NFE2L REGULATION BY ENDOPLASMIC RETICULUM STRESS

By

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ABSTRACT

Cell survival requires adaptive responses to transient increases of reactive oxygen species (ROS) during stress. The nuclear factor-erythroid 2-like factors (Nrf1 and Nrf2) and Maf cofactors transactivate genes with antioxidant response elements (ARE) to coordinate distinct metabolic pathways following ROS. Nrf2 predominantly responds to oxidative stress and electrophiles to regulate glutathione biosynthesis, while Nrf1 regulates proteasome induction. This thesis shows nuclear accumulation of the longer forms of Nrf1 (p120- and p95-Nrf1) in response to ER stress mediated by tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) in HEK293T cells. Nrf2, as opposed to Nrf1, was induced by the oxidative stressor antimycin A (AA) in the absence of BiP induction while both Nrfs accumulated in the nuclei from DTT-mediated redox stress. Cell stress was monitored using the H_2DCFDA, MTT and PI assays. These results are the first to indicate Nrf1 responds to ER stress distinctly from Nrf2 that responds to mitochondrial ROS.

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LIST OF ABBREVIATIONS

AA: Antimycin A

AD: Acidic domain

ARE: Antioxidant Response Element

ATP: Adenosine triphosphate

BiP: Binding Immunoglobulin Protein (GRP78 or 78-kDa glucose-regulated protein)

BTB: Bric-a-brac

bZIP: Basic leucine zipper

Ca^{2+}: calcium

***Cat*:** Catalase

cKO: Conditional knockout

CNC: Cap'n'collar

CncC: Cap'n'collar isoform C

CNS: Central nervous system

CTD: C-terminal domain

DCF: 2′,7′-Dichlorofluorescein

DCFH: Free Acidic Form of 2′,7′-Dichlorofluorescein

DTT: Dithiothreitol

Ep: Electrophile

ER: Endoplasmic Reticulum

ERAD: ER associated degradation

ETC: Electron transport chain

***Gcsh* and *Gcsl*:** Catalytic heavy and the regulatory light subunit of glutamate cysteine ligase

***Ggt*:** Gamma-glutamyl transpeptidase

***Gpx*:** Glutathione peroxidase

GRP: Glucose regulated protein

GSH: Reduced Glutathione

***Gss*:** Glutathione synthetase

***Gst*:** *G*lutathione S-transferase

HEK293T: Human Embryonic Kidney cells

H_2DCFDA: 5-(and-6)-Carboxy-2',7'-dichlorodihydrofluorescein diacetate

***Ho1*:** Heme oxygenase 1

I3PR:inositol 1,4, 5 triphosphate receptor

LCR-F1: Locus control region-factor 1

Maf: Musculoaponeurotic fibrosarcoma virus

MAM: Mitochondria-associated ER membranes

MRE: Metal response element

Mt: Metallothionein

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NehL: Nrf2-ECH homology like

NF-E2: nuclear factor-erythroid 2

NHB: N-terminal homology box

***Nqo1*:** NAD(P)H: quinone oxidoreductase 1

NST: Asn/Ser/Thr-rich

NTD: N-terminal domain

PERK: Protein kinase RNA-like endoplasmic reticulum kinase

PEST: Pro/Glu/Ser/Thr-rich

PI: Propridium iodide

PNGase: peptide:N-glycosidase

ROS/RNS: Reactive oxygen/nitrogen species

ROS: Reactive Oxygen Species

Skn-1: Skinhead 1

SR: Serine repeat

TCF11: Transcription factor 11

THP: Thapsigargin

TMc/TMi/TMp: C-terminal/intermediate/semihydrophobic transmembrane

TUN: Tunicamycin

UPR: Unfolded Protein Response

VDAC1: Voltage-dependent anion channel 1

$\Delta \psi$: mitochondrial membrane potential fluctuation

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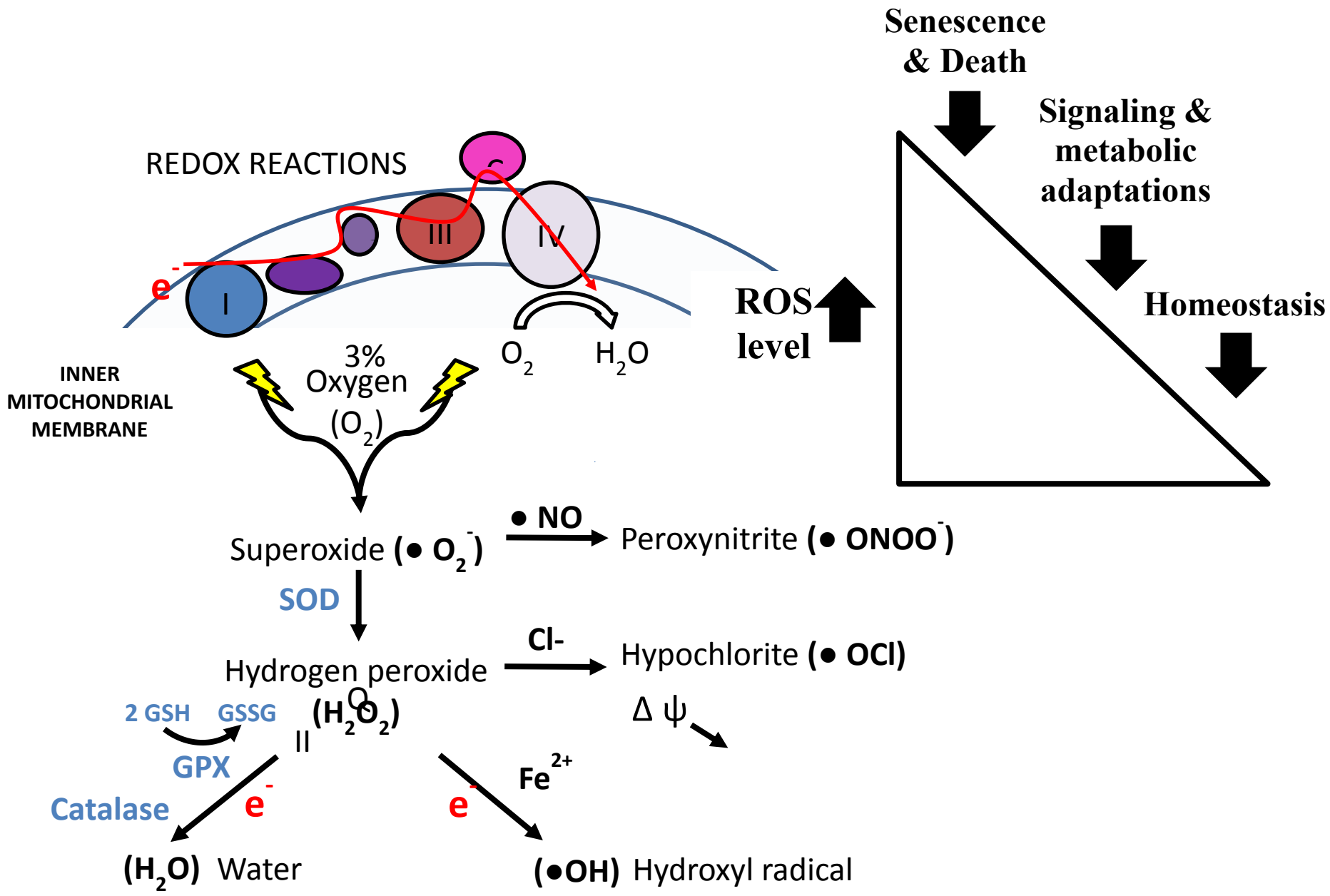
CHAPTER 1: INTRODUCTION

Well conserved core metabolic pathways, glycolysis and mitochondrial oxidative phosphorylation are used by all organisms in a manner that depends on extracellular conditions and cues, cellular needs, and stage of metabolic or circadian cycle (Sahar et al. 2009; Tu et al. 2005; Deberardinis et al. 2007). Homeostasis refers to the ability of a system, such as a cell or an organism, to maintain stability in the face of changing external conditions. The proper functioning and survival of cells depends on their ability to respond to stress that impinges on cellular homeostasis, by initiating either survival signaling pathways or cell death signaling. The choice of the stress response depends to a large extent on the nature and duration of the stress as well as the cell type, with large amounts of stress resulting in cell death. Exogenous factors and the ability of the cell to handle stress can determine the mechanism by which a cell dies (i.e., apoptosis, necrosis, pyroptosis, or autophagic cell death). The implications of an impaired cellular stress response are manifold to human physiology and metabolic diseases including diabetes, inflammation, cancer and aging (Wellen et al. 2010).

It is reactive oxygen species (ROS) that play signaling roles in stress adaptation and characterize various stress responses; diseases including diabetes and cancer are characterized by an elevation in (ROS) levels and an impairment of the cells to mediate stress response via a change in ROS levels. ROS, through radical-mediated mechanisms, damage DNA (base modification and single- and double-strand breakage), polyunsaturated fatty acids in lipids (lipid peroxidation), proteins (oxidation of amino acids) and can also deactivate enzymes by oxidation of their cofactors **[Figure 1]**. The activation of DNA repair and cell cycle arrest is an immediate response to DNA damage. The DNA damage signaling network functions to make calculated decisions, based on the status of DNA repair and the nature of DNA lesions to choose between life or death (Borges


