfolded protein response (UPR) [50]. When the UPR is activated, downstream signaling events lead to the activation of key transcription factors that regulate genes involved in promoting protein degradation pathways, especially autophagy [176]. One concern was that A6 could be potentially causing ER stress and subsequently activating the UPR, however, there was no indication of this occurring due to the absence of XBP1S expression and no phosphorylation of eIF2α at ser 52, two UPR markers that indicate the activation of ER associated degradation (ERAD) machinery and signifies that cap-dependent protein translation is suppressed due to an accumulation of proteins in the ER lumen, respectively (Figure 4.4B). Another mechanism that governs autophagy activation is the mTORC1 complex. Typically, mTORC1 functions to constantly suppress autophagic initiation, however, inhibition of mTOR with compounds such as rapamycin releases the hold on the formation of the ULK complex, allowing initiation to occur. A6 did not alter the phosphorylation of S6 ribosomal protein (S6RP), which is a downstream marker to determine the activity of the mTORC1 complex. Additionally, A6 did not alter the phosphorylation status of Akt at ser 473, which is a direct phosphorylation target by the mTORC2 complex. These data suggest that A6 does not have any effect on mTOR activity, identifying that A6’s ability to induce autophagy is independent of mTOR and that the mechanism of action of A6 should continue to be pursued.

There are various aggregate-prone proteins associated with the pathogenesis of different neurodegenerative diseases. Aggregated α-synuclein is frequently found in Lewy bodies in patients with PD, but there is currently no way to mitigate this phenomenon [163]. Moreover, PD, in addition to other neurodegenerative diseases, display compromised protein degradation pathways, especially in the later stages, which largely contributes to the neurotoxicity associated with protein aggregation and eventual Lewy body formation [177]. As such, induction of autophagy has emerged as a potential opportunity to degrade mutant/aggregate-prone proteins before large aggregates trigger significant neurotoxicity and neuronal death. Due to the finding that A6 can induce autophagy, differentiated SH-SY5Y cells were treated with A6 for 24 h and protein levels of α-synuclein were measured. Remarkably, A6 was able to reduce the amount of α-synuclein more so than Trehalose, a well-described autophagy inducer capable of clearance of α-synuclein mutants as well as mutant huntingtin containing a polyglutamine expansion (polyQ74) [178]. This is potentially due to the fact that Trehalose may take a longer time to initiate autophagy, implying that A6 could be a more potent and rapid inducer.

In order to prove that novel autophagy inducers can clear mutant proteins via autophagy, stable inducible cell lines capable of switching on the transgene of interest are used. Here, we have described that differentiation of SH-SY5Y cells displays moderately increased protein levels of α-synuclein compared to normal SH-SY5Y cells, possibly making use of biologically relevant amounts of α-synuclein as opposed to overexpressed transgene expression in inducible

systems. Additionally, the preferred degradation pathways for α-synuclein are well described [179,180]. α-synuclein is a substrate for both chaperone mediated autophagy (CMA) as well as the proteasome. If these primary mechanisms of degradation are non-functional (for instance if α-synuclein begins to accumulate and form oligomeric species), autophagy becomes the central mechanism of degradation. Interestingly, the two main familial α-synuclein mutants, A53T and A30P, are not CMA substrates, making the cell rely more on autophagy than for WT α-synuclein [181]. Therefore, the use of A6 could be more effective in clearing these mutant species over WT α-synuclein, while still benefitting from the protective effects of NRF2 induction.

Late-stage aggregation of α-synuclein leads to the formation of fibrils and it is still a controversial topic as to whether fibril formation is the toxic species, or if it is a cellular protection mechanism due to smaller oligomeric species having the most toxicity. Once fibrils are formed, they cannot be degraded by CMA or the proteasome, leaving autophagy as the only degradation mechanism possible. The difficulty associated with addressing these concerns is the fact that fibrils must first be generated *in vitro*, and then introduced into the cells. Our data argue that early treatment with A6 could attenuate fibril formation due to the clearance of soluble α-synuclein.

In conclusion, we have described an analog of withaferin A (A6) that is capable of inducing both autophagy and the NRF2 transcriptional program. Due to the complexity of the pathophysiological progression of PD, the use of A6 in differentiated SH-SY5Y cells was used to determine if it could be a good

candidate for chemoprevention against PD. Further work on this topic should focus on the mechanisms that drive induction of autophagy and NRF2, in addition to providing neuroprotection in cell culture or animal models of PD. The use of a molecule with these properties could be of extreme interest in the clinic, as there are very limited treatment options for patients with neurodegenerative diseases.

