NUCLEAR FACTOR ERYTHROID 2 RELATED FACTOR-1 (NRF1) MEDIATES CELASTROL-INDUCED GENE REGULATION, DEPENDING ON ITS HETERODIMERIC INTERACTIONS

A Thesis Submitted to the College of Graduate and Postdoctoral Studies
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In the Department of Anatomy, Physiology and Pharmacology
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

By

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Abstract

Oxidative stress has been recognized as critical in human aging and the progression of many chronic diseases, including cancer. Cells undergo oxidative stress when the overproduction of reactive oxygen species (ROS) within the cell outweighs its antioxidant defenses. As a defense mechanism, a series of cytoprotective genes is initiated and regulated by various transcription factors in order to minimize oxidative damage to the cell. NF-E2-related factor 1 (NRF1) is a CapN'Collar (CNC) transcription factor family member that plays a major role in regulating genes involved in defense against cell stress and damage. For example, NRF1 is a vital regulator of antioxidant and proteasome genes to counteract ROS and impaired protein homeostasis. Upon stress caused by impaired protein turnover, NRF1 undergoes endoplasmic reticulum to nuclear translocation and binds antioxidant response elements (ARE) located in close proximity to stress defense genes throughout the genome. The result is altered transcription of the associated gene. While this general concept has been established, the mechanism by which NRF1 is processed, selected for nuclear translocation rather than degradation, and the role of post-translational modifications is not understood. Moreover, previous studies show NRF1 must form a heterodimer with other transcription factors such as small musculoaponeurotic fibrosarcoma oncogene homolog (MAF) proteins to bind AREs and regulate gene transcription. The mechanism underlying NRF1 heterodimer formation and ARE binding and whether distinct heterodimers regulate distinct genes is unclear. I hypothesize NRF1 regulates proteasome and oxidative stress defense via specific heterodimer interactions.

In this thesis, I describe our identification of the nutraceutical celastrol as a stimulant for NRF1’s transcriptional activity. For centuries, celastrol has been used to treat inflammatory and chronic diseases and more recently found to influence a multitude of stress pathways and suppress chymotrypsin-like activity of the proteasome. I then proceeded to use celastrol to investigate my hypothesis. Using cultured Hep3B cells, I show celastrol elicits dose-dependent inhibition of the proteasome and this increases the level of a cleaved NRF1 protein product known to regulate transcription. Using clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 based technology to produce NRF1 loss-of-function cells, I show celastrol induces transcription of proteasome and oxidative stress defense genes in an NRF1-dependent manner. Likewise, quantitative polymerase chain reaction measurement of immunoprecipitated chromatin revealed that NRF1 binds to AREs in stress defense genes GCLC, GCLM, HO1, NQO1A in a manner that corresponds with transcription regulatory profiles. Moreover, ablation of NRF1 heterodimers MAFG, MAFK, or MAFF resulted in refractory responses to celastrol that partially and non-redundantly matched NRF1 deficiency.

Collectively, results of my research support a model whereby NRF1 heterodimerization with MAFK regulates one gene sub-set or program, whereas heterodimerization with MAFG or MAFF regulates another one. In other words, specific heterodimeric interactions coordinately stimulate or repress the transcription of a group of target genes.
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List of abbreviations

bZIP Basic leucine zipper (bZIP)
CNC Cap'n'collar
NRF1 Nuclear factor erythroid-derived 2-related factor 1
ORF Open reading frame
ER Endoplasmic reticulum
ERAD Endoplasmic-reticulum-associated protein degradation
ROS Reactive oxygen species
RNS Reactive nitrogen species
siRNA Short interfering RNA
UB Ubiquitination
GCLC Glutamate-cysteine ligase catalytic subunit
GPX1 Glutathione peroxidase 1
BAT Brown adipose tissue
PGC-1B Peroxisome proliferator coactivator-1β
TAD Transactivation domain
AD1 Acidic domain 1
NGLY1 N-glycanase 1
DDI2 DNA-damage inducible 1 homolog 2
ARE Antioxidant response elements
PKC Protein kinase C
CK2 Casein kinase 2
GSK3O Glycogen synthase kinase 3
UPS Ubiquitin proteasome system
BTZ Bortezomib
CFZ Carfilzomib
MARE MAF recognition element
ATF4 Activating transcription factor 4
NQO1 NAD(P): quinone oxidoreductase 1
β-NF β-naphthoflavone
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-BHQ</td>
<td>Tert-butyl-hydroquinone</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>ME</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>PRDX</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>GCLM</td>
<td>Glutamate-cysteine ligase regulatory subunit</td>
</tr>
<tr>
<td>Gss</td>
<td>Glutathione synthetase</td>
</tr>
<tr>
<td>GCLC</td>
<td>Glutamate cysteine ligase catalytic subunit</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase type 1</td>
</tr>
<tr>
<td>SLC7A11</td>
<td>Solute Carrier Family 7 Member</td>
</tr>
</tbody>
</table>
CHAPTER 1:
Introduction/Literature Review
1.1: Structure, function and properties of NRF1

Transcription factors are deoxyribonucleic acid (DNA)-binding proteins that regulate transcription by interacting with DNA sequences in the genome (1). Basal transcription factors maintain basal transcription activity, whereas regulatory transcription factors are induced or suppressed to alter gene transcription in a dynamic fashion, which is usually dependent on environmental influences. Transcription factors can be classified into families based on DNA-binding domain homology, which together with other domains play a critical role in coordinating transcriptional regulation. Mechanisms that regulate transcription factor activity including its synthesis, cellular localization, post-translational modification, degradation, and interactions with other factors (2). Through these mechanisms, transcription factors underlie all aspects of mammalian biology.

One sub-family of transcription factors especially relevant to this thesis are the nuclear factor, erythroid 2-related factors that contain a basic leucine zipper (bZIP) domain and 43 amino acid motif called the Cap'n'collar (CNC) domain (3). Like other bZIP family proteins, they contain a leucine-rich helical region of 30–40 amino acids that enable dimerization with other bZIP proteins, and a basic amino acid-rich DNA-binding domain (4). CNC-type bZIP proteins differ in the N-termini but all share the CNC domain, which was first identified in the Drosophila cap and collar gene and is located directly N-terminal to the bZIP DNA-binding domain (5). The first identified member was p45 Nuclear Factor Erythroid 2 (NFE2), discovered for its ability to interact with activating protein 1 (AP1) to control the human beta-globin locus (6). Additional members that have since been identified include Nuclear Factor, Erythroid 2-Related Factor-1 (NRF1; gene name NFE2L1), NRF2 (NFE2L2), NRF3 (NFE2L3) and distantly related members BTB Domain And CNC Homolog 1 (BACH; gene name BACH1) and BACH2 (BACH2). Interestingly, CNC-bZIP proteins have been broadly linked to cellular stress adaptation in numerous vertebrate species, including humans. NRF2 is by far the most studied, whereas the role of the other factors is poorly understood. The focus of this thesis is on NRF1 and its role in regulating transcription of genes that counteract cellular stress.

The full length NRF1 protein is ubiquitously expressed and synthesized as an endoplasmic reticulum (ER) membrane-bound protein that can be processed and translocate to the nucleus to promote cellular homeostasis (7). The NRF1 gene is located on chromosome 17q21.3, whereas the
mouse Nrf1 gene is located on chromosome 11 (8, 9). There are nine exons in the NRF1 locus, which have alternative first exons (1a and 1b) and terminal exons (6 and 6a), and two potential polyadenylation sites (10). Multiple isoforms of NRF1 exist, which result from differential slicing and alternative translation (11). The role of most of these isoforms is unknown as the product is usually undetectable. However, a full-length isoform that is approximately 120 kDa and an N-terminally truncated product from this isoform that is 95 kDa have been ubiquitously found and appear to play a predominant role in regulating stress adaptation in cells. The longest isoform, sometimes referred to as TCF11, contains 772 amino acids whereas the predominant isoform, sometimes referred to as NRF1α, has 742 amino acids and is derived from alternatively spliced mRNA that lacks exon 4 in the TCF11 transcript (10). Despite this difference NRF1α and TCF11 exhibit similar transactivation activity (12), and TCF11 has only been found in humans. Moreover, the products of either have an apparent weight of approximately 120 kDa on Western blots. A shorter isoform referred to as LCRF1 is 572 amino acids and approximately 65 kDa. An isoform referred to as NRF1β is generated through alternative in-frame translation initiating at an internal perfect Kozak start signal located within and around the four methionine codons between positions 289 and 297, and this isoform exhibits weak transactivation activity and it is possible that it acts as an inhibitor of NRF2 activity (13-15). Even smaller isoforms referred to as NRF1γ and NRF1δ that encode 36-kDa and 25-kDa protein products, respectively, have also been described (9, 16). For reference, a cartoon depiction of NRF1α, NRF1β, LCRF1, and NRF1γ is in Figure 1.1 below. As mentioned above, very little is understood regarding the shorter isoforms. One study attempted to gain insight by producing Hek293 cells lines compatible for tetracycline-inducible expression of NRF1α, NRF1β, or NRF1γ (11). Transcriptomic analysis revealed that NRF1α and NRF1β are capable of transcriptional programming. However, as NRF1α has consistently been identified as the key factor underlying this gene’s physiological roles, the remainder of this thesis will focus only on the full length 120 kDa isoform that binds to the ER membrane and its cleaved 95 kDa product that regulates gene transcription and will be referred to as simply NRF1.
Figure 1.1: The membrane-topogenic vectorial behaviour of NRF1 controls its post-translational modification and transactivation activity. Due to the N-linked glycosylation of the NST domain, Glycosylated and Non-glycosylated NRF1 migrate during electrophoresis at masses of 120 kDa and 95 kDa, respectively. Further processing by selective proteolysis can result in multiple cleaved forms of 85 kDa. The 55-kDa form, which was initially designated LCR-F1 (called NRF1β herein), is produced by in-frame translation and selective proteolysis and may be rapidly degraded to yield short dominant-negative isoforms of 36-kDa (called Nrf1γ) (11). Illustration created with Biorender.com.

Loss-of-function studies have been key for deciphering the physiological role of NRF1. Deficiency of NRF1 causes reduced antioxidant, detoxification, and proteasome genes, leading to oxidative stress and disruption of protein folding and ER-associated protein degradation (ERAD) and accumulation of ER stress causing misfolded proteins (17, 18). The common thread for the outcomes of NRF1 deficiency is suggestive that NRF1 counteracts oxidative damage produced by reactive oxygen species (ROS) and reactive nitrogen species (RNS), restores redox balance, and promotes proper protein turnover and lipid homeostasis. In many cases the effect is severe. Whole body gene deletion causes embryonic lethality (19). Deletion from liver causes steatohepatitis, hepatocellular carcinoma, and lipid accumulation which coincides with impaired metabolism and proteostasis (20-22). Deletion from brain causes neurodegeneration (23) and from brown fat causes metabolic disease and impaired cold tolerance (21). Overall, these findings reveal stress adaptive function of NRF1 plays a critical physiological role.
A growing body of work suggests NRF1 resolves imbalances between damaged protein accumulation and degradation and how it is affected by cellular ROS. Oxidative damage to cellular proteins can substantially increase when cells are under metabolic stress or have high synthetic demand. This will be discussed in greater detail below in section 1.2 and 1.3, which highlight the mechanism underlying NRF1 transcriptional activity and its sensitivity to proteasome activity.