Senescence & Death
Signaling & metabolic adaptations
Homeostasis
ROS level
REDOX REACTIONS
I
III
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INNER MITOCHONDRIAL MEMBRANE
3% Oxygen (O_2)
O_2
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Superoxide (• O_2^-)
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SOD
Hydrogen peroxide (H_2O_2)
Cl-
Hypochlorite (• OCl)
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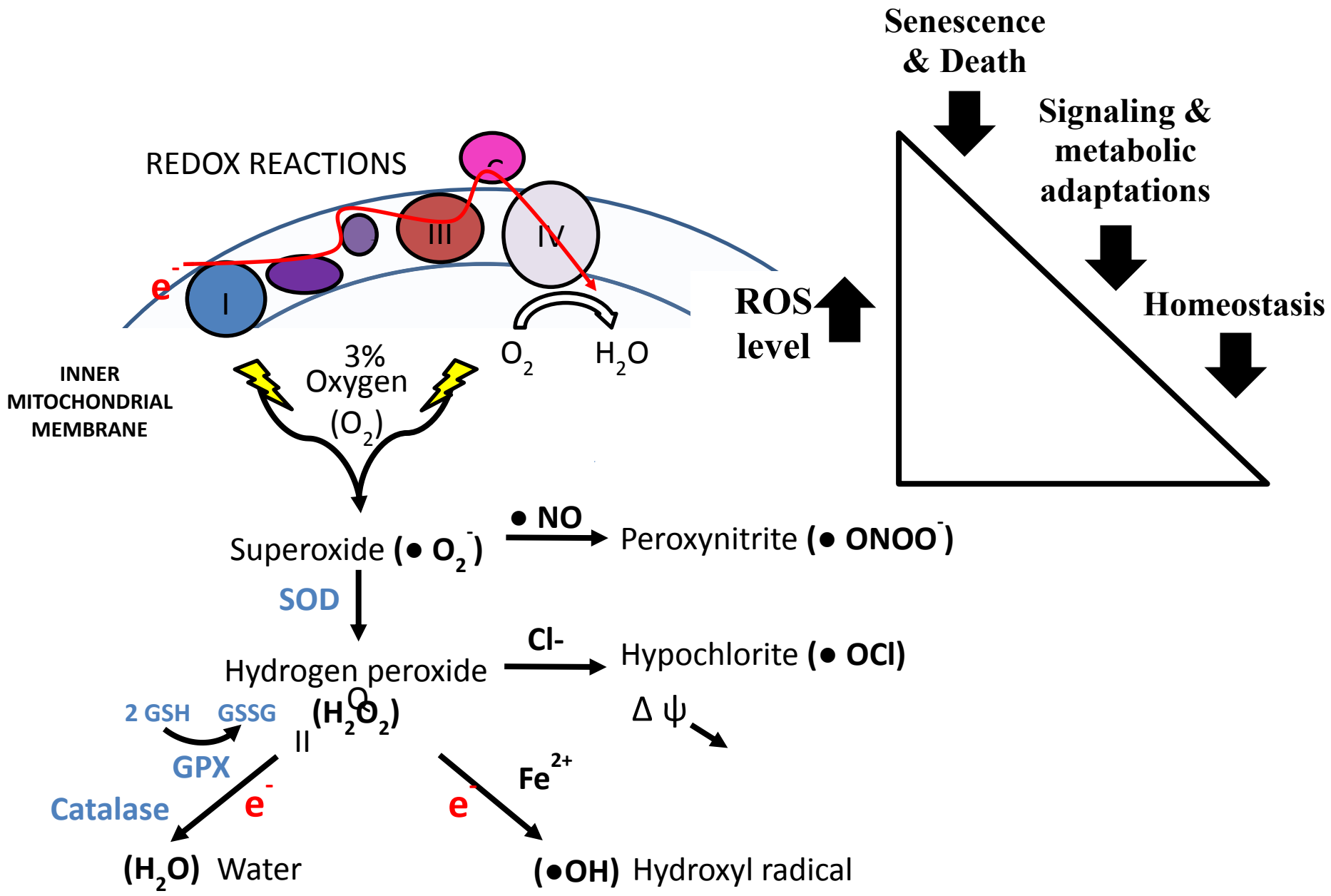
2 GSH
GSSG
GPX
Catalase
e-
Fe^{2+}
e-
(H_2O) Water
(•OH) Hydroxyl radical


Senescence & Death
Signaling & metabolic adaptations
Homeostasis
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I
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INNER MITOCHONDRIAL MEMBRANE
3% Oxygen (O_2)
O_2
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Peroxynitrite (• $ONOO^-$)
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Hydrogen peroxide (H_2O_2)
Cl-
Hypochlorite (• OCl)
Δ ψ
2 GSH
GSSG
GPX
Catalase
e-
Fe^{2+}
e-
(H_2O) Water
(•OH) Hydroxyl radical

Figure 1. The level of ROS within the cell determines cell fate.

Low levels of ROS are essential for cellular homeostasis, and are associated with a basal rate of electron leak from the electron transport chain (ETC) during oxidative phosphorylation. Transient rises in ROS mediate cell signaling follow changes in mitochondrial membrane potential ($\Delta \psi$) , through the controlled modification of stress-sensitive proteins and factors. These will in-turn mediate transcriptional responses in metabolic adaption to stress including the synthesis of cytoprotective genes, DNA repair pathways, and changes to metabolic substrate usage for controlled ROS production. If the stress and ROS levels are high, as is the case of insufficient antioxidant system response, the cell prevents the propagation of DNA damage and oncogenesis through initiation of cell death responses or senescence.

et al. 2008). The activation of apoptosis or senescence occurs later in time, in order to prevent metabolic deregulation and cancer depending on the degree of damage (Borges et al. 2008).

Lipid peroxidation is most harmful to cell viability, where ROS is involved in the destruction of double bonds within polyunsaturated fatty acids and directly mediates the formation of more reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal (Ayala et al. 2014). The products of lipid peroxidation, particularly 4-HNE, catalyze membrane disintegration through radical-mediated chain reactions that attack new double bonds and propagate ROS (Ayala et al. 2014). 4-HNE has high toxicity and will rapidly reactions with thiols and amino groups (Schaur et al. 2003). 4-HNE at low concentrations of 10-100μM gradually reduce cell viability as measured with the MTT assay in HepG2 cells, reflecting apoptotic cell death below 40 μM and necrotic cell death at higher levels (Chaudhary et al. 2010).

Reactive aldehydes similarly to ROS, function as a second messengers in cell signaling involved in regulation of several transcription factors sensible to stress such as nuclear factor erythroid 2-related factor 2 (Nrf2), activating protein-1, NF-κB, and peroxisome-proliferator-activated receptors, in cell proliferation and/or differentiation, cell survival, autophagy, senescence, apoptosis, and necrosis (Ayala et al. 2014). Diseases of metabolic nature can generally be attributed to the impaired regulation of a variety of stress-sensitive proteins and transcription factors, for instance protein tyrosine phosphatases and kinases, redox transcription factors as well as DNA repair systems, which function in controlling ROS and cell homeostasis (Wellen et al. 2010; Cortassa et al. 2014; Zhang et al. 2014c; Han et al. 2012; Kwak et al. 2004; Mitsuishi et al. 2012).

Nrf 1 and 2 are the main transcription factors that regulate redox homeostasis belonging to the family of CNC (cap‘n’collar)/bZIP (basic-region leucine zipper) transcription factors. The

CNC family of proteins includes the *Caenorhabditis elegans* skinhead-1 (Skn-1) protein (Bowerman et al. 1992; 1993), the *Drosophila melanogaster* cap'n'collar-isoform C (CncC) protein (Mohler et al. 1991; 1995), and the vertebrate activators nuclear factor-erythroid 2 (NF-E2) p45 subunit (Chan et al. 1993b), transcription factor 11 (TCF11) (Johnsen et al. 1996; Kobayashi et al. 1999), locus control region-factor 1 (LCR-F1 or Nrf1β) (Farmer et al. 1997; Caterina et al. 1994), nuclear factor erythroid 2 like 1 (Nrf1 or NFE2L1) (Andrews et al. 1993; Chan et al. 1993a; 1993b; Zhang et al. 2006; 2007; 2014a), Nrf2 (NFE2L2) (Moi et al. 1994), and Nrf3 (NFE2L3) [**Figure 2]** (Kobayashi et al. 1999; Chénais et al. 2005; Chevillard et al. 2011). It also includes the repressors, BTB and CNC homolog 1 (Bach 1) (Blouin et al. 1998; Ohira et al. 1998) and Bach 2 (Muto et al. 1998; Sasaki et al. 2000).

In adaptation to redox stress, cells activate a multi-layered induction or battery of cytoprotective genes via the Nrf factors (Hirotsu et al. 2012b). These redox pathways are tightly regulated so that basal activity is low and exposure to toxic xenobiotics or oxidants simultaneously activates the expression of multiple genes. The characteristic CNC domain binds to the Antioxidant or Electrophile Response Element (ARE) using the consensus sequence 5'-TGACNNNGC-3' where N is any nucleotide) (Rushmore et al. 1991). AREs are *cis*-acting enhancer sequences found in promoters of many enzymes involved in antioxidant responses, xenobiotic metabolism and inflammatory responses (Hayes et al. 2001). Nrf 1 and 2 represent the primary factors mediating ARE gene expression through heterodimerization with other bZIP factors, such as small Maf (musculoaponeurotic fibrosarcoma virus) proteins (Igarashi et al. 1994; Biswas et al. 2010; Jaiswal 2004; Sykiotis et al. 2010; Johnsen et al. 1998; Nguyen et al. 2000, 2003; Kannan et al. 2012). Skn-1 is unique due to the Skn domain that promotes high-affinity DNA binding by a basic region monomer (Carroll et al. 1997).

Figure 2. The cap'n'collar family of transcription factors and their common structural domains.

The human TCF11, Nrf1, Nrf2, Nrf3, LCR-F1 and NF-E2 p45 proteins are shown in comparison to the *Drosophila melanogaster* CncC and *Caenorhabditis elegans* Skn-1 proteins, as well as the Nrf2 inhibitors, Bach1 and Bach2. All family members have a cap'n'collar (CNC) and basic-leucine zipper (bZIP) domain present in the C-terminal domain (CTD) of the protein. The N-terminal domain (NTD) of many family members contains the N-terminal homology box 1 and 2 (NHB1 and NHB2 respectively) regions. Activators have Acidic Domains 1 and 2 (AD1 and AD2 respectively). The AD2L domain is present in Nrf3 only. Asn/Ser/Thr-rich (NST) and Serine Repeat (SR) domains are present in some, but not all, activators. The NST regions contain the sites for glycosylation in the family members that contain them. One or two Pro/Glu/Ser/Thr-rich (PEST) sequences are present in many family members. Transmembrane domains include transmembrane 1 (TM1), the intermediate transmembrane (TMi), the amphipathic semihydrophobic transmembrane (TMp) and the C-terminal transmembrane (TMc) domains. This family of proteins are also characterized by up to 7 highly conserved Nrf2-ECH homology like domain (NehL) domains (labeled Neh1L to Neh7L or Neh1 to Neh7). The DIDLID/DLG element and ETGE motif regulate protein stability in the family members in which they are found. The Nrf2 repressors, Bach1 and Bach 2, contain a broad complex, tramtrack, bric-a-brac (BTB) domains which are atypical of other CNC family member proteins. Taken from Bugno et al. 2015.

ARE genes include the catalytic heavy and the regulatory light subunit of glutamate cysteine ligase (*Gcsh* and *Gcsl*) for the rate limiting step in the glutathione synthesis pathway, glutathione synthetase (*Gss*), gamma-glutamyl transpeptidase (*Ggt*), class Alpha and Mu glutathione S-transferase (*Gst*) isoenzymes, NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), heme oxygenase 1 (*Ho1)*, catalase (Cat), metallothionein-1 and -2 (*Mt1* and *Mt2* respectively), the heavy and light subunits of ferritin, peroxiredoxin MSP23, and glutathione peroxidases (G*px*) (Primiano et al. 1997; Ishii T et al. 2000; Chen et al. 2003; Leung et al. 2003; Yang et al. 2005; McMahon et al. 2001; Chanas et al. 2002; Kensler et al. 2007; Zhu et al. 2005, Ohtsuji et al. 2008).

Importantly, only Nrf1 knockout (KO) mice are embryonic lethal, due to impaired hematopoiesis and ROS elevation from impaired expression of antioxidant genes such as *Gsh, Gclc, Gclm, Mt1* and *Mt2* under the cytotoxic effects of tumor necrosis factor during development (Leung et al. 2003, Kwong et al. 1999, Chen et al. 2003). Nrf2 KO mice on the other hand have no apparent phenotypes, except for increased sensitivity to ROS stress in a methionine-diet dependent manner (Sugimoto et al. 2010). Nrf1 and Nrf2 have distinct yet overlapping roles in antioxidant response elements (ARE) gene regulation. In this thesis, the distinct activation mechanism by which Nrf1 mediates ROS signaling from Nrf2 will be investigated and discussed, particularly under chronic endoplasmic reticulum (ER) stress. The contribution of Nrf1 vs. Nrf2 in ROS homeostasis and signaling will be discussed in terms of ROS production, ROS mitigation and prevention as well as repair mechanisms within the cell. As such, the signaling roles of ROS will first be discussed, followed by the structure, function and distinct gene regulation of Nrf transcription factors in adaptation to stress.