4.6 Acknowledgements

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Chapter 5

Current perspectives and future directions

It has been extensively shown that NRF2 is a key factor in disease progression. On the one hand, NRF2 activation has been shown to prevent the onset of chronic diseases, but on the other hand, NRF2 can provide a critical resistance to chemotherapy in neoplasia and thus enable malignant transformation. This fact highlights the importance of targeting NRF2 depending on disease context. In other words, if a healthy individual does not present with a disease, targeting NRF2 with phyto- or synthetic chemicals can boost the basal antioxidant response and facilitate the removal of potentially mutagenic species that pose a major threat to everyone. Conversely, because NRF2 is frequently found to be misregulated in certain cancers, inhibition of NRF2 has been shown to be largely effective in order to enhance the cytotoxic effects of first-line chemotherapeutic agents. In this regard, biopsy of tumors followed by sequencing for hot-spot mutations in key regulators of the NRF2 pathway should be considered for cancers that have been previously associated with constitutive NRF2 activation. This importance and complexity of NRF2 in disease progression prompted the presented studies in this dissertation in an attempt to understand additional modes of regulation of NRF2.

After careful consideration of the data presented, in addition to the continuously expanding body of literature, it is clear that there are numerous mechanisms that govern how NRF2 is regulated and that additional research is needed in order to fully understand the precise modes of regulation in certain

biological contexts for effective disease intervention. Here, a novel mechanism of NRF2 regulation by TSC22D4 has been uncovered. While there have been previous reports showing positive regulation of NRF2 by 'ETGE' containing proteins, the fact that the function and regulation of TSC22D4 itself remains elusive is of particular interest. Additionally, the mechanism by which brusatol, the first ever NRF2 inhibitor, exerts its inhibitory action on NRF2 has been explicated. Due to this finding, NRF2 has been shown to be particularly sensitive to inhibition of protein translation, potentially shedding light on the mechanism of action of additional NRF2 inhibitors, which largely report reduced NRF2 protein levels rather than reduced NRF2 activity. Finally, it has been well established that NRF2 and autophagy are interconnected, but it has been shown for the first time that enhancement of both of these pathways may be a new therapeutic strategy to combat the progression of neurodegenerative diseases, especially for those neurodegenerative diseases where the pathogenesis is mediated by proteins that form intracellular aggregates and severe neuronal toxicity.

In Chapter 2, TSC22D4 was shown to positively regulate the NRF2 pathway in an 'ETGE' dependent manner, similar to other 'ETGE'-containing proteins that have been previously reported. Despite the growing list of proteins that contain this motif, very few reports on these have highlighted the molecular mechanisms that are responsible for regulation. More often than not, many of these proteins, though capable of binding to KEAP1, have not been identified to be regulated at the protein level, and thus only contribute to the basal level of NRF2 in the cell, depending on cell type. Conversely, TSC22D4 was found to be

an intermediate between PR signaling and NRF2, a link that has been previously unexplored. Because TSC22D4 can be transcriptionally up-regulated by the PR, we have identified MPA as an indirect NRF2 activator. However, it does not seem that regulation by the PR is the primary signal that directs TSC22D4 expression due to the fact that only small changes in mRNA and protein levels were detected. Therefore, future studies should focus on the key mechanisms that drive the expression of TSC22D4 because there is little knowledge on the role of TSC22D4. In doing so, this could provide a new link between NRF2 and a field where there is currently no established connection. In addition to TSC22D4 itself, the relationship between NRF2 and hormone receptor signaling has been addressed. Previous research has strongly indicated a negative regulation of NRF2 by hormone receptors such as ERα, ERRβ, and GR. It remains to be determined how these ligand-activated hormone receptors can inhibit NRF2-mediated signaling, but the majority of the evidence suggests physical interaction between ligand-bound steroid hormone receptors and NRF2. More importantly, the estrogen and progesterone receptors are major drivers in hormone-driven malignancies; however, the literature regarding NRF2 and E2 is contradictory given that stimulation with E2 and other structurally related compounds induces the antioxidant response in an NRF2-dependent manner. Future studies could address correlations between hormone-driven malignancies and NRF2 activation, especially in cases where constitutive activation of NRF2 has been achieved. Such data could help to identify rational combination-chemotherapy approaches to treat these cancers.