1.2: Post-translational modification of NRF1 and its processing and translocation into the nucleus

Newly translated NRF1 is translocated into the ER lumen via a transactivation domain (TAD) in its N-terminus and connecting TAD sequences that comprise acidic domain 1 (AD1), NST glycodomain, AD2 and possibly serine-repeat (SR) domain. Thus, the TAD mediates NRF1 subcellular localization to the ER membrane upon translation. Then, the NST domain of TAD is glycosylated, resulting in an inactive/full-length form (24). Full-length NRF1 is ubiquitinated by ER-associated E3 ubiquitin ligase HMG-CoA Reductase Degradation 1 Homolog (HRD1) and retro-translocated into the cytosol by actions requiring the ATPase p97 (25). Subsequently, in order for NRF1 to become active it must undergo N-linked deglycosylation by N-glycanase 1 (NGLY1) and a proteolytic cleavage between amino acid 103 and 104, which trims off the N terminal region involved in ER membrane localization (26, 27). It has been discovered that the protease involved in this latter process is the aspartic protease DNA-damage inducible 1 homolog 2 (DDI2), a member of the aspartic protease family (28, 29). The proteolytically cleaved and deglycosylated NRF1 product containing amino acids 104 to 741 is capable of transcriptional regulation. Under a steady state, a large portion of NRF1 is degraded by the 26S proteasome in the cytosol, but in response to oxidative stress or proteasome insufficiency, it escapes from degradation and instead translocate to the nucleus (30).

Interestingly, the N-linked deglycosylation step has been found to play an intricate role in NRF1 action. During deglycosylation of NRF1, which occurs at several asparagine (Asn) residues, NGLY1 also removes the Asn’s amine group, resulting in Asn residues that are typically positively charged becoming aspartate (Asp), which are typically negatively charged (31, 32), and this can have important functional consequences in its ability to control transcript regulation (33). For example, NRF1 that is
glycosylation-defective, due to Asn being replaced by glutamine residues, exhibits a decreased transcription activity, whereas NRF1 in which Asn are replaced with Asp residues mimic the high transcriptionally active deglycosylated state (32). Furthermore, NGLY1 inhibition causes a defect in NRF1 activity (31). Hence, there are several important steps for the formation of the transcriptionally active NRF1, further highlighting its important biological role. A schematic of the main processing steps is shown in Figure 1.2.

Additional mechanisms that are not clearly established but may play a role in regulating NRF1 are as follows. First, in contrast to N-linked deglycosylation there may also be a role for O-linked glycosylation of serine or threonine residues, similar to a phosphorylation event (7). For example, O-GlcNAcylation of NRF1 can occur via O-linked N-acetylglucosamine transferase and this may facilitate its stabilization and consequent upregulation of proteasomal subunit genes, in addition to N-glycosylation effects. The transcription activity of NRF1 may also be regulated by phosphorylation. In ARE-driven luciferase assays examining NRF1, it was indicated that an increase in NRF1 transactivation activity occurred when cells were treated with the phosphatase inhibitor okadaic acid, and a protein kinase C (PKC) inhibitor, staurosporine, was capable of suppressing this activity, suggesting that phosphorylation by PKC has a positive effect on NRF1 activity (34). In addition, casein kinase 2 (CK2) and glycogen synthase kinase 3 (GSK3) have been found to directly phosphorylate NRF1 at serine 497 to suppress its transcriptional activity. At this stage, more research is needed to clarify the mechanism and physiological relevance.
Figure 1.2: An explanation of the activation pathway of NRF1 and its domain structure. In the ER lumen, full-length NRF1 is glycosylated, which is then retrotranslocated to the cytosol by VCP/p97. The membrane-bound NRF1 in the ER is then de-N-glycosylated by NGLY1. In the cytosol, NRF1 is cleaved by the protease DDI2 to the active transcription factor. Normally, the proteasome immediately degrades NRF1, so it is maintained at low levels in the cell during a basal unstressed state. In cells with insufficient proteasome capacity due to chemical inhibition or an overload of misfolded proteins, active NRF1 accumulates and migrates to the nucleus, where it heterodimerizes with cofactors (small MAF proteins), binds to chromosomal targets, and activates the synthesis of PSMs (35). Illustration created with Biorender.com.

1.3: Inhibition of proteasome via proteasome inhibitor mediate processing and activating the nuclear form of NRF1

Maintenance of cellular health, function, and survival depends on proteostasis, which is a homeostatic balance between protein production with degradation. Most protein degradation is mediated by the ubiquitin proteasome system (UPS) (35, 36), and the UPS has been shown to influence cellular processes such as apoptosis, inflammation, antigen presentation, growth, and survival (37, 38). Moreover, when the proteasome is inhibited or defective, misfolded proteins accumulate and cause cell
stress and damage (39). The UPS is a megacomplex molecular machine composed of a 19S regulatory particle (RP) and 20S core particle. An ATP-dependent binding process occurs between the 19S RP and the 20S proteasome (40) either at one end or both ends to form a 26S or 30S proteasome, respectively (41, 42). The RP has a base and a lid comprised of ten subunits and is responsible for recognizing, unfolding, and translocating substrates. Four base subunits are non-ATPases (Rpn1, Rpn2, Rpn10, and Rpn13) that participate in recognition of polyubiquitylated substrates (43). Rpn1 and Rpn2 interact simultaneously with polyubiquitylated substrates and the proteasome (44), while Rpn10 and Rpn13 bind polyubiquitylated substrates only. Other base subunits (Rpt1-Rpt6) are AAA-ATPases that contribute to ATP hydrolysis, as this energy consuming process is necessary for the unfolding, opening, and entry of substrates through the 20S proteasome (45, 46). Meanwhile, the lid consists of nine different subunits (Rpn3, Rpn5–9, Rpn11, Rpn12, and Rpn15 (Dss1/Sem1), which form a horseshoe-shaped structure. A key feature of the lid is the deubiquitylation of the incoming substrates by the structural DUB Rpn11, a highly conserved metalloprotease (47, 48), which also maintains a sufficient pool of free ubiquitin (Ub) molecules in the cells. In contrast to the RP, the 20S proteasome has three different catalytic activities: 1) caspase-like/PGPH, 2) trypsin-like, and 3) chymotrypsin-like (49, 50). The substrate binding pockets determine the specificity of the active site. In order to form binding pockets, the catalytic subunit interacts with its neighboring β subunit (51). Therefore, the proteasome is not simply a collection of independent proteases but rather a unique multicatalytic enzyme that is active when fully intact. The chymotrypsin-like site (β5) preferentially cleaves after hydrophobic residues, the trypsin-like site (β2) preferentially cleaves after basic residues, and the caspase-like site (β1) preferentially cleaves after acidic residues (52, 53).

Several proteasome inhibitors have been described, which generally target the active sites of the 20S proteolytic core. Typically, these are covalent electrophilic inhibitors that react with the active site, of which there are several peptide aldehydes (ALLN, MG132, and MG115), peptide boronates (PS-341 or bortezomib), epoxyketones (carfilzomib and epoxomicin), and a metabolite isolated from streptomyces called lactacystin (54). As a research tool, MG132 is most widely used. MG132 binds to the β5 subunit of the 20S proteasome in a reversible manner in the low nM range, and at higher µM concentrations also bind the β1 and β2 subunits to effectively inhibit all catalytic sites of the 20S proteasome (55). Bortezomib (BTZ) is an inhibitor that also binds to β5 subunits and to lesser extent β2 and β1 subunits (56, 57). BTZ was the first proteasome inhibitor to receive FDA approval and is used to treat newly diagnosed multiple myeloma (58). Interestingly, a 2002 study showed multiple
myeloma cells treated with BTZ adapted by upregulating proteasome subunit genes (58). This later became known as the bounce-back effect and is thought to underlie BTZ resistance that develops in patients with multiple myeloma.

In two seminal works, NRF1 was identified to mediate the bounce back (59). Follow up work shows NRF1 induces transcription of genes involved in the proteosome complex (60). Moreover, liver of mice with hepatocyte Nrf1 deficiency were found to suffer significant damage following injection of proteasome inhibitor, whereas liver of wild type controls were healthy and had restored proteasome activity (17). In response to reduced proteasome activity, NRF1 accumulates and promotes expression of ARE-dependent genes. This has been confirmed in multiple tissues and cells and likely plays key roles in stress adaptation. A schematic of the "proteasome bounce-back response" and its control by NRF1 is shown in figure 1.3. However, despite the pervasiveness of bounce-back responses, several details regarding the mechanism underlying this phenomenon and the transcription factor that mediates it remains unclear. Moreover, NRF1 appears to play a role in cancer and other diseases, and several drugs and compounds that manipulate the proteasome are under clinical investigation. A table of such compounds is shown in table 1.1. So, clarifying the mechanism by which NRF1 operates and the relationship of proteasome modulating agents to NRF1 action may provide clinically informative insight.
Table 1.1. Distinct effects of some drugs or chemicals on NRF1-related molecules and diseases.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Salient features or mechanisms of action</th>
<th>Diseases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRR139, an NGLY1 inhibitor</td>
<td>WRR139 directly inhibits Ngly1 to disrupt the processing of NRF1, its subcellular localization and its transactivation.</td>
<td>Multiple myeloma</td>
<td>(31)</td>
</tr>
<tr>
<td>CFZ, a β5-inhibitor; and LU-102, a β2-specific inhibitor</td>
<td>Co-inhibition of β5 and β2 by treatment of CFZ and LU-102 causes cell death through sufficiently blocking the production of active NRF1 factor and the compensatory production of new proteasomes.</td>
<td>Triple-negative breast cancers (TNBC)</td>
<td>(61)</td>
</tr>
<tr>
<td>p62 inhibitor</td>
<td>Expression of p62 mediated by NRF1 alleviates its ‘bounce-back’ response to the toxicity of proteasome inhibitors, but this cytotoxicity may be elevated by preventing p62 post-translational modifications.</td>
<td>Neurodegenerative diseases</td>
<td>(62)</td>
</tr>
<tr>
<td>DDI-2 inhibitor</td>
<td>DDI-2 may contribute to the proteolytic cleavage of NRF1 to yield a mature activate isoform of this CNC-bZIP factor.</td>
<td>Multiple myeloma</td>
<td>(29)</td>
</tr>
<tr>
<td>Rotenone, an inducer of NRF1</td>
<td>Rotenone induces the ER-to-nuclear translocation of TCF11/NRF1, leading to a rapid induction of proteasome expression and de novo synthesis, such that Nrf1 promotes cell viability under stressed conditions.</td>
<td>Parkinson's disease</td>
<td>(63)</td>
</tr>
<tr>
<td>Icariin, an inducer of NRF1</td>
<td>Icariin induces the expression of Hrd1/synoviolin through NRF1-mediated transcription, so as to protect neurons from ER stress-induced apoptosis.</td>
<td>Neurodegenerative diseases, such as Alzheimer's disease</td>
<td>(64)</td>
</tr>
<tr>
<td>IU1 &amp; IU1–47, two USP14-specific inhibitors</td>
<td>Two inhibitors promote proteasome-mediated proteolytic degradation of misfolded proteins, albeit it is unknown whether and/or how they influence NRF1-mediated 26S proteasomal function and/or contents.</td>
<td>Proteotoxic diseases</td>
<td>(65)</td>
</tr>
<tr>
<td>Cholesterol, a regulator of NRF1</td>
<td>Cholesterol regulates NRF1 turnover, proteolytic processing, subcellular localization, and transactivation activity. Conversely, the cellular defense against cholesterol accumulation is disrupted by NRF1 deficiency.</td>
<td>Fatty liver disease, neurodegeneration, and atherosclerosis</td>
<td>(21)</td>
</tr>
</tbody>
</table>
Figure 1.3. **Bounce-back response.** Under normal conditions, ERAD machinery continuously degrades NRF1. When proteasome inhibition occurs, NRF1 accumulates in the ER and translocates to the nucleus, where it promotes the expression of target genes, including those encoding proteasome subunits. Proteasome gene induction is characterized by negative feedback mechanisms known as 'bounce-back responses' (58, 66). Illustration created with Biorender.com.