1.1 Reactive oxygen species (ROS) production in metabolism and stress

Reactive oxygen species (ROS), are reduced molecules of oxygen including superoxide and its derivatives. ROS are continuously produced from free radicals derived from cellular reduction-oxidation or redox processes within the cell that react with other molecules of high reducing potential, particularly oxygen. The majority of ROS production occurs within the mitochondria in oxidative phosphorylation as well as in the endoplasmic reticulum (ER) during oxidative protein folding and processing. These redox processes define the function of these organelles. By compartmentalizing protein folding events and energy production, cells may protect themselves from being exposed to nonspecific oxidation events (Rand et al. 2009).

The mitochondrial electron transport chain (ETC) is a key source of ROS. During oxidative phosphorylation, the ETC functions to pump protons across the inner mitochondrial membrane for the generation of an electrochemical gradient used in adenosine triphosphate (ATP) synthesis. The energy for ATP synthesis is derived from the high reducing potential of oxygen. It is complexes I-IV which harvest the reducing potential of oxygen in a series of non-enzymatic steps. Since these steps are non-enzymatic, ROS production via the ETC occurs following nutrient imbalance that can impinge on non-enzymatic flow of electrons, for instance by a reduction in the final electron acceptor, oxygen, or depending on the CoQH2/CoQ, NADH/NAD+ ratios which are used as electron sources (Murphy et al. 2009). The reduction of particularly complex I and III allows for electron leakage and association with molecular oxygen, generating superoxide, the molecular source of all derived ROS within the cell. Superoxide rapidly dismutates into hydrogen peroxide or alternatively, in the absence of superoxide dismutase activity, superoxide may combine with nitric oxide to produce peroxynitrite (Zhu et al. 2005, Martínez et al. 2009). In the cell, NADPH oxidase, cyclooxygenases and 5-lipoxygenase also contribute to ROS production.

The endoplasmic reticulum produces 25% of the ROS in the cell (Tu and Weissman, 2004). The ER is a huge organelle primarily functioning in protein folding and processing by several ER chaperone proteins as well as lipid and membrane biosynthesis. When electrons are transferred from a protein's thiol groups in cysteine residues to protein disulfide isomerase (PDI), disulfide bonds are formed. ER oxidoreducin (ERO1) oxidizes PDI and reduces molecular oxygen, resulting in hydrogen peroxide production in a flavin-dependent reaction.

Following physiological and pathological stimuli, including increased translation during cell growth, there is an increase in the number of unfolded proteins, allowing hydrophobic patches to surface that are normally buried internally in the native folded state. These hydrophobic surfaces trigger aggregation and can sequester normal proteins compromising their functionality. The ER triggers the unfolded response (UPR) in the response to an accumulation of unfolded proteins. The UPR pathway activation is sensitive to the ER oxidative redox environment for protein folding by PDI/ERO1 or via alteration of Ca^{2+} and ATP levels needed for chaperone function. Specific and limited PDI oxidation by ERO1α is essential to avoid ER hyperoxidation during ER stress (Görlach et al. 2015).

Three main transducers, PERK, IRE1 and ATF6 mediate the ER stress response to restore proteostasis. PERK, ATF6 and IRE1 are activated sequentially mediated by the release of the GRP78/BiP molecular chaperone from their ER luminal domains in preference for binding of unfolded proteins. BiP promotes protein folding using an ATPase domain. The UPR results in the suppression of protein translation via the PERK pathway, the facilitation of the refolding of unfolded proteins by the induction of ER glucose-regulated proteins or BiP, and the activation of the ER-associated degradation (ERAD) and autophagy for the clearance of terminally unfolded proteins that have accumulated in the ER via the ubiquitin-proteasome pathway (Sano et al. 2013).

BiP also seals the pore of the translocon (TLC) involved in transfer of damaged proteins during ERAD such that during ER stress, the TLC regulates ER Ca^{2+} leakage (Hammadi et al. 2013). The degree of Ca^{2+} leak *via* TLC is connected to GRP78 expression and apoptosis (Hammadi et al. 2013). Thus the UPR restores ER and thus cell proteostasis by controlling which transcripts are translated within the ER, the induction of chaperones in protein folding and finally through the upregulation of protein and organelle clearance pathways. Despite extensive characterization of the regulatory signaling for the UPR, the determination of cell fate and morphological changes due to damage caused by ER stress are not well understood. Nonetheless, adaptations of subcellular compartments to redox changes in the cell will critically determine survival and cell death fates (Zhang et al. 2012; Bravo et al. 2011; Li et al. 2008, Little et al., 1994, Sano et al. 2013; Cullinan and Diehl, 2004).

Organelle interactions play a key role in ROS crosstalk. During cell stress, the mitochondria can take up the leaked Ca^{2+} previously stored in the ER. Since the ER forms continuous membranes with the outer membrane of the nuclear envelope and has transient physical and functional interactions with the mitochondria, Ca^{2+} can be taken up directly through mitochondrial associated membranes (MAM) s or indirectly from the cytosol Görlach et al. 2015. MAMs consist of voltage-dependent anion channel (VDAC1), GRP75, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol 1,4,5-triphosphate receptor (I3PR), and other proteins transiently present in a macromolecular complex at the ER–mitochondria interface (Szabadkai et al. 2006; Verfaillie et al. 2012). Constitutive Ca^{2+} transfer from the ER to mitochondria is needed for the maintenance of baseline bioenergetics as it is Ca^{2+} that controls the rate of energy production in oxidative phosphorylation (Green and Wang, 2010; Szabadkai et al. 2006; Gorlach et al. 2015). ROS and calcium signaling are bidirectional, wherein ROS can regulate

cellular calcium signaling, while calcium signaling is essential for ROS production (Gordeeva et al., 2003). Consequently, mitochondrial Ca^{2+} uptake stimulates ROS production during ER stress which may then cross-talk with ER ROS leading to ROS amplification (Peng and Jou, 2010).

It is becoming increasingly evident that MAMs control autophagy in a ROS-dependent manner, emphasizing the role of ROS crosstalk in organelle clearance during stress (Artero-Castro et al. 2015; Azad et al. 2009; Ma et al. 2011; Hamasaki et al. 2013; Hailey et al. 2010; Betz et al. 2013; Bouman et al. 2011). ROS can induce autophagy through several distinct mechanisms involving Atg4, catalase, and the mitochondrial ETC, particularly complex III where antimycin a (AA), a complex III inhibitor was shown to inhibit autophagy (Ma et al. 2011; Azad et al. 2009). During autophagy, the cell catabolizes oxidized organelles and proteins, allowing for the recycling nutrients but also diminishes the formation of peroxidized lipids that propagate radicals, as discussed earlier. MAMs contain redox-sensitive and nutrient-sensitive interactions such as oxidative-stress regulated ERO1 association with MAM (Gilady et al. 2010) and Akt-dependent (following growth factor signaling) hexokinase 2 binding to MAM or VDAC1 (Gottlob et al. 2001). Hexokinase can increase the coupling of glucose metabolism to oxidative phosphorylation and inhibit apoptosis (Gottlob et al. 2001). The regulation of MAMs and autophagy is therefore critical in understanding adaptation to stress and survival under different nutrient conditions.

1.2 Countering cell ROS with antioxidant systems

In the cell, enzymatic scavengers (e.g., superoxide dismutases, catalase, glutathione peroxidase, glutathione reductases, and peroxiredoxins) and low-molecular-weight reductants (e.g., vitamin E, GSH, and ascorbate) function to counteract oxidative stress within the cell. GSH is the most abundant antioxidant in the cell and can be regenerated by the hexosamine biosynthetic

pathway, other antioxidants and antioxidant enzymes. Intracellular concentrations of glutathione can be in the millimolar range. The high levels of glutathione allow for oxidized glutathione to readily oxidize other GSH molecules instead of other biomolecules. Therefore glutathione prevents the oxidation of other cellular components as the main antioxidant in the cell. The ratio of GSH to glutathione disulfide GSSG, is a measure of over-all oxidative stress. In numerous cell types, GSH depletion is an early hallmark in the progression of cell death in response to a variety of apoptotic stimuli (Franco et al. 2009).

GSH is especially abundant where redox buffering capacity is most critical, within in the mitochondria and in the endoplasmic reticulum (ER) (Jones et al. 2010). In the ER, GSH is a buffer against hyperoxidizing conditions and also plays a direct role in the isomerization of disulfide bonds by maintaining the mammalian oxidoreductases in a reduced state (Cuozzo and Kaiser, 1999; Jessop and Bulleid, 2004). Mitochondria maintain their redox state via the combined actions of glutathione/thioredoxin scavenging antioxidant systems (Cortassa et al. 2014). While most studies focus on oxidative stress within organelles, elevated GSH has recently been shown to have pro-oxidative consequences within the mitochondria due to deregulation of mitochondrial redox coupling systems; promoting a more reducing GSH redox potential with N-acetyl-l-cysteine or overexpression of glutamate cysteine ligase GCLC/GCLS, the rate limiting enzyme in GSH production, initially triggered reductive stress followed by pathogenic mitochondrial oxidation at decreased levels of ROS *in vitro* (Zhang et al. 2012). Thus an appropriate balance of GSH: GSSG is imperative for proper organelle function.

1.3 Nuclear factor-erythroid 2 p45 subunit-related factor (Nrf) 1 and 2 structure,function and regulation

Nrf1 plays a distinct yet overlapping role in ARE gene regulation with Nrf2. Nrf2 is the dominant transcription factor involved in the basal and stress-inducible expression of a number of antioxidants and phase 2 enzymes in vivo and in vitro (Itoh et al. 1997, McMahon et al. 2001, Zhu et al. 2005). These genes include the GSH biosynthesis pathway genes, especially GCLC, the rate limiting enzyme in GSH synthesis utilizing glutamate and cysteine for production. Nrf1 plays a minor role in the transactivation of GSH biosynthesis genes, however its function is essential to ROS homeostasis since Nrf1 KO are embryonic lethal (Chen et al. 2003).

The distinct role of Nrf1 was elucidated using Nrf1 liver conditional knockouts (cKO) mice (Xu et al. 2005, Ohtsuji et al. 2008, Hirotsu et al. 2012a, 2014) with a phenotype portrayingprogressive development of inflammation, non-fatty liver steatosis, and ultimately hepatocarcinoma in 100% of the animals studied (Xu et al. 2005). This phenotype was rescued by proteasome gene as well as glutathione overexpression emphasizing that Nrf1 dysfunction is attributed to oxidative stress and genetic instability stemming from the inability to promote protein clearance following increased growth factor signaling during liver development (Lee et al. 2013; Oh et al. 2012). Distinct Nrf1 regulation of the proteasome seems to be both at transcriptional, downstream of mTORC1/SREBP1 signaling (Zhang et al. 2014c) and post-translational levels (Tsuchiya Y et al. 2013).

Nrf1's coordinate regulation of proteasome genes has also been associated with neurodegeneration (Radhakrishnan et al. 2010, 2014; Sha et al. 2014; Steffen et al. 2010; Tsuchiya et al. 2013; Lee et al. 2011, 2013; Kobayashi et al. 2011; Oh et al. 2011). Nrf1 expression correlates with the upregulation almost all base and lid proteasome subunits in a cell-type dependent manner,

along with proteasome-related genes Pa200, Usp14, Pomp, the p97-complex, components of the ER stress response and antioxidant genes such as Gsta4 (Sha Z et al. 2014; Steffen J et al. 2010; Tsuchiya Y et al. 2013).