Because there is limited knowledge on the function/regulation of TSC22D4, the studies performed in Chapter 2 also aimed to address these concerns because of the apparent relationship with NRF2. In addition to these, work from the Herzig lab has recently provided evidence for TSC22D4 as a factor that can, in part, regulate metabolic gene expression and insulin sensitivity in a cancer cachexia phenotype and obesity-related insulin resistance, respectively. In the first study, it was shown that hepatic metabolic dysfunction is triggered by tumor growth, resulting in cancer cachexia, otherwise known as a 'wasting' condition characterized by loss of adipose tissue and skeletal muscle mass. In search of a molecular determinant of this process, Jones *et al* described TSC22D4 as a molecular regulator of very-low-density-lipoprotein (VLDL) secretion and hypobetalipoproteinemia [142]. TSC22D4 was found to be up-regulated in the livers of patients with cachexia. Interestingly, overexpression of TSC22D4 in healthy livers led to decreased VLDL secretion as well as reduced lipogenic gene expression. It has been well described that NRF2 also negatively regulates genes involved in lipogenic processes. As such, TSC22D4 expression could potentially be a factor that governs NRF2 protein levels, helping cells to respond to fluctuations in energy sources and further work could determine if these two factors are dependent upon one another in certain disease contexts. In the second study, inhibition of hepatic TSC22D4 prevented and/or reversed hyperglycemia, glucose intolerance, and resistance to insulin in mouse models of diabetes. Conversely, genetic or pharmacological activation of NRF2 has been shown to lower blood glucose, improve insulin secretion, and improve insulin

sensitivity, indicating that there is a negative correlation between the roles of TSC22D4 and NRF2 in diabetes. Üstünel *et al* identified an increased expression of *TSC22D4* in the livers of diabetic patients, indicating that additional molecular mechanisms that govern NRF2 expression/activity may be involved [182]. Further work on this topic should focus on direct transcriptional targets of both TSC22D4 and NRF2 in this disease context in order to understand how these factors contribute to diabetes progression.

The work performed in Chapter 3 definitively identified the mechanism of action of brusatol, the most effective NRF2 inhibitor to date. Originally published by our lab in 2011, brusatol was shown to potently inhibit NRF2 protein levels, as well as NRF2 target gene expression. As such, brusatol sensitized A549 cells to the cytotoxic effects of cisplatin, arguing for the use of brusatol as an adjuvant therapy to treat cancers that present with misregulated NRF2. Unfortunately, the target of brusatol remained elusive, and the mechanism of NRF2 inhibition was not known.

Because of the importance of identifying the first known NRF2 inhibitor, understanding the mechanism of action proved to be vital. Brusatol was identified from an ARE-driven reporter-screening assay, which did not warrant target identification. In order to assess the most likely mechanism of action, A549 cells were treated with brusatol, and changes in the transcriptome were evaluated. In comparison with publicly available gene set enrichment data sets, mRNA changes with brusatol treatment shared a striking similarity to those treated with the translation inhibitor cycloheximide (CHX). As mentioned earlier, NRF2 is a

factor that is constantly being transcribed/translated, but also degraded via the KEAP1-RBX1-CUL3 E3 ubiquitin ligase complex. Due to this post-translational regulation, NRF2 is highly susceptible to fast turnover, so the cell continues to create more NRF2 in case oxidative or electrophilic stress is encountered. It became apparent that NRF2 protein levels are profoundly impacted by translation inhibition, concluding that the majority of identified NRF2 inhibitors may affect translation. As a result, additional assays to evaluate the effects on translation, as performed in Chapter 3, should be employed when continuing to screen for NRF2 inhibitors. Despite this assertion, brusatol is still an effective adjuvant chemotherapeutic capable of sensitizing cancer cells with constitutive NRF2 activation to chemotherapies because these cancer cells heavily rely of NRF2 for protection. In other conditions, brusatol would also be effective in helping to kill cancer cells that also rely on cellular factors with short half-lives (i.e. HIF1α, β-catenin).