1.4: **Heterodimerization of NRF1 with small MAFs proteins can regulate its target genes**

In the nucleus, NRF1 regulates transcription of genes, including proteasome subunit and oxidative stress defense genes. Such action is engaged in response to proteasome insufficiency. However, for this adaptation to manifest, nuclear NRF1 must also form a functional heterodimer with small MAFs or other bZIP proteins, as upon binding to an antioxidant response element (ARE) this heterodimer complex recruits the molecular machinery underlying gene transcription (7). AREs are found in regions of many cytoprotective antioxidant and proteasome subunit genes, have a core sequence of 5′-TGACnnnGC-3′, and have been recognized to play a critical role in the oxidative stress response (67-69). The ARE consensus sequence, TGA(G/C)nnnGC, was initially identified as a binding site for v-maf oncprotein and resembles the MAF recognition element TGCTGA(G/C)TCAGCA (70), which highlights the close relationship between MAFs and NRF proteins. Large MAFs contain activation domains and small MAFs lack activation domains, and only the small MAFs heterodimerize with NRF1. Three small MAFs known to heterodimerize with NRF1
are MAFF, MAFG, and MAFK, and each can also form homodimers or heterodimers with each other (71). The functional role of each is uncertain. For example, a recent study showed triple deletion of MafF, MafG, and MafK in mice resulted in embryonic lethality, similar to the phenotype of Nrf1 deficiency, whereas mice with MafF and MafK deficiency but heterozygous for MafG were still viable and fertile (72). These and other findings have led to a view that MAFF, MAFG, and MFK are functionally redundant (73). However, as discussed in the rationale for my hypothesis below, I propose that distinct small MAFs may be able to render distinct transcriptional programming by NRF1.

MAFF, MAFG, and MAFK encode homologous bZIP proteins that have a molecular weight of approximately 18 kDa (74). As NRF1 shares a conserved bZIP domain with other CNC-bZIP proteins and contains charged amino acids at its dimerization interface, it is predicted that NRF1 forms a heterodimer with these small MAF proteins at this interface (75). Experimental data have shown a heterodimer composed of NRF1 and MAFG are capable of activating transcription (76). It is noteworthy that other complexes may also occur. For example, NRF1 has been proposed to form a heterodimer with activating transcription factor 4 and cAMP response element binding protein (77) as well as activating protein 1 (AP1) transcription factors such as FOSB, JUN, JUND, and ATF2 (78). In this case, there could be a wide array of NRF1 complex formations that elicit a multitude of stress adaptations or other homeostatic functions. However, evidence for NRF1 forming a heterodimer with non-small MAF proteins is less established than with the small MAF proteins, and so, while interesting, will be considered outside the scope of this thesis that aims to address only the function of NRF1 heterodimerization with small MAF proteins.

NRF1 may regulate target genes by selectively interacting with ARE binding sites. Various transcription factors are capable of binding to AREs. Indeed, it was initially believed AP1 heterodimers composed of JUN and FOS were responsible for ARE-dependent transcription (79). An analysis of binding motifs for the NRF2-, p45-NFE2, and BACH1-sMAF heterodimers revealed that a sequence of RTGACTCAGCA (80-82), with the CNC factors recognizing the R side and sMAF recognizing the GC side. It is suggested CNC-sMAF heterodimers bind a CNC-sMAF-binding element: RTGA(C/G)NNNGC (83). In this way, this element is distinguishable from elements that preferentially host MAF homodimers. Through these sites, both CNC-sMAF heterodimers and sMAF homodimers may participate in the regulation of gene transcription. Moreover, a switch between CNC-sMAF heterodimers and sMAF homodimers has been reported (84), and there is a correlation between the
expression of different subsets of cognate genes and the presence of distinct cis-regulatory consensus sequences within enhancer and promoter regions. Given these findings for other CNC factors, differential recruitment of NRF1 along with heterodimeric partners (e.g., sMAF, c-JUN, JUND, or c-FOS), may determine the transcriptional expression of ARE-driven genes. However, at present time it has not been established that NRF1 can heterodimerize with different sMAFs or other bZIP proteins to regulate distinct sets of cognate genes.

To understand whether NRF1 may regulate gene sets through distinct heterodimer pairings, it is useful to consider which genes have been shown to be regulated by NRF1 or at least contain AREs and assess whether this can reveal gene groupings related to specific types of stress defense. One set of genes NRF1 has been firmly established to regulate are the subunits comprising the proteasome complex such as PSMA1, PSMA2, PSMA3, and PSMB1 (27, 30, 60, 85), which underlie the stress response to insufficient protein turnover. Furthermore, AREs that may be targeted by NRF1 have been identified in the following genes linked to protection against oxidative stress (19, 22, 47, 86-89): 1) NAD(P): quinone oxidoreductase 1 (NQO1) which is responsible for reductive detoxification of quinones, 2) heme oxygenase 1 (HO-1) and ferritin heavy chain 1 (FTH1) which are involved in detoxifying labile and reactive heme iron, 3) metallothionein 1E (MT1E) which detoxify heavy metals such as copper and zinc, 4) solute carrier family 7 member 11 (SLC7A11), glutamate-cysteine ligase catalytic subunit (GCLC), and glutamate-cysteine ligase modifier subunit (GCLM) which are involved in glutathione (GSH) biosynthesis, 5) glutathione s-transferase alpha 4 (GSTA4), glutathione peroxidase (GPX1), and glutathione disulfide reductase (GSR) which utilize GSH to counteract reactive oxygen species (ROS), and 6) superoxide dismutase 1 (SOD1) and 2 (SOD2) as well as thioredoxin reductase 1 (TXNRD1) which are also involved in counteracting ROS. A schematic illustrating how some of these enzymes contribute to stress defense is shown in figure 1.4 and 1.5 below.
Figure 1.4. The NRF1-regulated cytoprotective defense system. Through the coordinated regulation of GSH and TXN production, utilization and regeneration, NADPH regeneration, heme and iron metabolism, ROS and xenobiotic detoxification, NRF1 can provide the cytoprotective defense system in the cell (86). Illustration created with Biorender.com.
Figure 1.5. ROS detoxification is driven by GSH and TXN antioxidant pathways. GSH and TXN are the two main antioxidant pathways. The metabolite NADPH can be used by TXNRD to regenerate TXN, a protein that acts as an antioxidant intermediate in reducing peroxiredoxins (PDXs). By reducing ROS levels directly, GSH, derived from glutamate, cysteine, and glycine, also requires NADPH for regeneration (90).

In addition to defense against proteasome insufficiency and oxidative stress, there are AREs in genes that encode metabolic enzymes supporting these defenses and may also counteract other stressors (22, 88). This includes malic enzyme 1 (ME1) which generates NADPH for lipid synthesis, isocitrate dehydrogenase (NADP(+))1 (IDH1) which generates NADPH for peroxisome functions, peroxisome proliferator-activated receptor gamma coactivator 1 beta (PGC1B) which is a transcription co-regulator with the lipid sensing peroxisome proliferator-activated receptor transcription factors, and lipin 1 (LPIN1) which is a phosphatidic acid phosphohydrolase involved in triglyceride synthesis. Collectively are three possible clusters involved in cell stress defense that may be controlled by NRF1; one that controls proteasome activity, one that detoxifies ROS, and one that supports metabolism. For this thesis, I will investigate the role of NRF1 in responding to cellular stress and regulating genes related
to these clusters, and whether specific small MAFs contribute to NRF1-mediated transcriptional responses.

1.5: Hypothesis and Objectives

I hypothesize that NRF1 regulates transcription of genes underlying proteasome activity, antioxidative stress defense, and cell metabolism through specific heterodimer interaction with small MAF proteins. Based on this hypothesis, I predict activating NRF1 transcriptional activity with a stressing agent will result in increased transcription of corresponding genes, and that NRF1 deficiency will prevent induction of these genes. Moreover, I predict MAFF, MAFG, or MAFK deficiency will impact transcriptional induction of these same genes, but not all the same genes. Below are my objectives for investigating the hypothesis.

Aim 1: Characterize the effect of celastrol on NRF1 and proteasome function. Researchers in the Widenmaier lab have identified celastrol as an NRF1-activating agent. The mechanism is unclear, but is of interest because celastrol is a natural agent known to have medicinal properties (42, 43). In this aim, I investigate how celastrol activates NRF1 in cultured Hep3B cells and show it involves inhibition of proteasome activity.

Aim 2: Investigate transcriptional effects of celastrol on stress defense genes, in the presence and absence of NRF1, MAFF, MAFG, and MAFK. Transcription effect of celastrol on possible NRF1-target genes mentioned in section 1.4 are investigated in wild type Hep3B cells and in cells lacking NRF1, MAFF, MAFG, or MAFK. The results are profiled to interpret whether there is a co-dependency of NRF1 and one of the other small MAFs in regulating transcription of defenses related to proteasome activity, anti-ROS, or cell metabolism.