Quite a bit of evidence is pointing to the distinct role of Nrf1 in the regulation of autophagy: 1) mTORC1 pathway not only transcriptionally regulates Nrf1 (Zhang et al. 2014c) but is a transcriptional regulator of autophagy (Martina et al. 2012); 2) Hirotsu et al. (2012a) demonstrated that Nrf1 distinctly regulates Lipin1 and PGC1 β; Lipin-1-related myopathy in the mouse is associated with a blockade in autophagic flux and accumulation of aberrant mitochondria (Zhang et al. 2014i); 3) the expression of Herpud1, involved in the UPR under ER stress (Ho DV et al. 2015); 4) in addition to the induction of many proteasomal genes, Nrf1 induces the proteasome activator PA200, the proteasome-associated deubiquitinating enzyme Usp14, POMP for proteasome maturation, serine 497 of Nrf1 is phosphorylated by CK2 to mitigate proteasome dysfunction and the formation of p62-positive juxtanuclear inclusion bodies (Tsuchiya et al. 2013); and finally 5) Nrf1 induction of the induction of p97 complex and ERAD machinery in an Hrd1-dependent manner (Tsuchiya et al. 2013), where p97 is essential to autophagy (Ju et al. 2010).

Nrf1 has been shown to regulate metabolic gene transcription in an opposing manner to Nrf2. Nrf2 induces glycolysis consistently with its function as an oncogene, diverting glucose carbons into the the pentose phosphate pathway and resulting in an increased production of NADPH (Heiss et al. 2013). Nrf1 suppresses genes in glycolysis (via *Slc2a2* and *Gck*) and gluconeogenesis (via *Fbp1* and *Pck1)* as determined in one or four copy Nrf1 transgenic mice (Hirotsu et al. 2014). As a result, Nrf1 increased utilization of acetyl CoA and increased energy charge or the ATP/ADP ratio, but not from glucose and amino acid catabolism. Nrf2 increases the

energy charge and acetyl-coA levels *in vitro* through redirecting glucose and glutamine into anabolic pathways, particularly following sustained PI3K-Akt signaling (Mitsuishi et al. 2012; Holmström et al. 2013).

Nrf1 regulation of metabolic targets is associated with diabetes, not only via glycolysis but also via the control of ROS homeostasis within pancreatic β-cells. In agreement with Hirotsu et al. (2014), Nrf1 knockdown in mouse pancreatic β-cells lead to the induction of multiple glucose-metabolizing enzymes, including *Glut2*, *Hk1*, *Gapdh*, and *Ldh1*, leading to elevated glycolysis and lactate production (Zheng et al. 2015). β-cell dysfunction occurred as a consequence, marked by increased ROS, energy charge, and glucose-stimulated insulin secretion, resulting in excessive insulin secretion that is associated with diabetes (Zheng et al. 2015).

ARE promoter crosstalk was first demonstrated *in vivo* when Nrf2 derepressed Nrf1 on the ARE promoter of the *xCT* gene, a cysteine/glutamate antiporter responsible for amino acid uptake (Tsujita et al. 2014). This emphasizes the opposing roles of Nrf1 and Nrf2 in ARE gene regulation are most likely influenced by differential promoter affinities of Nrf transcription factor complexes on ARE promoters (Ohtsuji et al. 2008). This is in addition to the temporal aspect of gene regulation by the CNC family members (Bugno et al. 2015).

In vitro studies support Nrf1 involvement in the response to small amounts of stress and Nrf2 response to larger, more acute stresses. Low-dose X-ray irradiation of normal human skin fibroblast HS27 cells showed that 0.05 Gy-irradiation increased only Nrf1 activation, but 0.5 Gy induced both Nrf1 and Nrf2 activation of *Ho-1*, manganese superoxide dismutase and NQO1 antioxidant genes (Lee et al. 2013). In another study Song et al. 2015 by utilizing the *Mt1* promoter in a luciferase reporter following Nrf1 or Nrf2 knockdown HepG2 cells examined the dependence

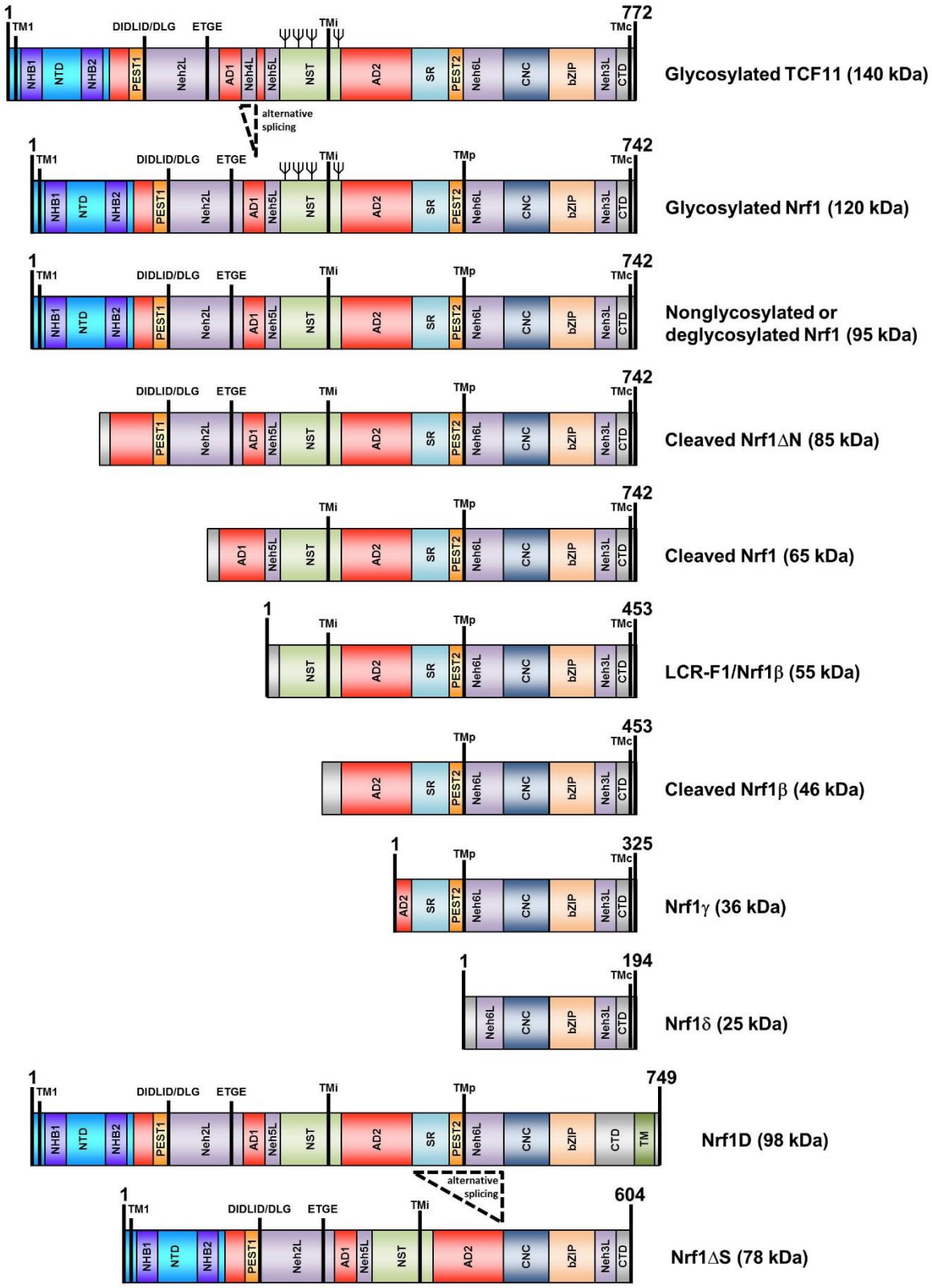
1
772
TM1
DIDLID/DLG
ETGE
TMi
TMc
NHB1
NTD
NHB2
PEST1
Neh2L
AD1
Neh4L
Neh5L
NST
AD2
SR
PEST2
Neh6L
CNC
bZIP
Neh3L
CTD
Glycosylated TCF11 (140 kDa)
alternative splicing
742
TMp
Glycosylated Nrf1 (120 kDa)
Nonglycosylated or deglycosylated Nrf1 (95 kDa)
Cleaved Nrf1ΔN (85 kDa)
Cleaved Nrf1 (65 kDa)
453
LCR-F1/Nrf1β (55 kDa)
Cleaved Nrf1β (46 kDa)
325
Nrf1γ (36 kDa)
194
Nrf1δ (25 kDa)
749
TM
Nrf1D (98 kDa)
604
Nrf1ΔS (78 kDa)

Figure 3. The multiple forms and cleaved products of Nrf1.

Full length and fully glycosylated Nrf1 (p120) is created by alternate splicing of TCF11 (p140) which removes the Neh4L domain. Nonglycosylated Nrf1 (p95) is then proteolytically processed (possibly by a partially inhibited proteasome) to form all other cleaved products (p85, p65, p55, p46, p36 and p25). p55, p36 and p25 are also known as LCR-F1/Nrf1β, Nrfγ and Nrfδ respectively. Nrfγ and Nrfδ act as dominant-negative inhibitors of the CNC proteins as they compete with all other family members. Nrf1D and Nrf1ΔS are found to be alternatively expressed forms of Nrf1. Nrf1ΔS is created by alternative splicing of Nrf1D which removes the SR, PEST2 and Neh6L domains. All domains and motifs are labeled as in Figure 1. From Bugno et al. 2015.