Despite brusatol's effectiveness *in vivo*, identification of the next generation of NRF2 inhibitors is essential. Further screening platforms should focus on identifying compounds capable of prohibiting NRF2-sMAF heterodimerization, which would yield ultra-specific inhibitory effects on NRF2 transcriptional activity. Additionally, another potential avenue to pursue would be to target NRF2 binding to the ARE, however, the fact that there is no consensus ARE sequence could encumber this process, potentially leading to the identification of molecules with limited inhibitory action. Nevertheless, brusatol could still be an effective treatment option in certain cases, such as the KRAS

G12D lung cancer mouse model or for NSCLC. Tumors that are extremely reliant on NRF2 signaling are significantly affected by inhibition of protein translation because NRF2 is reduced so quickly. Because of this, brusatol could still be effective for treating tumors with high NRF2, but more specific inhibitors of the NRF2 pathway will be needed in case NRF2 is induced through non-canonical mechanisms such as the one presented in Chapter 2 or if people are exposed to chronic, low levels of arsenic.

The use of natural products that target NRF2 as chemopreventive agents to suppress carcinogenesis has gained a lot of interest, however, there is not much attention geared towards using these agents to prevent the onset of neurodegenerative diseases. In Chapter 4, an NRF2 reporter screen to identify novel NRF2 inducers from a library of purified natural products revealed a compound structurally related to withaferin A. Further characterization of this analog (termed A6) revealed an ability to initiate the autophagy pathway.

Because autophagy is a bulk degradation process, the power of autophagic induction has been harnessed to enhance the clearance of aggregate-prone proteins that cause neurotoxicity associated with the pathogenesis of various neurodegenerative diseases. Additionally, autophagy, as well we other protein degradation mechanisms, are deregulated in various neurodegenerative diseases and could very likely be a causative factor in their progression, and reinstatement of proper autophagic flux could be beneficial. Of particular interest was the small neuronal protein α-synuclein due to the fact that it is a substrate of autophagic degradation. α-synuclein is thought to play a major

role in the pathophysiological progression of Parkinson's Disease (PD) because it is the main component found in Lewy bodies. Accordingly, activation of NRF2 has also been shown to offer neuroprotection in cell culture and *in vivo* models of PD. As a result, the inductive properties of autophagy and NRF2 could offer extreme neuroprotection in animal models that recapitulate the hallmarks associated with PD.

Thus far, only a small effort has tried to address the mechanism of action of A6. Kensler's group recently published data that suggested withaferin A induces the NRF2 transcriptional response, but in a manner independent of KEAP1. They provided evidence to suggest that withaferin A acts by activating Akt, which suppresses the GSK-3β/β-TrCP mechanism of NRF2 degradation. Interestingly, we did not observe this phenomenon with A6. We also examined if A6 had any effect on mTOR signaling because of the fact that mTOR is the master regulator of autophagy. According to the data presented above, A6 did not have any effect on the phosphorylation of Akt at ser 473, which is a well-established target of mTORC2 complex activity. Additionally, A6 did not have any effect on the phosphorylation of S6RP at ser 235/236, a downstream marker of mTORC1 activity, demonstrating that A6 does not inhibit the action of mTORC1, which largely governs autophagic initiation. This demonstrates that A6 could activate autophagy through an mTOR-independent mechanism and that other cellular factors involved may be involved. Taken together, future efforts should focus on the up-stream factors that govern autophagic induction independent of mTORC1. It is important to consider that autophagy is a stress-responsive

pathway, so it is likely that A6 could be inducing some additional form of stress that has not yet been tested, making target elucidation of extreme importance. Once a true target of A6 is identified, quantitative structure activity relationship studies will be needed to fine-tune the molecule to achieve specificity in order to increase potency and minimize toxicity.

One additional concern that should be met with pursuing A6, is the fact that it is an inducer of the NRF2 pathway. As explained earlier, there is a good side, as well as a ‘dark side’ to NRF2 signaling. NRF2 is a factor that provides a growth advantage to cancer cells, and can thus facilitate malignant transformation. Because the etiology of PD is unknown, and previous experiments have suggested that clearance of α-synuclein can only occur when it is in soluble forms, this would require chronic, persistent utilization of A6 to prevent the onset of PD over the course of a lifetime. Therefore, *in vivo* approaches should address if A6 can in fact facilitate malignant transformation, and if so, determine if this phenomenon is dependent upon NRF2.

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