Aim3: Confirm if NRF1 binds AREs in target genes identified in Aim 2. I used quantitative polymerase chain reactions (qPCR) of anti-NRF1 chromatin immunoprecipitated (ChIP-qPCR) samples to investigate if NRF1 occupied AREs in target genes and how this is affected by celastrol.
CHAPTER 2 – Materials & Methods
2.1: Cell culture, trypsinization, and drug treatment

Hep3B cells (American Type Culture Collection (ATCC) HB-8064) derived from liver tissue of a human with hepatocellular carcinoma, Hepa 1-6 cells (ATCC CRL-1830) from mouse liver hepatoma, and immortalized murine embryonic fibroblasts (iMEFs; gift from Hotamisligil Lab) was cultured in complete media consisting of Dulbecco's Modified Eagle Medium (DMEM) (catalog: 11966-025, Gibco, Grand Island, NY) supplemented with 10% cosmic calf serum (CCS, catalog: SH3008704HI, Cytiva HyClone™) at 37°C in a humidified incubator that was maintained at a CO₂ level of 5%. The attached cells were trypsinized with 0.25% trypsin (catalog: 25200056, Gibco™). The number of viable cells was counted using the trypan blue exclusion method with a hemocytometer. Hep3B cells were plated one day before treatment with celastrol (catalog: C0869, Sigma-Aldrich), which was dissolved in dimethylsulfoxide (DMSO; catalog: BP231-100, BioReagents™), in 6-well plates at a density of 1x10⁶ cells/well and a volume of 2 mL pre-warmed media. The following final drug concentrations were used: celastrol (0.25, 0.5, 1, 2, 4 μM). Hep3B cells incubated with vehicle (DMSO) in DMEM medium served as untreated control.

2.2: Luciferase reporter assays (Conducted by Sherin McDonald)

Hepa 1-6 cells and iMEF cells (described in chapter 3) were infected with lentivirus encoding ARE promoter-driven firefly luciferase gene from SABiosciences (catalog: LUC1000) that also expressing a puromycin resistance cassette. After transduction, cells with genomic integration of the cassette were selected by adding 2 μg/ml puromycin to media for at least one week. Surviving cells were grown and stocks stored in liquid N₂. This luciferase system was used to assess NRF1 transcriptional activity via luciferase activity as a surrogate. Induction of luciferase expression was monitored by measuring luciferase activity with a luciferase assay system kit (catalog: E1501, Promega), according to manufacturer instructions. For this, cells were seeded in 24-well plates at a density of 5x10⁴ to 1x10⁵ cells/well. Compound agents were examined for their potential ability to stimulate luciferase expression, as described in figure 3.1. Notably, this work was completed by at the time research assistant Sherin McDonald. The compounds tested were apigenin, curcumin, genistein, ECGC, and celastrol at concentrations ranging from 0.02 to 2.56 μM for a 6-hr treatment period.
2.3: Establishing NRF1, MAFK, MAFG, and MAFF knock-out cell lines

Stable cas 9 expressing Hep3B cell lines (Hep3B<sup>cas9</sup>) were generated in the Widenmaier lab by Sherin McDonald and Dr. Lei Li. These cells were used in my thesis research to employ CRISPR-cas9 based indel mutations that caused nonsense mutations in the genes for deletion of NRF1, MAFF, MAFG, and MAFK. First, Hep3B<sup>cas9</sup> cells were generated by transducing with cas9 expressing lentivirus that contain a blasticidin resistance cassette. After blasticidin selection, isolated single clones were collected using a cloning cylinder (sigma, TR-1004). Each clone was grown and examined for cleavage efficiency. Two were identified as having high efficiency, and each was grown and stocks stored in liquid N2. Hep3B<sup>cas9</sup> cells were then used by me to generate cell lines lacking NRF1, MAFF, MAFG, or MAFK. This was done by infecting cells with lentivirus encoding a puromycin resistance cassette and expressing single guide RNAs (sgRNA) targeting the transcription start site for NRF1 (GTGGATACCATAAGGCATCCA), MAFF (AGCCACCGTTTTGAGTGTG), MAFK (GACTAATCCCAAACCGAATA), and MAFG (GGACGATCTCCTCCTTGAGAC). These products were procured from LentiArray™ Lentiviral sgRNAs (Thermo Fisher Scientific) (Assay ID: CRISPR897042_SGM, CRISPR747887_LV, CRISPR1055811_LV, CRISPR800571_LV, respectively) (catalog: A32042). For introducing the sgRNAs, first Hep3B cas9 cells were grown in DMEM. Subsequently, 0.9 × 10<sup>6</sup> cells were seeded into a 6 cm plate for viral infection by adding polybrene (4 μg/ml). 1×10<sup>8</sup> TU (Transducing Unit)/mL (multiplicity of infection (MOI) used as 0.5) of sgRNAs were used for transduction. Another 6 cm plate without sgRNA containing polybrene was used as the negative control. Cell viability was checked every two days for up to two weeks. Selection processes performed by adding 3ug/ml puromycin plus 1ug/ml blasticidin and following by observing complete cell death for negative control after seven days. Finally, 15-20 isolated surviving clones that were picked using a cloning cylinder (sigma, TR-1004) and grown into cell line stocks were examined for loss of function nonsense mutation via Western blot of the targeted protein of interest.

2.4: Western blotting

Western Blotting and Antibodies

Western blots were used to assess NRF1 activation. After the endpoint of cell treatments, media was aspirated and cells washed with ice-cold phosphate buffered saline (PBS; catalog: 30028.02,
Cytiva). Then, PBS was aspirated and cells lysed in 200 ul of ice-cold RIPA lysis buffer (50 mM Tris-HCl PH 7.5, 150 mM NaCl, %1 NP-40, 1mM EDTA, 1mM EGTA, 0.1% SDS, 2.5 mM Na-pyrophosphate, and 1:50 protease inhibitor. Lysate was incubated at 4°C for 30 minutes, and then spun at 16,000g, 4°C for 15 minutes in a centrifuge. The protein-containing supernatant was transferred to a new tube and stored at -80°C until used for Western blots.

Equal protein loading/lane was employed in my Western blots. Protein concentration was quantified using the BCA assay (catalog: 23225, PierceTM, ThermoFisher Scientific, Ottawa, Ontario, Canada) protocol. When preparing to load gels, 20 μg of protein was incubated with 6X sodium dodecyl sulfate (SDS) loading buffer containing 5% beta mercaptoethanol and then boiled at 95-100°C for 5 minutes. Sample was then loaded into gel (catalog: NW04120BOX, ThermoFisher Scientific, 4-12% polyacrylamide concentrations) next to the lane loaded with 5 ul of PageRuler™ Prestained Protein Ladder (catalog: 26616, ThermoFisher Scientific), which served as protein molecular weight marker. Electrophoresis was run for 1 hour and 30 minutes at 100 V. Protein was then transferred to nitrocellulose membrane, 0.45 μm (catalog: 1620115, Bio-Rad) for 1 hour and 30 minutes at 10 V using a transfer system. Once complete, the membrane was blocked with 5% milk in tris buffered saline and 0.5% tween-20 in 1x PBST (50 ml PBST 10x + 450 ml d H2O) with gentle shaking for 1 hour at room temperature. For protein detection the membrane underwent overnight incubation with primary antibody diluted 1:1000 in PBST containing 5% milk at 4°C, with gentle shaking. On the next day, the membrane was washed 3-5 times for 5 minutes with PBST and then incubated with secondary antibodies, which were diluted with blocking buffer (1:5000) include PBST containing 5% milk at room temperature for 1 hr with gentle shaking at room temperature. Primary antibodies used are as follows: 1) TCF11/NRF1 (D5B10) Rabbit mAb, Cell Signaling, #8052, 2) Anti-Ubiquitin Mouse mAb (Milipore Sigma, FK2), and 3) GAPDH (Cell Signaling, (D16H11) XP® Rabbit mAb #5174). Secondary antibodies used are as follows: 1) Anti-mouse IgG, HRP-linked Antibody (Cell Signaling, mAb #7076), 2) Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling, mAb #7074). The secondary antibody was covalently attached to horse radish peroxidase (HRP). So, Western blot detection of protein of interest was done by incubating membrane with chemiluminescent substrate (catalog: 34580, Thermo Scientific™) working solution, which was added by mixing equal parts of the Stable Peroxide Solution and the Lumino/Enhancer solution. Chemiluminescent signal was detected and recorded using a CCD camera-based imager (BIO-RAD Imager).
2.5: Nuclear fractionation

Cells were washed once with ice-cold PBS, then incubated in Buffer A (10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 0.34 M sucrose, 10% Glycerol, 0.1% Triton X-100, 0.5 mM DTT, 1:50 protease inhibitor) for 5 minutes while on ice. Nuclei were collected in the pellet by low-speed centrifugation (1500 x g, 4 min, 4°C). The supernatant was further clarified by high-speed centrifugation (1300 x g, 4 min, 4°C) to remove all debris and insoluble aggregates. Pellet were washed once with buffer A without 0.1% Triton X-100 and then lysed in 200 μl of buffer B (20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.05% NP-40, 25% Glycerol, 0.5mM DTT, and 1:50 protease inhibitor). After 10 min incubation on ice, soluble nuclear proteins were separated from chromatin by centrifugation (2000 x g, 4 min).

2.6: RNA isolation and quantitative reverse transcriptase PCR

Complementary DNA (cDNA) was generated from cells to measure the level of gene transcript as a surrogate of gene expression regulation by NRF1. RNA was extracted from cells using 1ml Trizol RNA reagent (catalog: 15596026, Thermo Fisher Scientific) (1 mL) for 1 × 10⁵ cells directly to the culture dish. Then 0.2 mL of chloroform was added, the sample was mixed and left to stand and separate and then centrifuged the samples for 15 minutes at 12,000 x g at 4°C. Using filtered tips for this and all subsequent steps, approximately 300 ul of the aqueous phase was carefully collected and then transferred to a RNAase-free tube. 0.5 mL of RNAase-free isopropanol was added, the sample was gently mixed, and underwent centrifugation for 10 min at 12,000xg, 4°C. At this stage, the RNA formed a precipitated pellet. The supernatant was discarded and pellet resuspended in 1 mL of 75% ethanol in RNAase-free water. Sample again underwent centrifugation for 10 min at 7500 x g at 4°C. The supernatant was discarded and pellet dried at room temperature for 5 minutes. The pellet was then resuspended in 50 ul of RNase-free water (catalog: B1500S, New England Biolabs). RNA quantification was done by spectrometry using a NanoDrop 2000 (Thermo Fisher Scientific), also allowing purity check via the A260/A280 ratio (≥2.0).

The Maxima® First-Strand cDNA Synthesis Kit (catalog: K1641, Thermo Scientific) was used for cDNA synthesis in two-step reverse transcriptase. This kit contains both oligo(dT)18 and random
hexamer primers to prime first-strand cDNA synthesis. 1 μg of template RNA was used, and to complete the reaction the following reagents were added to RNA template in a sterile, RNase-free tube: 10X dsDNase buffer, dsDNase and after mix gently and micro-centrifuge the samples, incubate them for 2 minutes at 37°C in water bath, and briefly centrifuge and place on ice. Then, the following components were added to the tube: 5X reaction mix, Maxima enzyme mix, and nuclease free water. The mixture was placed in a thermocycler and run the program as follows: 25°C for 10 min, 37°C for 60 min, 70°C for 10 min, 4°C hold.