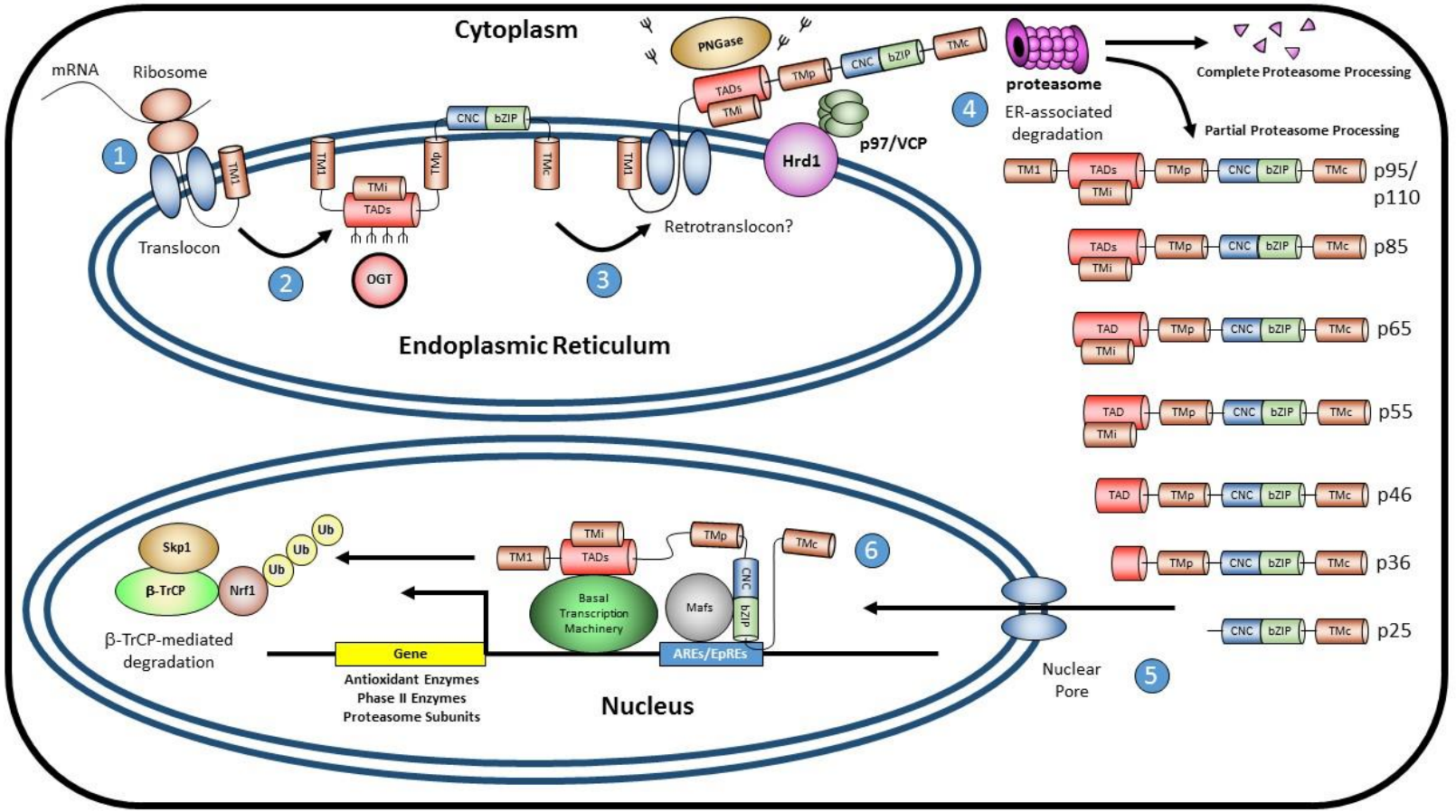
Cytoplasm
mRNA
Ribosome
1
TM1
Translocon
2
TMi
TADs
OGT
CNC
bZIP
TMp
TMc
3
Retrotranslocon?
PNGase
4
Hrd1
p97/VCP
Endoplasmic Reticulum
proteasome
ER-associated degradation
Complete Proteasome Processing
Partial Proteasome Processing
p95/ p110
p85
TAD
p65
p55
p46
p36
p25
Ub
Skp1
β-TrCP
Nrf1
β-TrCP-mediated degradation
Gene
Antioxidant Enzymes
Phase II Enzymes
Proteasome Subunits
Basal Transcription Machinery
Mafs
bZip
AREs/EpREs
6
Nucleus
Nuclear Pore
5

Figure 4. **Proposed mechanisms by which Nrf1 activates ARE responsive gene expression.**

Translation by the ribosome at the rough ER allows Nrf1 to be inserted directly into the ER once it moves through the translocon. **2.** The NST domain and TADs are transiently translocated into the ER lumen where they are glycosylated to form p120-Nrf1. **3.** p120-Nrf1 is repartitioned across the ER membrane where it is deglycosylated in the cytoplasm by PNGase, generating p95-Nrf1. In ER-associated degradation (ERAD) **4**, Nrf1 associates with the p97 complex and is partially processed by a partially inhibited proteasome to generate the de glycosylated and truncated versions of the full-length protein (p95/p110, p85, p65, p55, p46, p36 and p25), which are assisted though the nuclear pore **5.** Of these, p46, p36 and p25 are inhibitors of ARE function. **6.** Active and inactive forms of Nrf1 interact with small Maf proteins though their CNC-bZIP domains and associate with the AREs. The TADs of active forms of Nrf1 associate with the basal transcription machinery and activation of antioxidant and phase II detoxification enzyme gene expression can occur. From Bugno et al. 2015.

of *Mt1* gene expression on Nrf1 following ROS-inducing copper treatment. It was found that Nrf2 was predominantly responsible for *Mt1* gene induction acutely (within 4 hours of copper treatment in HepG2 cells) following Nrf1 knockdown. Inconsistently with luciferase assay, Nrf1 was responsible following Nrf2 knockdown, in the induction of *Mt1* gene expression in qPCR experiments even following 24 hrs of copper treatment, which was not observed for Nrf1 knockdown cells. This suggested that Nrf2 is acutely regulating *Mt1* gene and Nrf1 is doing so chronically. The authors also offered the explanation that, in response to copper toxicity, Nrf2 can bind with metal response element (MRE) binding site(s) in the upstream promoter region of the *Mt1* gene to allow more potent (albeit acute) gene induction in Nrf1 vs Nrf2 knockdown cells (see Hayes and McMahon 2001 for MRE regulations of redox genes). Song et al. did not address the bZIP heterodimeric cofactor MafG, which can itself be induced by hydrogen peroxide (Crawford et al. 1996) and thus presumably introduce a temporal dimension into transcriptional activation through the ARE (Hayes and McMahon 2001). Even so, by offering a comparison with Nrf2, this study offers some insight into temporal regulation of genes by CNC proteins in cells subject to stress.

1.4 Nrf1 activation mechanism relative to Nrf2 dictates Nrf functional cross-talk

Unlike Nrf2, which is localized in the cytosol, Nrf1 is inserted into the endoplasmic reticulum (ER) co-translationally. In contrast to Nrf2 that has been extensively studied, Nrf1 activation mechanism determining transcriptional function is still not very well understood. The dynamic interaction between Nrf1 and the ER membrane is of crucial importance to understand Nrf1 form generation and function, and has been comprehensively described by Drs. John Hayes, Zhang and co-workers (Zhang et al. 2007; Zhang et al. 2007; 2013; 2014a; 2014b).

The N-terminal homology box 1 directs the anchorage of the newly synthesized Nrf1 to the ER membrane, through the transmembrane region 1 (TM1) and further transient translocation of adjacent transactivation domains (TADs) into the lumen results in glycosylation of Nrf1 to produce an inactive glycoprotein of 120 kDa (Zhang et al. 2006; Wang and Chang, 2006; Zhang et al. 2007). The TADs are retrotranslocated into the cytoplasm with the help of the ERAD component p97 under proteotoxic stress (Radhakrishnan et al. 2014). In the cytosol, the NST domain is deglycosylated by peptide:N-glycosidase (PNGase) (Zhang et al. 2013), and is associated with low-glucose conditions (Zhang et al. 2014c). Deglycosylation is rapidly followed by proteasomal cleavage, and is the rate-limiting step in the generation of the active p110-Nrf1 and p95-Nrf1 forms that interacts with the basal transcription machinery and allows for the transactivation activity of Nrf1 (Zhang et al. 2014a-c). Moreover, p110-Nrf1 and p95-Nrf1 are proteolytically cleaved to produce multiple cleaved forms that act as both activators (p85) partial repressors (p65, p55/LCR-F1/Nrf1β) and dominant negative repressors (p46, p36/Nrfγ, p25/Nrfδ) of the ARE that lack the TAD (Wang et al. 2007; Zhang et al. 2013). The p65-Nrf1 and smaller forms may also be generated by internal translation (Zhang et al. 2013) **[Figure 2]**. Apart from glycosylation, Nrf1 function and stability can be regulated by post-translational mechanisms such as phosphorylation (Tsuchiya et al. 2013; Chepelev et al. 2011; Biswas et al. 2013), and ubiquitination (Steffen et al. 2010; Biswas et al. 2011; Tsuchiya et al. 2011; Sha et al. 2014). Three distinct ubiquitin ligase systems exist (Hrd1, Fbw7 and β-TrCP) that can promote Nrf1 proteasomal degradation (Biswas et al. 2011; Tsuchiya et al. 2011; Steffen et al. 2010; Sha et al. 2014; Chowdhry et al. 2013) **[Figure 4]**. The nuclear form of Nrf1 is faster-migrating than the cytosolic form and this is hypothesized to be due to differential post-translational modifications (Steffen et al. 2010).

The final gene expression profile of Nrf1 target genes will thus converge on the type of Nrf form is induced transcriptionally, Nrf1 post-translational modifications and ROS or stress events levels that determine the ratios of Nrf isoforms to alter ARE gene activation in unique manners. For example, the truncation of the N-terminus of TCF11 unexpectedly showed decreased proteasomal gene expression (Steffen et al. 2010) in contrast to Wang and Chang findings (2006). It is therefore important to remember that while the N-terminus acts to tether Nrf1 to the ER, Nrf1 cleavage does not always confer gene activation **[Figure 5]**. This is evidenced with upregulation and downregulation of genes *in vivo* following Nrf1 liver cKO, the proposed repressive dominant negative role of Nrf1 short products and p65 on all Nrf isoforms (Wang et al. 2007; Zhang et al. 2014b) as well as the repressive and inducing roles of Nrf1 in metabolism that contrast Nrf2 (Hirotsu et al. 2014). Determining the context when and which Nrf1 forms are generated will help determining the regulation of Nrf1 cleavage by unknown proteases (Bugno et al. 2015).

Zhang Y et al. (2007) found no SP1 and SP2 sites in Nrf1 that would allow for Nrf1 ER intramembrane proteolysis, and thus hypothesized a cleavage-independent mechanism for Nrf1 nuclear localization given the lack of Nrf1 nuclear entry by ER stressing agents (Zhang et al. 2009). Nrf1 was suggested to be translocated from the ER directly to the inner nuclear membrane to regulate ARE targets via its full length p120 form (Zhang Y et al. 2007; 2013). However this model is only supported by cytosolic fractionation experiments and is in conflict with studies that show maximal proteasome inhibition through various agents, blocks nuclear import of Nrf1 and target gene activation (Sha Z et al. 2014).