Amplification of cDNA was done to measure transcript levels via quantitative reverse transcriptase PCR, using a Step One Plus PCR machine (ABI). In each reaction mixture was 2 μL of 1/100 diluted cDNA, 3.5 μL of water, and 7.5 μL of 2x SYBR Green master mix reagent (catalog: A25741, Applied Biosystems™), making for 15-μL reactions, which were run in duplicate. GAPDH mRNA level was used to normalize qPCR results, and relative expression was calculated using the delta-delta Ct method, also known as the $2^{-\Delta\Delta Ct}$. Fold change in expression was determined from the difference between the averaged expression levels relative to the control.

2.7: Proteasome activity assay

Proteasome activity was measured using the Proteasome Activity Fluorometric Assay Kit II (catalog: J4120), UBPBio, USA). Two 15 cm dishes of Hep3B cells were grown in DMEM. Confluent cells were washed twice with ice cold 1X PBS, then cells were collected as a pellet in a 15 ml conical tube and stored at -80°C. At time to complete proteasome activity assay, the cell pellet was resuspended in 2 ml ice-cold cell lysis buffer (40 mM Tris, pH 7.2, 50 mM NaCl, 2 mM BME, 2 mM ATP, 5 mM MgCl2, 10% glycerol). Cells were sonicated using a 550 Sonic Dismembrator (Fisher Scientific) as follows: power output of 3, 15 seconds/time for three times, and the tube placed on ice for 2 minutes after each sonication. Then, lysed cells underwent centrifugation at 17,000 x g for 20 min, 4°C. Protein concentration of the supernatant was determined using the BCA assay.

Proteasome enzymatic activity was determined by fluorometric assays using the substrates Suc-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc-LLVY-AMC), Boc-Leu-Arg-Arg-7-amino-4-methylcoumarin (Boc-LRR-AMC), or Ac-Nle-Pro-Nle-Asp-7-amino-4-methylcoumarin (Ac-
nLPnLD-AMC). This enabled assessment of chymotrypsin like activity, trypsin like activity, and caspase-like activity respectively. Inhibitory effect of MG132 and celastrol directly on the proteasome was determined by adding MG132, celastrol, or vehicle to lysates. In contrast, cells were also treated with MG132 and celastrol to assess effect of cellular treatment on proteasome activity. Proteasome activity was assessed by adding 200 μl 1X Assay Buffer, 50 μl lysate, and 50 μl of substrate (Suc-LLVY-AMC, Boc-LRR-AMC, or Z-LLE-AMC) into a black 96 well plate. The release of fluorogenic AMC was monitored at 360/40 nm excitation and 460/30 nm emission. The following parameters were introduced for a 15 min kinetic assay: 1 minute per reading interval, sensitivity setting at 62, probing from the bottom of the plate, and shaking the plate for 5 seconds before the first reading to mix samples. A Biotek Synergy HT Microplate Reader from Marshal Scientific was used to measure fluorescence.

2.8: Chromatin immunoprecipitation (ChIP-qPCR)

ChIP-qPCR was done to assess ARE binding by NRF1 in gene promoters. Briefly, four confluent 15 cm dishes (1x10^7 cells per dish) were used per sample. Protein was cross-linked to DNA by adding methanol free 16% formaldehyde to a final concentration of 1%. Then glycine was added to a final concentration of 125 mM to each dish and then incubated with shaking for 5 minutes at room temperature. Then, cold PBS + 1mM PMSF was added to scrap the cells and transferred into the 50 mL tube. Sample underwent centrifugation for 5 min, 4°C, 2,000 x g and supernatant removed. Then, buffer A containing 1mM PMSF and 1mM DTT was used to add 2 ml per 15 cm dish and incubated it on ice for 10 min. Then centrifuge the samples for 5 min, 4°C, 2,000 x g, and discard the supernatant. Then, the cell pellet was lysed with DNAzol (catalog: 10503027, Invitrogen™) (1 mL per 15 cm plate) by re-pipetting and incubating for 15 min at room temperature. To extract the DNA protein complexes, 0.5 ml of ice-cold 100% ethanol was added per 1mL of DNAzol. Precipitation of the pellet was done by centrifugation at 1792 x g at 4°C for 10 minutes. Pellet was washed with 1 mL ice-cold 75% ethanol per 1 ml of DNAzol. Then centrifugation was performed at 1792 x g at 4°C for 10 min. After air drying the pellet, the pellet was resuspended in 1 ml of 50 mM Tris-HCL buffer (PH 7.4). Then, 200 ul of 2% SDS and 500 mg Urea were added to each 15 cm plates. Sample was transferred to a 15 mL tube. An equal volume of 5M NaCl was added and sample was incubated at 37°C for 30 minutes. 200 ul of 3 M sodium acetate was added to the samples, and then incubation at -20°C for 1 hr to precipitate DNA and DNA-protein complexes. Sample underwent centrifugation at 1792 x g at 4°C for 10 minutes.
Supernatant was aspirated and pellet washed 3 times with 5 mL ice-cold 75% ethanol. Then after dissolving the pellet with 950 ul of IP buffer (150 mM NaCl, 50 mM Tris-HCl (PH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1% vol/vol). Sonication of DNA was performed using Bioruptor Pico by setting 15 cycles of 30 sec ON/30 sec OFF at 4°C.

ChIP reactions were done using material prepared above. A 50 ul aliquot of total DNA was added to 150 ul 1x ChIP elution buffer (75 ul 2X chip elution buffer + 75 ul dH2O), which is used for elution of chromatin. The concentration of sheared chromatin was determined by purifying DNA with a PCR purification kit (K702, source) and the measuring purified DNA with nanodrop. Aliquots of 30 ug DNA was set aside for each IP reaction, as well as input sample. For ChIP, 2ug of anti-NRF1 or anti-IgG antibody pre-conjugated to ChIP-Grade Protein G Magnetic (catalog: 9006, Cell Signaling Technology) was added to 30 ug DNA in IP buffer with 1X protease inhibitor cocktail II. The antibody magnetic bead pre-conjugation step was done by adding 30 ul of protein G Magnetic Beads per 2 ug of antibody and incubating in IP buffer for 2 hr at 4°C with rotation. This was magnetically precipitated and washed with 1 mL of IP buffer prior to using in the ChIP reaction. Antibody conjugated beads and chromatin sample were incubated overnight at 4°C with rocking. Sample was magnetically precipitated and washed with 1 ml IP buffer at least 3 times to generate the IP sample. For reversal of cross-linking, 150 ul of the 1X ChIP elution buffer was added to a 5% input sample and eluted chromatin from the antibody IP and incubated for 30 minutes at 65°C with vertexing (1,200 rpm). Protein in samples was eliminated by adding 6 ul of 5M NaCl and 2 ul of proteinase K (20 mg/mL) and incubating at 65°C overnight. A PCR purification kit (Thermo, Genejet PCR purification kit, K0702) was used for sheared chromatin DNA purification.

qPCR was done using primers to specific genomic regions to assess whether anti-NRF1 ChIP showed binding of NRF1 to these regions. 2 ul of each ChIP sample was used for the qPCR reaction. For designing the primers, the location of the potential gene ARE regions that protein is bound to was found through the Eukaryotic Promoter Database (https://epd.expasy.org/epd). Then we used the BLAST-like alignment tool (BLAT) option from the UCSC website (https://genome.ucsc.edu/). The genetic region entered for primer was around 400 bp surrounding the putative ARE site. The target PCR product size was approximately 70-150bp (Table 2.2). After performing ChIP-qPCR, average Ct values of ChIP samples were normalized by a 5% input sample. Delta Ct values were calculated by
simply subtracting the Ct values of the antibodies of interest from the input Ct value. The % of input value was generated based on the delta Cts and then relative differences in fold change were calculated by comparing groups to control for each antibody (i.e., anti-NRF1 and anti-IgG).

2.9: Statistical analysis

The data were presented as fold change mean values ± SD, each representing three independent experiments performed in duplicate. Statistical comparisons among groups were performed using one/two-way ANOVA, as described in figure legends. The qPCR results were analyzed using Tukey's multiple comparison test. These analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, USA).
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<td><strong>MT1E</strong></td>
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<td>CCGACATCAGGCACACAGCAGCT</td>
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<tr>
<td><strong>SLC7A11</strong></td>
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<tr>
<td><strong>IDH1</strong></td>
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<td>CCTCTGCTTTCTACTGGCTTGC</td>
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<tr>
<td><strong>SOD1</strong></td>
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<td>CCACAAGCCAAAGCACTTCCAG</td>
</tr>
<tr>
<td><strong>SOD2</strong></td>
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<td>AACCTGAGCCTTGGACACCAAC</td>
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<tr>
<td><strong>GSR</strong></td>
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</tr>
<tr>
<td><strong>MT1</strong></td>
<td>GGCTATATACAAACTGCAAAAGGC</td>
<td>GGTAGATGGGCGGGTTTGGAG</td>
</tr>
</tbody>
</table>

**Table 2.1.** NRF1 target genes RT-qPCR primer sequences.
Table 2.2. Primers designed for ChIP-PCR.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>FWD primer sequence (5’→3’)</th>
<th>REV primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXNRD1</td>
<td>CCCTCACGTGATGACAACAGC</td>
<td>CATAGCCGCCAACCTAA</td>
</tr>
<tr>
<td>HO-1</td>
<td>CCCTGAGCAGGCACTGAGTA</td>
<td>CACCTGGTATCCGCTTGGAC</td>
</tr>
<tr>
<td>GCLC</td>
<td>GAGGCCCTGTCGAATAAAACA</td>
<td>GTCGCAAGTGAGGGAGCAG</td>
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<tr>
<td>NQO1</td>
<td>CTGAGCCTAGGGCACCACAG</td>
<td>GTCCAGGGATCAAGTGGGCA</td>
</tr>
<tr>
<td>GCLM</td>
<td>AGTGGTGGTGGTCTTGGGTA</td>
<td>ATTGAACGACCTTGGCCTG</td>
</tr>
</tbody>
</table>
CHAPTER 3 – NRF1 activation by celastrol through proteasome inhibition
3.1: Luciferase activity assay for chemical screening

The central goal of my research was to test whether NRF1 regulates transcription of genes underlying proteasome activity, anti-oxidative stress defense, and metabolism through specific heterodimeric interactions with distinct small MAF proteins. To test this hypothesis, I deemed I would need a tool compound capable of stimulating NRF1 transcriptional activity and expression of genes related to proteasome activity, anti-oxidative stress defense, and/or metabolism. Previous research by Sherin McDonald, while a research assistant in the lab, was able to identify a tool compound that may be suited for this purpose. Below, I will describe that work.

First, Sherin undertook a small screen of compounds reported to have therapeutic benefit (91-93) and proteasome inhibitory effectiveness. These agents were apigenin, curcumin, genistein, epigallocatechin-3-gallate (ECGC), and celastrol. To test whether they may activate NRF1, Hepa 1-6 cells expressing ARE promoter-driven luciferase (ARE-luciferase) were treated with a dilution series of compound ranging from 0.04 – 4 μM for 6 hours. The proteasome inhibitor epoxomicin was used as a positive control for NRF1 activation, whereas CDDO-methyl was used as a positive control for NRF2 activation. A schematic is below, and the result is shown in Figure 3.1A. Notably, celastrol exhibited the highest potency, as it increased ARE-luciferase activity by 30-fold at 4 μM and at the 1 – 2 μM dose the levels were comparable to positive controls. Then, to test whether the effect of celastrol may be due to NRF1 activation rather than NRF2 activation, Sherin generated ARE-luciferase expressing immortalized murine embryonic fibroblasts (iMEFs) that were wild type (WT) or deficient for NRF1 (NRF1 KO) or NRF2 (NRF2 KO). Each line was treated with 2 μM celastrol for 6 hours and then luciferase activity underwent examination. As shown in Figure 3.1B, celastrol induced ARE luciferase in WT cells to a greater degree than NRF1 KO and NRF2 KO cells, showing that ARE-luciferase activation by celastrol involves NRF1 and NRF2. This was interesting to us because celastrol has important therapeutic properties (91, 94, 95). Moreover, while celastrol was already known to activate NRF2, our finding is the first demonstration that celastrol may activate NRF1. So, I pursued this line of work by investigating how celastrol may activate NRF1. This work will be described for the remainder of chapter 3. How I then used this agent to investigate my hypothesis is described in chapter 4.
Luciferase assay for chemical screening
(Sherin McDonald)

Transduction
Hepa 1-6

Immortalized MEFs
(iMEFs)

Cell Lysate

Measurement of Luciferase Activity

Figure 3.1: Luciferase-based reporters as a measure of antioxidant response element (ARE) driven genes activity to identification of celastrol (Conducted by Sherin McDonald).

A) Luciferase-based reporters as a measure of ARE driven gene activity after treating Hep1-6 cells with Apigenin, Curcumin, Genistein, ECGC, and celastrol at different concentrations (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 μM) for 6 hr. Fold increasing of ARE- luciferase activity showed at different concentrations of chemical compounds by two-way ANOVA with Sidak’s multiple comparisons tests.

B) Nrf1 and Nrf2 immortalized MEFs expressing ARE luciferase were treated with celastrol as indicated for 6 hr. Luciferase-based reporter systems, using Nrf1+/+, Nrf1−/−, or Nrf2−/− immortalized MEFs. Fold increasing of ARE- luciferase activity showed in WT cells compared to NRF1 and NRF2 ko cells by two-way ANOVA with Tukey multiple comparison test.
3.2: Celastrol increases the level of cleaved NRF1 and its nuclear localization

The first step was to investigate the processive steps by which NRF1 is transcriptionally activated in celastrol treated Hep3B cells. As I described in section 1.2, activation of the 741 amino acid ER-membrane bound NRF1 typically involves its dislocation from the ER, deglycosylation by NGLY1, cleavage between amino acids 103-104 by DDI2, and mobilization into the nucleus. Hence, a surrogate detection of NRF1 activation can be done by measuring levels of the full length and 95 kDa cleaved NRF1 isoform as well as its abundance in the nucleus. To examine this, Hep3B cells were treated for 6 hours with 0, 0.25, 0.5, 1, 2, and 4 μM celastrol and whole cell lysate was collected for Western blot analysis. Antibody targeting GAPDH was used as a loading control. Celastrol at 2 and 4 μM doses, consistent with ARE-luciferase data, enhanced cleaved NRF1 levels by 3-fold (Figure 3.2A and 3.2B) and there was an apparent decline in full length NRF1 levels. Then, since we considered this effect of celastrol may be caused by proteasome inhibition, the effect of known proteasome inhibitor bortezomib (BTZ) on cleaved NRF1 level also underwent examination in Hep3B cells. As shown in Figure 3.2C and 3.2D, BTZ at 5 to 40 nM doses dramatically increased cleaved NRF1 level. Taken together, these findings show that NRF1 in Hep3B cells exhibits typical responsiveness to proteasome inhibition and that celastrol elicits a response by NRF1 that is comparable to the proteasome inhibitor BTZ.
Figure 3.2: Dose-response experiment performed by western blot for measuring the cleaved form protein levels of NRF1 after inducing with celastrol and Bortezomib (BTZ). A) HEP3B cells were treated with different dosages of celastrol in dose-response experiments for 6 hr. Cells were lysed and subjected to immunoblot analysis visualizing the full-length, cleaved form of NRF1 and GAPDH as the loading control. B) Cleaved NRF1 expression protein level was quantified from the integrated luminescence of corresponding cleaved (95 kDa) bands normalized to GAPDH, and the cleaved form of NRF1 was quantified as shown as fold changes. C) Treatment of HEP3B cells with different concentrations of BTZ for 6 hr induces the processing of NRF1 at 5 nM or higher. D) Data in the bar graph are the fold changes of three independent measurements. *p < 0.05 compared with the control for each time. Asterisks (*) indicate a significant difference (p<0.05) compared to the control, **p ≤ 0.01, ***p ≤ 0.001.

Next, I sought to determine if celastrol treatment enhances the translocation of NRF1 from the cytosol into the nucleus in a similar manner to proteasome inhibitor. For this, Hep3B cells were treated with vehicle control, 2 μM celastrol, or 20 nM BTZ for 6 hr. At this point, cells were collected and then nuclei and cytoplasmic fractions were harvested and underwent Western blot analysis. Antibodies detecting IRE1 and Lamin A/C were used as a loading control and to verify the quality of the fractionation into cytoplasmic and nuclear compartments, respectively. In comparison to control, celastrol increased nuclear level of cleaved NRF1, as did BTZ (Figure 3.3A and 3.3B). However, levels were much greater in BTZ treated cells, indicating that if celastrol inhibits the proteasome it is only modest in comparison to BTZ. Altogether, data in section 3.1 show celastrol induces NRF1-dependent
ARE-promoter activity and NRF1 cleavage and accumulation in the nucleus. Thus, celastrol is a suitable compound to study the hypothesis.

Figure 3.3: Celastrol stimulates nuclear translocation of Nrf1 in HEP3B cells. A) The cells were then treated with 2μM of celastrol and 20 nM of BTZ for 6 hr. The cells were lysed for the preparation of cytoplasmic and nuclear extracts. IRE1α and Lamin A/C were used as cytoplasmic and nuclear markers, respectively. B) The intensity of the Western blot signal was normalized to the corresponding signal of IRE1α and Lamin A/C for cytoplasmic and nuclear samples, respectively, and expressed as fold change over the intensity of controls without celastrol and BTZ treatment. Quantitative data from three independent experiments.

3.3: Measuring proteasome activity after treatment with celastrol

Given the rationale of the screen with proteasome inhibiting compounds (section 3.1) that led to identifying celastrol as an NRF1-activator, I assessed the effect of celastrol on proteasome activity. As the proteasome degrades poly-ubiquitinated protein substrates, I reasoned celastrol treatment at doses that activate NRF1 may increase poly-ubiquitinated protein level. Hep3B cells were treated for 6 hours with celastrol at the same dose range as Figure 3.2A and 3.2B. Whole cell lysate was collected and Western blot done with anti-ubiquitin antibody. Antibody targeting GAPDH was used as a loading control. As shown in Fig. 3.4A and 3.4B, the level of ubiquitinated protein increased in cells treated with 2 and 4 μM celastrol, but not less, which largely correspond to the NRF1-activating doses found in section 3.1 and 3.2. Thus, these findings support celastrol may inhibit the proteasome and that, by doing so, may engage NRF1 transcriptional activity. To expand on this, for the remainder of section 3.3 below I examined the effect of celastrol on the enzymatic rate of chymotrypsin-like, trypsin-like and caspase-like proteasome activities.
**Figure 3.4: Quantifying the ubiquitinated protein level in a dose-response experiment after 6 hr treatment with different dosages of celastrol.** A) Quantifying the ubiquitinated protein level in a dose-response experiment after 6 hr treatment with different dosages of celastrol, total cell lysates were subjected to western blot analysis with anti-ubiquitin and anti-GAPDH antibodies. B) Western blots were normalized with the GAPDH as a loading control, and data are presented as the mean of treatment groups compared with the control group for three independent experiments *p < 0.05 indicates a statistical difference from the control.

To interrogate the proteasome, I performed kinetic assays assessing proteasome-dependent degradation rate of the following: 1) succinyl-Leu-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC), which is specific for chymotrypsin-like activity, 2) ter-butyloxy carbonyl-Leu-Arg-Arg-AMC (Boc-LRR-AMC), which is specific for trypsin-like activity, and 3) carbonyl benzyl-Leu-Leu-Glu-AMC (Z-LLE-AMC), which is specific for caspase-like activity. Soluble proteasome-containing lysate was collected from a batch of untreated Hep3B cells under normal media conditions, then aliquoted and stored in the freezer for *in vitro* assays. Using this material, 0.5 or 2 μM celastrol or vehicle control was added to thawed lysate. A group containing 100 μM MG132 was added as a control for maximum proteasome inhibition. Then, substrate for chymotrypsin-like activity (Suc-LLVY-AMC), trypsin-like activity (Boc-LRR-AMC), or caspase-like activity (Z-LLE-AMC) was added and the proteasome degradation rate of substrate underwent examination. As expected, MG132 almost completely blunted proteasome activity, whereas there were high activities found in vehicle control
treated lysate (Figure 3.5A-C). Treatment with 0.5 µM celastrol reduced chymotrypsin-like activity by approximately 50% but did not significantly reduce trypsin-like or caspase-like activity. In comparison, 2 µM celastrol reduced chymotrypsin-like activity to a greater extent and also significantly reduced trypsin-like and caspase-like activity. These findings show celastrol exhibits dose-dependent inhibition of proteasome activities, with greater effect on chymotrypsin-like activity than on trypsin-like and caspase-like activity.

**Figure 3.5: Proteasome activity assay after treating cell lysates in-vitro with MG132 and celastrol (0.5 and 2 µM).** Treatment of Hep3B cells directly in vitro with the Mg132 (100 µM), celastrol (0.5, and 2 µM). The bar graph used to show different proteasome activity based on relative fluorescent units (RFU) of A) Suc-LLVY-AMC, B) Boc-LRR-AMC, and C) Z-LLE-AMC. In these figures, error bars represent the means ± SEM. One-way ANOVA with Dunnett multiple comparison test.