Nrf2 is held in the cytosol by Keap1, where it is continuously ubiquitinated and broken down by the proteasome (Jaiswal et al. 2004; Taguchi et al. 2011; Kansanen et al. 2013; Uruno et

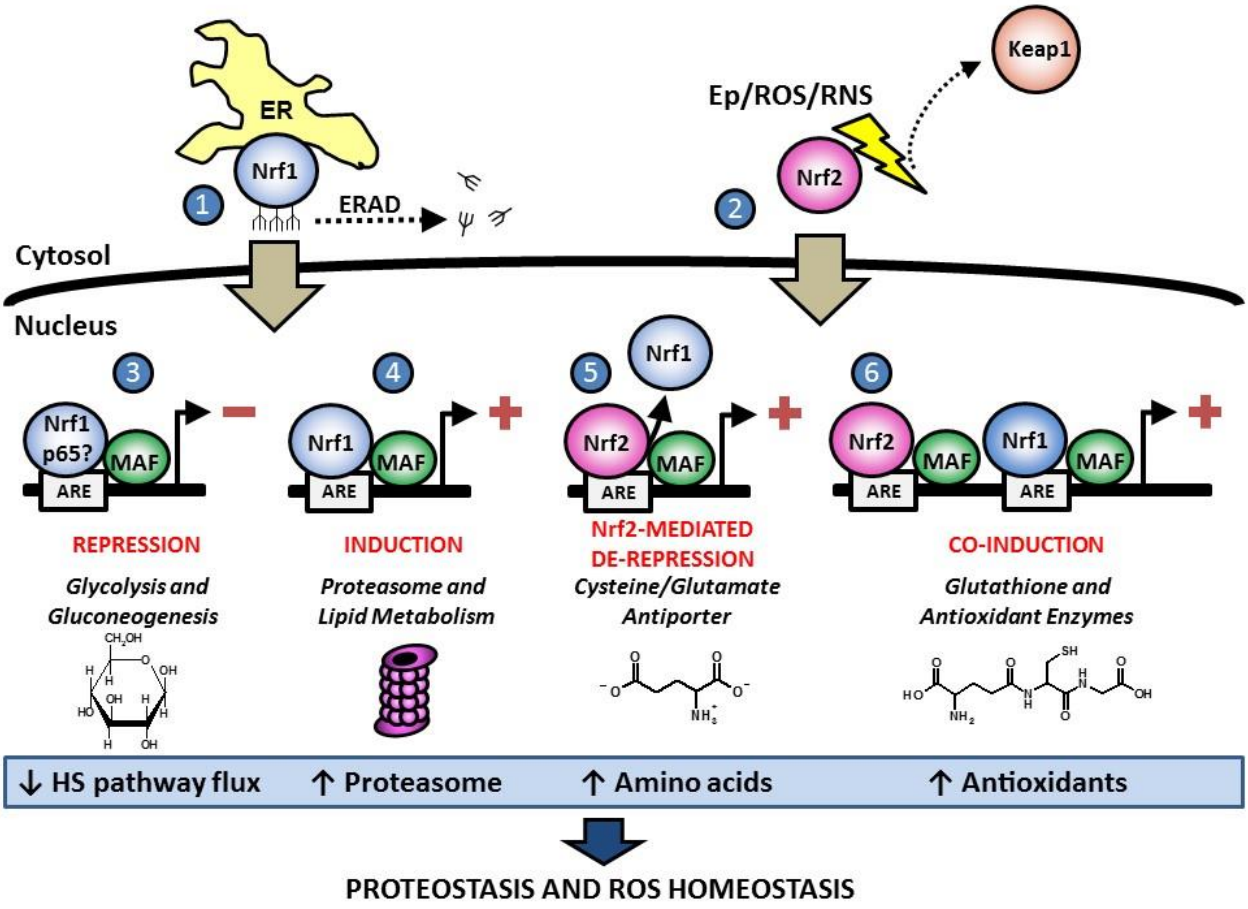
ER
Nrf1
1
ERAD
Keap1
Ep/ROS/RNS
Nrf2
2
Cytosol
Nucleus
3
Nrf1
p65?
MAF
ARE
4
Nrf1
MAF
ARE
5
Nrf1
Nrf2
MAF
ARE
6
Nrf2
MAF
Nrf1
MAF
ARE
REPRESSION
Glycolysis and
Gluconeogenesis
INDUCTION
Proteasome and
Lipid Metabolism
Nrf2-MEDIATED
DE-REPRESSION
Cysteine/Glutamate
Antiporter
CO-INDUCTION
Glutathione and
Antioxidant Enzymes
↓ HS pathway flux
↑ Proteasome
↑ Amino acids
↑ Antioxidants
PROTEOSTASIS AND ROS HOMEOSTASIS

Figure 5. Distinct roles of Nrf1 and Nrf2 in maintaining proteostasis and ROS homeostasis. **(1.)** Nrf1 nuclear localization is dependent upon deglycosylation by PNGase and ERAD within the ER. **(2.)** This is in contrast to Nrf2, whose nuclear localization is dependent on Ep/ROS/RNS modification of cysteine residues within Keap1 and Nrf2. **(3.)** In the nucleus, smaller forms of Nrf1 (p65) may repress all ARE function (including those of Nrf2). These effects are seen primarily on components of glycolysis and gluconeogenesis. **(4.)** Depending upon the cell type and the ARE-inducible gene, Nrf1 may repress or induce specific genes (specific genes for proteasome components), independently of Nrf2 **(5.)** Nrf2 induction by stresses takes precedence over Nrf1 for ARE binding in Nrf2-mediated de-repression. An example of a gene involved in Nrf2-mediated gene repression is the glutamate/cysteine antiporter. **(6.)** Co-induction of AREs with both Nrf1 and Nrf2 is also possible for many antioxidant and glutathione synthesizing enzymes. Nrf1 distinctly regulates proteostasis and ROS homeostasis, under transcriptional control of mTORC1 and post-translational modifications (PTMs). Bugno et al. 2015.

al. 2015; Hayes and Dinkova-Kostova et al. 2014). Electrophiles (Ep), reactive oxygen species (ROS), reactive nitrogen species (RNS) and heavy metals react with critical cysteines in Keap1 (Uruno et al. 2015) and Nrf2 (He et al. 2009) and disrupt the Keap1-Nrf2 interaction, allowing Nrf2 to travel to the nucleus and activate ARE-induced gene expression. Masayuki Yamamoto's group has shown that a CUL2-associated Keap1 homodimer binds to a single Nrf2 protein at its DLG (low affinity) and ETGE (high affinity) sites in a "cherry bomb" formation (Taguchi et al. 2011). Upon modification of specific thiols within Keap1 by Ep/ROS/RNS, conformational changes in Keap1 causes release of Nrf2 from the low affinity DLG site and the cessation of ubiquitination (known as the "Hinge and Latch" mechanism). Alternatively, modification of other thiols on Keap1 causes CUL2-Keap1 dissociation and the cessation of ubiquitination. Both mechanisms stabilize Nrf2 and result in its translocation to the nucleus. The Neh2L domains of TCF11, p120 Nrf1 and p95 Nrf1 also contain the DLG/ETGE sequences. However, the Neh2L subdomain in Nrf1, unlike the Neh2 domain in Nrf2, binds to Keap1 with only 28% of the affinity as for Nrf2 (Zhang et al. 2006; Kobayashi et al. 2004) as determined by yeast two-hybrid analysis. In contrast to Nrf2, neither Nrf1's protein stability, nor its nuclear localization is affected by the interaction with Keap1 (Zhang et al. 2006; Wang and Chang, 2006). With the exception of one report (Zhao et al. 2010), Nrf1 does not respond to pro-oxidants at the protein level, while the reports of increased Nrf2 response to pro-oxidant treatments at the level of the protein are widespread (Motohashi et al. 2004; Okawa et al. 2006; Yates et al. 2009; Zhang et al. 2006).

In this study, we investigated the hypothesis that nuclear factor-erythroid 2 p45 subunit-related factor (Nrf) 1 and 2 transcription factors act to independently regulate the redox state of the ER and mitochondria. As such, their functional roles may be redefined from overall redox homeostasis factors to organelle-specific redox factors, and their relative levels may in turn

mediate ROS homeostasis through the control of organelle interactions. It was predicted that in Human Embryonic Kidney 293T (HEK293T) cells, Nrf1 ER localization is underlying its activation mechanism in response to ER stress and that crosstalk occurring from mitochondrial ROS can regulate ER stress, and consequently impact Nrf1/Nrf2 relative nuclear protein levels.

We focused on Nrf1/2 ratios under chronic ER stress at viable doses more indicative of roles in adaptation to stress. UPR-inducing drugs were DTT, a reducing agent, THP, an ER Ca^{2+} deregulator/ inhibitor of SERCA pumps, and TUN, a glycosylation inhibitor (Shinjo et al. 2013). Antimycin A was used to stimulate ROS production by complex III in the ETC and served as a positive control for Nrf2 induction. The UPR response was assessed with BiP induction in HEK293T cells (Shinjo et al. 2013).

The first two objectives were to determine the dose of drugs used by assessing cell viability with the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTT assay and cell death with the propridium iodide (PI) assay. In objective two and three, the effects of each drug was assessed for oxidative stress using the 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) assay, as well as for ER stress using BiP Western blotting. In the last objective, Nrf1 and Nrf2 induction was quantified by Western blotting at drug doses that reflected stress, as determined by objectives 1-4.

It was determined that Nrf1 is only induced along with BiP in response to UPR drugs, while Nrf2 levels appear more associated with oxidative stress. Drug-induced ROS toxicity mediated by DTT was the only drug that induced both Nrf1 and Nrf2 significantly, thus confirming the roles of both transcription factors in redox homeostasis.

MATERIALS AND METHODS

2.1 Cell culture

Human embryonic kidney 293T cells (HEK293T) from the American Tissue Culture Collection, (ATCC, Manassas, VA) was cultured in complete media consisting of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), at 37 °C in a 5% CO_2 and 21% O_2 atmosphere (Thermo Forma Series II Incubator, Waltham, MA). Cells were passaged every 2-3 days. Briefly, Cells were rinsed in sterile PBS, 1mL Trypsin (0.25% in PBS) was added for 2-5 minutes followed with 9 mL of complete media.

2.2 Drug treatments

HEK293T cells were plated 2 days prior to treatment in 6-well plates, $4x10^5$ cells/ well in a total volume of 5mL pre-warmed media. AA was treated for 1hr just prior to harvest. The following final drug concentrations in 5mL media were used: TUN (0, 0.3, 1, 3, 10 µg/mL); THP (0, 0.3, 1, 3, 10 nM); DTT (0, 0.3, 1, 1.5, 3 mM) and AA (0, 2, 5 µM). All drugs were purchased from (Sigma, St. Louis, MO); (Appendix A). Stock concentrations were diluted into DMSO such that DMSO had a final concentration of 0.5%.

For PI, MTT and H_2DCFDA assays, 100µl of resuspended cells at 1.0×10^5 cells/mL, were plated in a 96-well plate and grown at 5% CO_2, 21% O_2, 37℃ (Thermo Forma Series II Incubator, Waltham, MA) for 48 hours. Stock drug concentrations were diluted 1 in 100 to give the same final concentrations as in 6-well plates.

2.3 Propidium Iodide (PI) assay

Dying cells have perforated membranes and the PI assay takes advantage of this in cell death determination. PI is membrane impermeable, and following cell death, it can enter cells and intercalate non-specifically with DNA. In aqueous solution, the dye has excitation/emission maxima of 493/636 nm.

Cells were washed 2x with PBS and exposed to PI (Sigma, 81845, St. Louis, MO) working reagent, diluted in PBS to 100μg/mL for 10 min. The cells were washed 3x with PBS and read using an excitation wavelength of 530nm (Epoch Bioteck, Winooski, VT).

2.4 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay

Cell viability or proliferation is measured through the MTT assay, as it is a general readout of cellular metabolism. MTT is reduced in a cell, either enzymatically or through direct reaction with cellular NADH or NADPH, turning it into an insoluble dark blue precipitate, which can be easily extracted with DMSO for quantification. The amount of signal generated is dependent on several parameters including the number of viable cells and their and metabolic activity, the concentration of MTT, and the length of the incubation period.

Ten microliters of MTT (5 mg/mL stock in ice-cold, autoclaved PBS) was added to each well containing 100 μL media and incubated in the dark at 5% CO_2, 21% O_2, 37℃ for 1 hour. The media was removed, the cells were rinsed in ice-cold PBS and then dissolved in 100 μL of DMSO. The absorbance was read at 570 nm with background subtraction at 630 nm (Epoch Bioteck, Winooski, VT).