Next, I examined whether celastrol treatment to cultured Hep3B cells, rather than isolated the proteasome-containing lysate above, also resulted in reduced proteasome activity. Hep3B cells were treated with 0.2 or 2 µM celastrol or vehicle control for 6 hours. From each group, soluble proteasome-containing lysate was collected, aliquoted and stored in the freezer. Using thawed lysate from this material, substrate for chymotrypsin-like activity (Suc-LLVY-AMC), trypsin-like activity (Boc-LRR-AMC), or caspase-like activity (Z-LLE-AMC) was added and the proteasome degradation rate of
substrate underwent examination (Figure 3.6A-C). Compared to control, 0.5 µM celastrol significantly reduced chymotrypsin-like activity but not trypsin-like or caspase-like activity. In comparison, 2 µM celastrol exhibited a potent inhibitory effect by almost completely blunting chymotrypsin-like, trypsin-like, and caspase-like activity. These effects were much more dramatic than observed when celastrol was added to lysates (Figure 3.5), suggesting celastrol can directly inhibit the proteasome and also impact other cellular functions in turn indirectly inhibit the proteasome to an even greater extent. Taken together, the results of section 3.3 clearly show celastrol has proteasome inhibitory capacity, and this occurs at NRF1 activating doses. Thus, my results suggest celastrol activates NRF1 by inhibiting the proteasome.

![Graphs A, B, C showing proteasome activity assay](image)

**Figure 3.6:** Proteasome activity assay after treating Hep3B with celastrol (0.2, 2 µM) for 6 hr. Treatment of Hep3B cells with celastrol (0.2, and 2 µM) for 6 hr. A) Suc-LLVY-AMC B) Boc-LRR-AMC C) Z-LLE-AMC was used here as the measure of activity of proteasome based on the relative fluorescent unit (RFU). In these figures, error bars represent the means ± SEM. One-way ANOVA with Dunnett multiple comparison test.
CHAPTER 4 – The regulatory role of NRF1 and its heterodimer partners on proteasomal subunit and oxidative stress defense genes expression in response to stimulation by celastrol
4.1: The regulatory role of NRF1 on proteasomal subunit and oxidative stress defense genes expression in response to stimulation by celastrol

Upon establishing celastrol as a suitable tool compound to activate NRF1, I then turned to investigate the hypothesis that NRF1 regulates transcription of genes underlying proteasome activity, anti-oxidative stress defense, and cell metabolism through specific heterodimer interaction with small MAFs. To do this, Hep3B cells deficient for NRF1 (NRF1 Ko), MAFF (MAFF Ko), MAFG (MAFG Ko), or MAFK (MAFK Ko) were generated using CRISPR/Cas9 technology (see chapter 2 for details). Then, the transcriptional response of these cells to celastrol was compared to regular wild type Hep3B cells. In accordance, if NRF1 induces expression of these genes upon celastrol treatment than NRF1 Ko cells should lack this response. Likewise, if NRF1 elicits this effect by heterodimerizing with MAFF, MAFG, or MAFK than transcriptional response in the respective Ko cells should behave similarly to NRF1 Ko cells.

To identify genes induced by celastrol in an NRF1-dependent manner wild-type and NRF1 Ko cells were treated with 2 µM celastrol or control media for 6 hours and quantitative PCR (qPCR) was done to measure transcription of possible target genes. Genes selected for examination were due to their involvement in proteasome activity, oxidative stress defense, or cell metabolism (Figure 4.1A-V). The results show NRF1-mediated celastrol induction of proteasome subunits genes (PSMA1, PSMA2, PSMA3, PSMB1), as this effect occurred in wild type cells but not in NRF1 Ko cells. Likewise, NRF1-mediated celastrol induction of some oxidative stress defense and metal transport genes such as glutamate-cysteine ligase modifier (GCLC) and catalytic subunits (GCLM), heme oxygenase (HO-1), glutathione reductase (GSR), metallothionein 1 (MT1), metallothionein 1E (MT1E), ferritin heavy chain 1 (FTH1), and solute carrier family 7 member 11 (SLC7A11). Moreover, NRF1-mediated celastrol induction of some cell metabolism genes such as Lipin1 (LPIN1), peroxisome proliferator-activated receptor gamma co-activator 1-beta (PGC1B), NADP-dependent malic enzyme (ME1), and Isocitrate dehydrogenase 1 (IDH1). In contrast, NRF1 was dispensable for celastrol induction of NAD(P)H Quinone Dehydrogenase 1 (NQO1) and thioredoxin reductase 1 (TXNRD1), and celastrol had no effect on glutathione S-transferase alpha 4 (GSTA4), glutathione peroxidase (GPX1), and superoxide dismutase (SOD1 and SOD2). Overall, these findings reveal several NRF1 regulated target genes in
response to celastrol with roles ranging from proteasome activity, oxidative stress defense, and metabolism.
Figure 4.1: Expression of transcriptional regulators associated with proteasome subunit and oxidative stress defense genes in wild-type and NRF1 deficient in normal and celastrol treated groups. (A-D) mRNA expression of proteasome subunit genes, and (E-V) relative gene expression of oxidative stress defense genes. Delta Ct values of 20s proteasome subunit and oxidative stress defense genes expression levels measured by qRT-PCR. The expression level of each mRNA was normalized to that of GAPDH mRNA. The data represent the means ± standard deviations of three independent experiments.

4.2: The regulatory role of MAFF, MAFG, and MAFK on proteasomal subunit and oxidative stress defense genes expression in response to stimulation by celastrol

To identify genes-induced by celastrol in a MAFF-, MAFG-, or MAFK-dependent manner wild-type, MAFF Ko, MAFG Ko, and MAFK Ko Hep3B cells were treated with 2 µM celastrol of control media for 6 hours and qPCR was done to measure transcription of NRF1-dependent genes, identified in Figure 4.1 (Figure 4.2A-V). In MAFG Ko cells compared to wild type cells there was a blunted celastrol response for all four proteasome subunit genes PSMA1, PSMA2, PSMA3, and PSMB1, all three metal transport genes FTH, MT1E, and MT1, all four metabolism genes LPIN1, PGC1B, ME1, and IDH1, but only a partial blunting of glutathione-linked ROS-defense genes GCLC and GCLM and no other celastrol-induced ROS-defense genes such as GSR and HO-1. By
contrast, in MAFK Ko cells there was a blunted celastrol response for two proteasome subunit genes PSMA1 and PSMA3 but not for PSMA2 and PSMB1, a blunted response for all three metal transport genes as well as ROS-defense genes GCLC, GSR and, HO-1 but not for GCLM, and a blunted response for cell metabolism genes LPIN1, PGC1B, and IDH1 but not for ME1. And lastly, in MAFF Ko cells there was a blunted celastrol response for three proteasome subunit genes PSMA1, PSMA3, and PSMB1 but not for PSMA2, a blunted response for metal transport genes MT1E and MT1 but not FTH, as well as a completely blunted response for all four ROS-defense genes GCLC, GCLM, GSR, and HO-1, and a blunted response for cell metabolism genes LPIN1, PGC1B, and IDH1 but not for ME1. Additionally, it was found that MAFF Ko, MAFG Ko, and MAFK Ko had blunted celastrol-induced N NQO1, which since NRF1 is not required for this effect (Figure 4.1I) is likely linked to their heterodimeric interaction with NRF2. Further in regard to NRF1-independent regulatory effects, each small MAF was not involved in the expression of GSTA4, SOD1, or SOD2 whereas MAFK was required for celastrol induced regulation of TXNRD1 and MAFF was required for celastrol induced regulation of GPX1. Overall, these findings reveal each sMAF protein has unique and overlapping roles, which are likely linked to formation of heterodimers with NRF1.
4.3: NRF1 binds to the regulatory regions of oxidative stress defense genes

The findings in Figure 4.2 prompted me to determine whether celastrol enhances NRF1 binding directly to the ARE sites for gene regulation. So, I found ARE sequences in the human genome for the \textit{GCLC}, \textit{GCLM}, \textit{TXNRD1}, \textit{HO-1}, and \textit{NQO1} genes, in accordance to previous reports (33, 34). To determine whether NRF1 directly bound these ARE motifs, I performed ChIP-qPCR analyses on chromatin extracted from Hep3B wild type and NRF1 Ko cells treated with 2 µM celastrol or control media for 6 hours, as described in section 2.8. As shown in Figure 4.3, using ChIP on NRF1 Ko control treated cells as baseline, celastrol caused a rise in NRF1 binding to the promoter of \textit{GCLC}, \textit{GCLM}, \textit{HO-1}, and \textit{NQO1} but not to \textit{TXNRD1}. Overall, these findings confirm direct regulation by NRF1 of
GCLC, GCLM, and HO-1 in celastrol treated cells, and it was interesting that there was also binding to the NQO1 promoter as celastrol induction of this gene was NRF1-independent.

**Figure 4.3:** NRF1 binds to the regulatory regions of oxidative stress defense genes. ChIP assays were performed with chromatin extracts from Hep3B cells using normal IgG (control) and anti-Nrf1 antibodies and analyzed by qPCR. (A-D) NRF1 is recruited to the AREs of the GCLC, GCLM, HO-1, \(NQO1\), and TXNRD1 genes. The graphs show the percentage of input for each gene under different conditions (Non-Treated and Treated with Celastrol) and the significance level of the differences (p-values). The location and position of the AREs are indicated in the panels.
NQO1 genes. E) NRF1 ChIP does not enrich an ARE site in TXNRD1 gene. P-value by two-way analysis of variance. Data are mean ± standard error of the mean.
CHAPTER 5: Discussion
5.1 Overview of the thesis research

Stress is a state of tension that has become an attractive topic for biomedical researchers. In cells, stress defenses enhance resistance to and promote survival during adverse conditions, such as oxidative stress, heat, hypoxia, genotoxicity, and nutrient deficiency (96). Two types of stress relevant to this thesis are reactive oxygen species (ROS) and toxic build-up of damaged protein (proteotoxicity). Under normal conditions, oxidants generate low levels of ROS as a by-product of cellular metabolism and biochemical processes within the membrane (97), and this has been shown to play a role in cellular processes by acting as secondary messenger in signal cascades that control cell growth, autophagy, inflammation, immunity, and redox regulation (98, 99). A large portion of ROS is generated by mitochondrial oxidative phosphorylation and protein folding and processing within the ER. When these ROS levels are chronically elevated it can impair these organelles, leading to challenges in energy metabolism and production of secreted and membrane bound proteins (100). To overcome this challenge, cells utilize stress defense mechanisms that guard against uncontrolled ROS production as well as proteotoxicity.

One stress defense factor that guards against excess ROS and proteotoxicity is NRF1. This transcription factor is initially localized and anchored to the ER membrane and undergoes cleavage and release from the ER in a manner determined by the same molecular machinery that identifies ROS-damaged proteins and delivers it to the proteasome for degradation via the ER-associated degradation (ERAD) pathway. When protein degradation by the proteasome is insufficient due to an overwhelming rise of damaged protein or proteasome inhibition, cleaved transcriptionally active NRF1 levels increase and undergo nuclear translocation. In the nucleus, NRF1 binds to AREs in the promoter of genes involved in anti-oxidant stress defense as well as ERAD and proteasome activity to promote an adaptive response (7). Collectively, this indicates the activity of NRF1 is coupled with ERAD and protein turnover capacity. As dysregulation of this pathway has been implicated in neurodegenerative disorders, cardiovascular diseases, and cancer (30, 53, 85, 101), further study is needed to understand the underlying molecular mechanism of action and the nature of potential links between NRF1 and disease.

There are hundreds to thousands of genes predicted to have a functional ARE (7). And so, a key unanswered mechanistic question is as follows. How does NRF1 select which ARE-promoter
driven gene or gene cluster to target for transcriptional regulation? It is known that NRF1 forms heterodimers with proteins such as small MAFs to bind AREs and regulate transcription and that there are many small MAFs as well as other transcription factors to which NRF1 can interact. In this thesis, I hypothesized that NRF1 regulates transcription of genes involved in proteasome activity, antioxidative stress defense, and metabolism through specific heterodimer interaction with distinct small MAFs. To investigate, I first characterized a newly identified activator of NRF1 named celastrol (described in chapter 3). Then, I tested if MAFF, MAFG, or MAFK contributed to NRF1-dependent, celastrol-induced gene transcription in distinct ways (described in chapter 4). Overall, my findings support the hypothesis. Below, I will elaborate on the evidence leading to this conclusion as well as limitations of my findings and future research directions. A schematic that highlights celastrol-induced genes and the heterodimer complex that appears to be responsible, based on evidence in this thesis, is shown in Figure 5.1.

As described in section 3.1, our group discovered the nutraceutical celastrol exhibits potent ARE-luciferase activating effect in Hepa 1-6 cells as well as in iMEFs. Moreover, this effect was partially reduced in NRF1 deficient iMEFs, suggesting NRF1 plays a role in mediating the effect. In support of this, I found that Hep3B cells treated with 2 and 4 μM celastrol had increased levels of the cleaved transcriptionally active NRF1 isoform and increased NRF1 in the nucleus (Figure 3.2 and 3.3). Given that these same doses of celastrol increased the levels of poly-ubiquitinated protein in Hep3B cells (Figure 3.4) and inhibited in vitro proteasome activity (Figure 3.5) and proteasome activity in cultured Hep3B cells (Figure 3.6), the most likely reason celastrol activates NRF1 is by inhibiting its turnover. Particularly since it is known that proteasome inhibiting drugs such as bortezomib activate NRF1 via the same mechanism (26, 27, 30), consistent with my own findings in figure 3.2. Interestingly, I found 0.2 μM celastrol partially inhibited chymotrypsin-like activity but did not inhibit trypsin-like or caspase-like activity, whereas 2 μM celastrol caused almost complete suppression of each (Figure 3.6). This may suggest NRF1 activation is coupled more tightly to trypsin-like activity or caspase-like activity than to chymotrypsin-like activity or that all three activities must be reduced to engage NRF1 activation. It is unclear how one might pursue this line investigation. Regardless, the results of chapter 3 demonstrate that celastrol is a potent NRF1 activating agent that likely acts through inhibiting the proteasome.
Upon identifying celastrol as a suitable tool compound, I proceeded to investigate the role of NRF1 and its heterodimerization with MAFF, MAFG, and MAFK in mediating transcriptional responses to celastrol. For this, I first treated control and NRF1 deficient Hep3B cells with celastrol and examined possible NRF1 target genes to identify which are transcriptionally up-regulated by celastrol in an NRF1-dependent manner (Figure 4.1). Then, I performed a similar examination using celastrol treated control, MAFF deficient, MAFG deficient, and MAFK deficient Hep3B cells to identify which NRF1-dependent genes is also dependent on one of these heterodimeric partners (Figure 4.2). From this I found the following.

1) NRF1 was required for celastrol to induce proteasome subunit genes PSMA1, PSMA2, PSMA3, and PSMB1. Interestingly, MAFG was also required for maximum induction of these genes, whereas MAFK and MAFF were only partial contributors.

2) NRF1 was required for celastrol to induce glutathione metabolism and antioxidant related genes GCLC, GCLM, HO-1, and GSR1. In this case, MAFF was required for maximum induction of these genes, MAFK was also a major contributor, and MAFG had only a minor role.

3) NRF1 was required for celastrol to induce metal transporter and cell metabolism related genes FTH1, MT1, LPIN1, PGC1B, and ME1. In this case, only MAFG was required for ME1 induction, all three are required for induction of MT1, LPIN1, and PGC1B and MAFF was dispensable for induction of FTH1.

4) NRF1 was not required for celastrol-induced transcription of NQO1, TXNRD1, SLC7A11, which may instead be NRF2 targets, and celastrol had no effect on the other potential targets - GSTA4, GPX1, SOD1, and SOD2. Taken together, these findings suggest the heterodimeric interaction of NRF1 and MAFG plays a prominent role in regulating proteasome subunits involved in the degrading damaged, misfolded, oxidized, and short-lived proteins as well as in metabolism and metal transport but has a minor role in glutathione-linked anti-oxidant defense. In contrast, the heterodimeric interaction of NRF1 and MAFF appears to be majorly involved in glutathione-linked anti-oxidant defenses, but not in proteasome regulation. The NRF1 and MAFK heterodimeric interactions may be partially linked to each function. A summary of the results is shown in figure 5.1 below.

Next, I employed ChIP-qPCR assays to investigate celastrol-induced enriched occupancy of NRF1 on AREs of its target genes. This revealed NRF1 may directly regulate the expression of the GCLC, GCLM, and HO-1 genes, since it was enriched in AREs and induced expression of these genes in response to celastrol. NRF1 was not found to bind to the ARE of TXNRD1, which served as a negative control gene based on our qPCR result in Figure 4.1.
Figure 5.1: Specificity and target gene selection of NRF1 and its heterodimer partners. As a result of proteasome insufficiency and oxidative and electrophilic stresses, NRF1-sMAFs activates several genes involved in the synthesis of proteasomes and antioxidant and xenobiotic-metabolizing enzyme genes. Although the findings of this study are encouraging, further investigation is required to determine whether the NRF1-sMAFs heterodimer can activate antioxidant enzymes and enzymes involved in xenobiotic metabolism.

5.2 Implications for future research directions

There is still much to be learned about the molecular basis of differential recruitment of NRF1 and its heterodimeric partners and its role in differential transcriptional of NRF1, but the results of this thesis provide a strong launching point for pursuing this line of investigation. Our results indicate promoting or repressing heterodimeric interactions of NRF1 with specific small MAF proteins may elicit targeted stress adaptive programming. Additional interference by other factors or competition with other CNC factors such as NRF2 and NRF3 is another possibility. In the end, it will be necessary to conduct further studies to elucidate the mechanisms and highly advanced omics technologies such as single-cell ChIP-seq and ChIP-qPCR as well as proteomic techniques and dimensional analyses could provide a comprehensive understanding.
At this stage and given the results described above and the initial question of ‘how does NRF1 select which ARE-promoter driven gene or gene cluster to target for transcriptional regulation’, I propose the following two areas be considered as the next research directions.

1) The finding that NRF1 and MAFG were required for induction of proteasome subunit genes \textit{PSMA1}, \textit{PSMA2}, \textit{PSMA3}, and \textit{PSMB1} should be interrogated further, as this implicates this heterodimer complex in guarding against proteotoxicity. Using ChIP-qPCR with anti-NRF1 and anti-MAFG antibody the ARE site NRF1 and MAFG interact with should be uncovered and determined whether they overlap. Moreover, cell viability and poly-ubiquitinylated protein levels should be compared between wild type, NRF1 deficient, and MAFG deficient cells treated with a range of doses of proteasome inhibitor as well as celastrol.

2) The finding that NRF1 and MAFF were required for induction of \textit{GCLC}, \textit{GCLM}, and \textit{GSR1} should be interrogated further, as this implicates these heterodimeric complexes in guarding against ROS. The products of these enzymes are involved in GSH production and metabolism to counteract oxidative stress. Using ChIP-qPCR with anti-NRF1 and anti-MAFF antibody the ARE site NRF1 and MAFF interact with should be uncovered and determined whether they overlap. In this thesis research, I identified NRF1-targeting AREs in the promoter of \textit{GCLC} and \textit{GCLM} (Figure 4.3). So, next step would be to determine whether MAFF also interacts with that same ARE. In addition to this, cell viability and ROS levels should be compared between wild type, NRF1 deficient, and MAFF deficient cells treated with a range of doses of hydrogen peroxide and other ROS generating agents.

Additional areas that could be considered are in control of cell metabolism. Indeed, NRF1 in liver has been shown to contribute to hepatic lipid, glucose, and lipoprotein metabolism (21, 22, 88, 102, 103). However, there was not a clear pattern of which small MAF was required for celastrol induced expression of metabolism genes \textit{LPIN1}, \textit{PGC1B}, and \textit{ME1}, so priority should be considered to options 1) and 2) above for now. Indeed, this work probably only scratches the surface, as there could be a broad spectrum of NRF1-dependent stress defense genes involved in cytoprotection, immunometabolism, and homeostasis.
5.3: Limitations of the research

As a limitation of our study, it should be noted the high dosage of celastrol that is not suitable for cell health and may cause cell toxicity and death so it might be good to use the lowest dosage of celastrol in the future work. Moreover, we did an immunoprecipitation experiment to confirm the interaction between NRF1 and each individual small MAF. Because the quality of IP data is heavily dependent on the sensitivity and specificity of the antibody that is used to pull down the protein complex of interest, but due to the sticky feature of the NRF1 antibody that we used, we couldn’t confirm our IP result due to the non-specific bands as appeared by the presence of different bands in a complex biological sample with various molecular weights. Additionally, our study currently cannot indicate the underlying mechanisms by which NRF1 heterodimeric interaction with each small MAF selects specific target genes and exerts their diverse biological functions, so advanced omics technologies, such as single-cell ChIP-qPCR, integrative multi-omics analyses, and genomics and proteomics techniques coupled with multi-dimensional analyses, could provide a comprehensive understanding of the spatiotemporal dynamics of NRF1-small MAF-mediated gene regulation. Additionally due to the complex environment in living organisms, including the interactions between different cell types, tissues, and organ systems, the regulatory role of NRF1 can vary between different cell types, and in vitro models may not fully capture this diversity, so NRF1 function in a more complex biological context may not be fully captured. In the future, we can continue gaining mechanistic insight and then relate this information to in vivo contexts in which NRF1 may have a crucial translational impact.
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