2.5 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay

H2DCFDA is a traditional fluorescent probe that is used to detect ROS generation. The diacetate form is cell permeable and is hydrolyzed by intracellular esterases to liberate the free acid DCFH. The highly fluorescent compound 2′,7′-dichlorofluorescein (DCF), (ʎ excitation = 498 nm; ʎ emission = 522 nm) is then formed following oxidation by ROS (i.e. HO* and ROO* , *NO and ONOO-). This assay can be used to indicate overall oxidative stress.

Cells were washed 2X with warm PBS (37^0C), and incubated for 30 min with 20µM H_2DCFDA in DMEM in the dark cell culture incubator. Cells were washed 2 more times and the plate was read using 488nm ʎ excitation and 525 nm ʎ emission (Epoch Bioteck, Winooski, VT). Cells were divided by total protein, corrected for background fluorescence in non-exposed cells, and finally normalized relative to DMSO-treated control fluorescence units/µg protein.

2.6 Cell harvest, Sample preparation and Western Blotting

2.6.1 Whole-cell lysis (RIPA)

Cells were rinsed two times on ice with ice-cold PBS, and lysed on ice, on-plate, using 200µl of RIPPA buffer (Pierce, Waltham, MA), supplemented with a Protease Inhibitor Tablet (Roche, 04693132001, Laval, QC; 1 PIT/50mL buffer). After transferring the lysate to Eppendorf tubes, cells were spun at 17000*x g* for 15 minutes after at least 30 min of lysis.

The BCA assay (Pierce, 23225, Waltham, MA) protocol was used to quantify protein concentrations. Fifty micrograms of whole-cell lysate sample and 20 micrograms of nuclear isolations were diluted 1:1 into 20-25µl of 2X SDS Sample Buffer (BioRad, 1610737, Hercules, CA), for SDS-PAGE. These were boiled for 4-5 minutes at 95-100°C, and loaded into a 10% gel, next to the All-Blue protein ladder to determine protein molecular weight (BioRad, 1610373,

Hercules, CA). The gel ran for 30 min at 100V, followed by 1hr at 150V (see Appendix A for Western Blot buffer compositions). The protein was transferred onto SDS transfer buffer-equilibrated nitrocellulose membranes (BioRad, 1620115, Hercules, CA).

2.6.2 Nuclear isolations

Nuclear isolations were done very similarly to (Holden et al. 2009) on ice with continual vortexing. Briefly, cells were rinsed with ice cold PBS prior to collection at 800 x *g* in pre-cooled eppendorf tubes. Cells were swollen in hypotonic Buffer 1 (10mM Hepes, 10mM KCl, 1.5mM $MgCl_2$, 1mM DTT, 0.5mM PMSF) with addition of protease inhibitor tablet (Roche, Laval, QC) for 10 minutes, NP40 was added for 5 minutes to a final concentration of 1% (*i.e.* Buffer 2) and vortexed. The nuclei were then spun down 5000xg for 5 min, washed 3x (Buffer 1) prior to lysis with RIPA buffer (Pierce, 89900, Waltham, MA) (*i.e*. Buffer 3), and frozen at -20^{0}C prior to protein determination using the BCA assay (Pierce, 23225, Waltham, MA). Absence of β-tubulin and BiP bands in the nuclei was used to confirm purity in Western blot analysis.

2.7 Antibodies used for Western blotting

Primary antibodies were diluted into TBST while secondary antibodies were diluted into TBST-milk as follows: anti-GRP78/BiP rabbit polyclonal (Abcam, Cambridge, MA) ab21685) 1:500; anti-Nrf1 (H4) mouse monoclonal (Santa Cruz, sc-28379, Dallas, TX), 1:500; anti-Nrf2 rabbit polyclonal (C20) (Santa Cruz, sc-722, Dallas, TX), 1:5000; anti-β-tubulin (E7) mouse monoclonal (E7, Developmental Studies Hybridoma Bank) 1:5000; goat anti-rabbit-HRP (Santa Cruz, sc-2004, Dallas, TX), 1:5000; goat anti-mouse-HRP (DAKO), 1:5000; anti-β-lamin goat

polyclonal (Santa Cruz, sc- 6217, Dallas, TX) 1 in 500; mouse anti-goat-HRP, 1:5000 (Santa Cruz, sc-2354, Dallas, TX).

2.8 Densitometry

The densitomtry was perfomed by the AlphaEase software. Boxes of equal and minimal areas were drawn for each band, and normalized to a background box. The IDV value was subtracted from the background IDV value to obtain a final IDV. Each IDV was normalized to controls. Normalized data was then analyzed for statistical significance.

2.9 Statistical Analysis

All statistical comparisons were made relative to the controls using Sigmaplot software (San Jose, CA) with one-way ANOVA followed by the Tukey's test, for multiple comparisons. All values displayed represent the mean ± the standard error of the mean (SEM) for a minimum of 3 independent experiments. Values were considered to be significantly different from one another if $p<0.05$.

CHAPTER 3: RESULTS

3.1 Drug effects on cell death, viability and ROS

A drug dosage was first chosen reflecting changes to cell death and viability. The PI assay determined no changes in cell death to all drugs, except for DTT at a dose of 3mM and higher (Figure 6). This reflects an end-point in oxidative-stress mediated cell death and suggests DTT was the most potent ROS inducer.

All drugs portrayed a declining viability trend in the MTT assay (Figure 7), but only TUN > 0.3 μg/mL and DTT > 0.3mM treatments were significantly affected.The H_2DCFDA assay depicted oxidative stress, indicative of an altered GSH: GSSG ratio (Figure 8). A significant increase in DCF fluorescence suggested that oxidative stress was seen for TUN 3 μg/mL and AA at 0.5 μg/mL, while DTT shown DCF fluorescence at cell death doses of 3mM and higher (Figure 8).

Since a decline in MTT reflects a decline in the number of mitochondria, concomitant oxidative stress and decline in viability in TUN and DTT treatments (Figure 7 and 8) may suggest oxidative-stress mediated decline of mitochondria, or autophagy induction. Autophagy did not occur in THP and AA treatments, where the change in viability was not significant.

AA only shown an increase in oxidative stress, which signifies mild or adaptive ROS signaling (Figure 7 and 8). This is in contrast to THP that shown a mild yet significant decline in oxidative stress and a mild decline in viability (Figure 7 and 8), reflective of a decline in the number of mitochondria following stress and an elevation in GSH: GSSG ratio. This may be a consequence of mild autophagy in THP treatment at the highest dose.

3.2 UPR is evidenced by BiP induction using Western blotting

Figure 9 depicted ER stress activation via BiP induction. BiP induction was very pronounced and significant in all doses of TUN, and portrayed increased trends in THP and DTT; THP dose of 10 nM was significant and toxic doses of DTT at 3 mM and higher (Figure 6) were significant for BiP (Figure 9). Importantly, AA did not induce ER stress following 1 hour of treatment, despite significant ROS elevation (Figure 8 and 9). All-together, this data suggests that ER stress was induced by UPR drugs and not via AA during signaling levels of ROS.

3.3 UPR and ROS impact Nrf1 and Nrf2 nuclear accumulation respectively

Figure 8 depicts Nrf2 levels that were only detected in nuclear isolations, at the 100kDA MW marker (Lau A et al. 2013). The Nrf2 band was validated by overexpression of Nrf2 plasmid in HEK293T cells (Appendix B). As anticipated, Nrf2 follows DCF fluorescence trends indicative of total ROS in the cell (Figure 8). Nrf2 was only significantly elevated at 1mM DTT and higher, with insignificant elevations during TUN and AA- mediated oxidative stress (Figure 8). Nrf2 also declined during cell death (Figure 6 and 10). While Nrf2 showed a non-significant elevated trend at an AA dose of 2µM, the relative BiP protein average did not change (Figure 9), suggesting that Nrf2 induction is independent of ER stress and confirms its role as the primary redox response transcription factor.

Nrf1 has active forms of high molecular weights above 95kDA (p95) that induce ARE gene expression, and inhibiting forms of 65kDA (p65) that repress gene expression. Figures 11, 12 and 14 show Nrf1 p120 and p95 to be significantly induced in response to UPR drugs, at doses that induced BiP (Figure 9), and not in response to AA in the absence of BiP; in fact the p95 trend was declining non-significantly in response to AA (Figure 14). Importantly, this signifies distinct

regulation of Nrf1 by ER stress, independent of ROS. This was validated by showing that TUN and THP drugs generate parallel trends in both the whole cell lysate and the nucleus for p95 and p120 (Figure 12), while similar non-significant trends were seen for p110 (Figure 13). The inhibitory p65 form of Nrf1 was not induced concomitantly with BiP levels (Figure 9 and 15).

DTT is a reducing agent but may also auto-oxidize its thiols to generate ROS. DTT is commonly used to induce ER reductive stress (Rand et al. 2006). DTT at 1mM significantly upregulated active forms of both Nrf1 and Nrf2. This was consistent with its strong redox deregulations as seen in the PI and viability assay (Figure 6 and 7). Nrf1 p95 induction by DTT at 1mM was more pronounced than at THP 3 nM at common levels for BiP induction (Figure 9), suggesting a more potent effect of DTT on the Nrf1 activation mechanism (Figure 16).

3.4 Nrf responds to ER stresses in a biphasic manner.

Nrf1 shows a biphasic response to all ER stresses around 4-6 hours post-treatment in contrast to AA where Nrf1 declined with time (Figure 15). Nrf2 is also responding in a biphasic manner however in a weak manner compared to Nrf1 following DTT and THP treatment, and clearly becomes undetectable similarly to controls prior to 18 hours of treatment. This result validates Nrf1 gene induction at 24 hours following ER stress, and not Nrf2. Furthermore, this indicates that Nrf1 and Nrf2 are chronic and acute ARE gene regulators in response to stress.

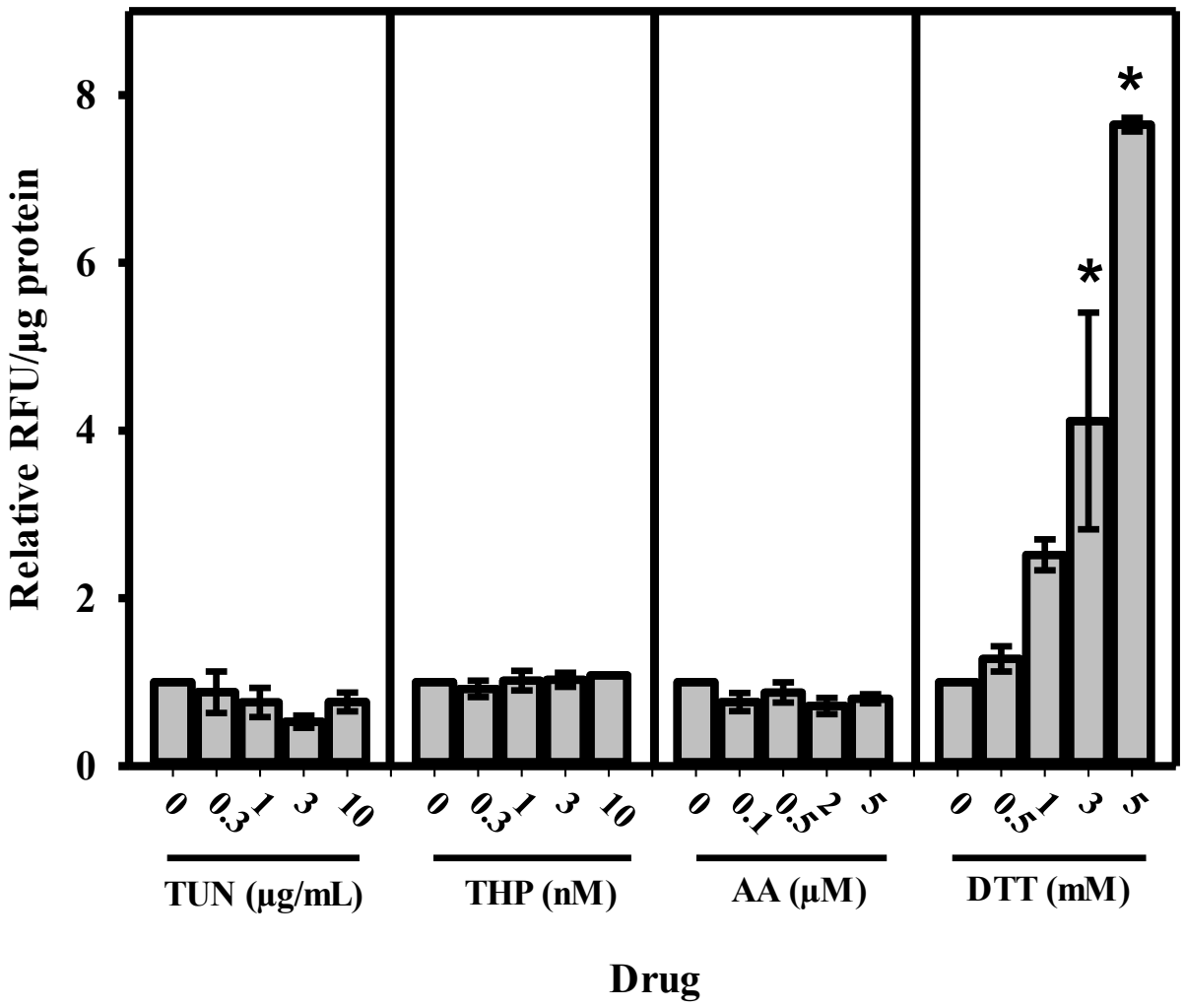
PI ASSAY
Relative RFU/µg protein
0
2
4
6
8
0 0.3 1 3 10
0 0.3 1 3 10
0 0.1 0.5 2 5
0 0.5 1 3 5
*
*
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 6. Cell death as measured by propidium iodide assay.

Propidium iodide (PI) assay shows cell death response in HEK293T cells after 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA). Relative fluorescence units (RFU) at 530nm (Epoch Bioteck, Winooski, VT) was normalized to total protein in each well.

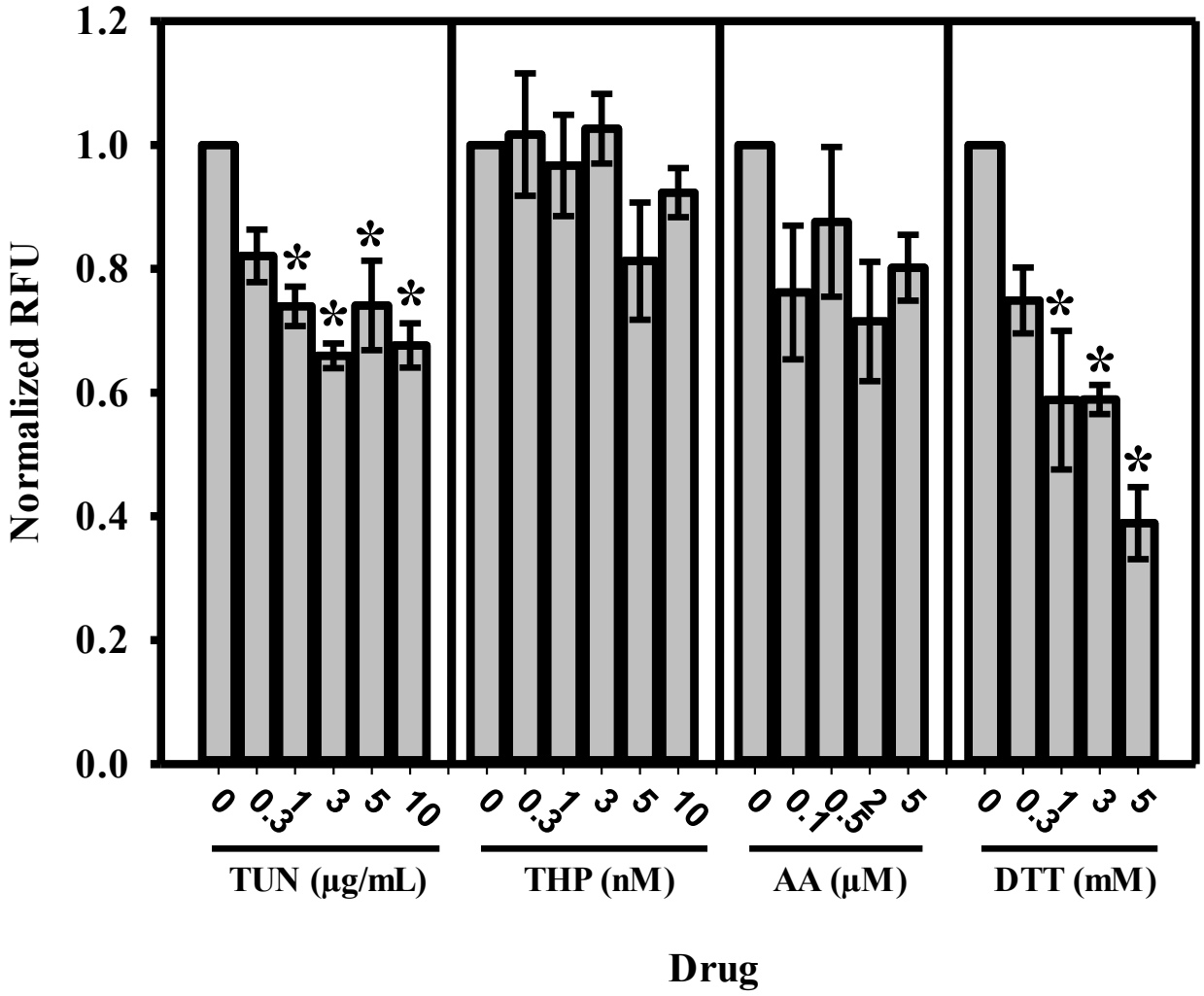
MTT ASSAY
Normalized RFU
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 0.3 1 3 5 10
0 0.3 1 3 5 10
0 0.1 0.5 2 5
0 0.3 1 3 5
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 7. Cell viability as measured by MTT assay.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay depicts viability response in HEK293T cells after 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA). The absorbance was read at 570 nm with background subtraction at 630 nm (Epoch Bioteck, Winooski, VT), and normalized to control fluorescence units.

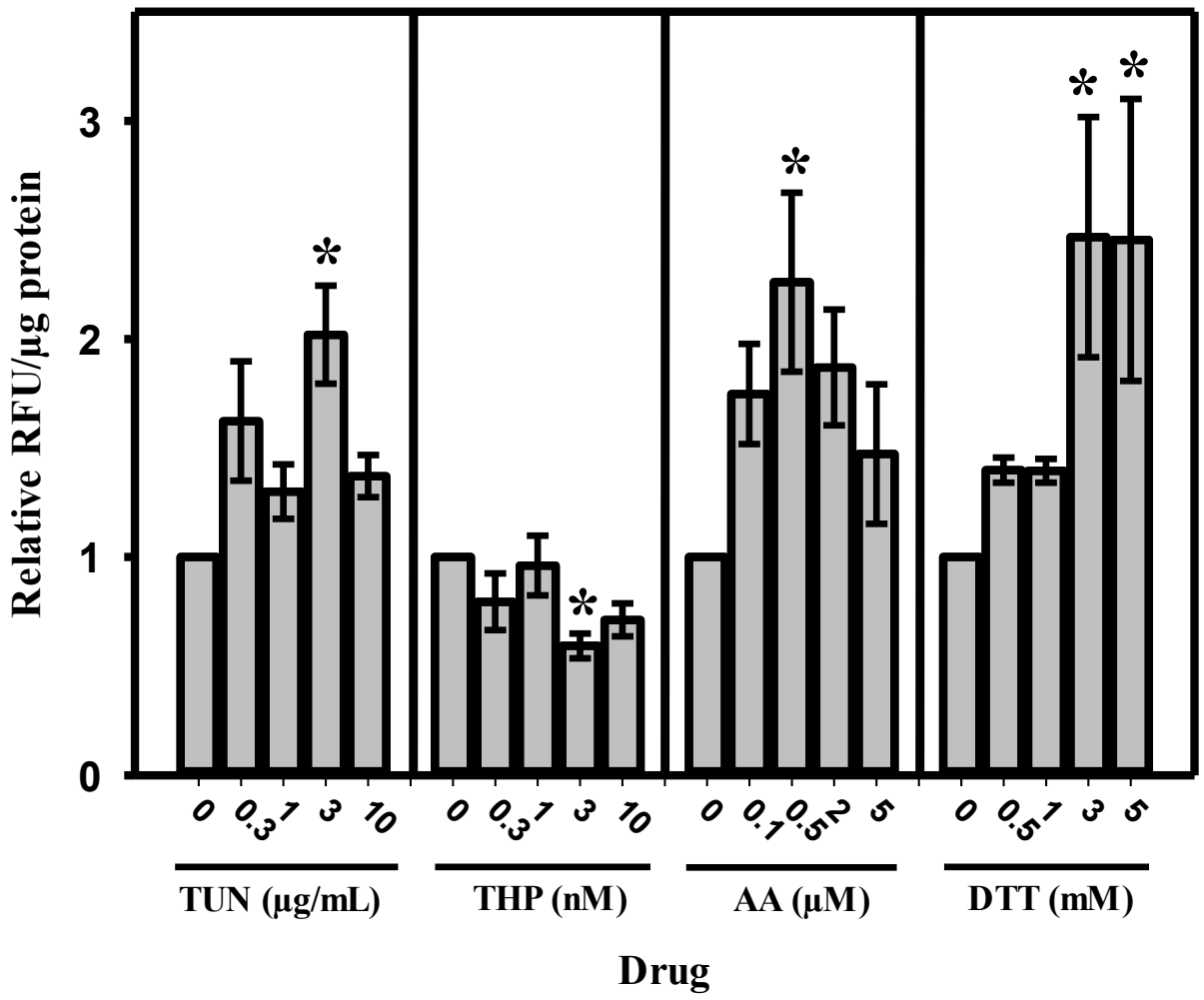
H2DCFDA ASSAY
Relative RFU/µg protein
0
1
2
3
*
*
*
*
0 0.3 1 3 10
0 0.3 1 3 10
0 0.1 0.5 2 5
0 0.5 1 3 5
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 8. Reactive oxygen species as measured by H_2DCFDA.

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay depicts ROS response in HEK293T cells after 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA). Relative fluorescence, 488nm ʎ excitation and 525 nm ʎ emission (Epoch Bioteck, Winooski, VT) was normalized to total protein in µg in each well.

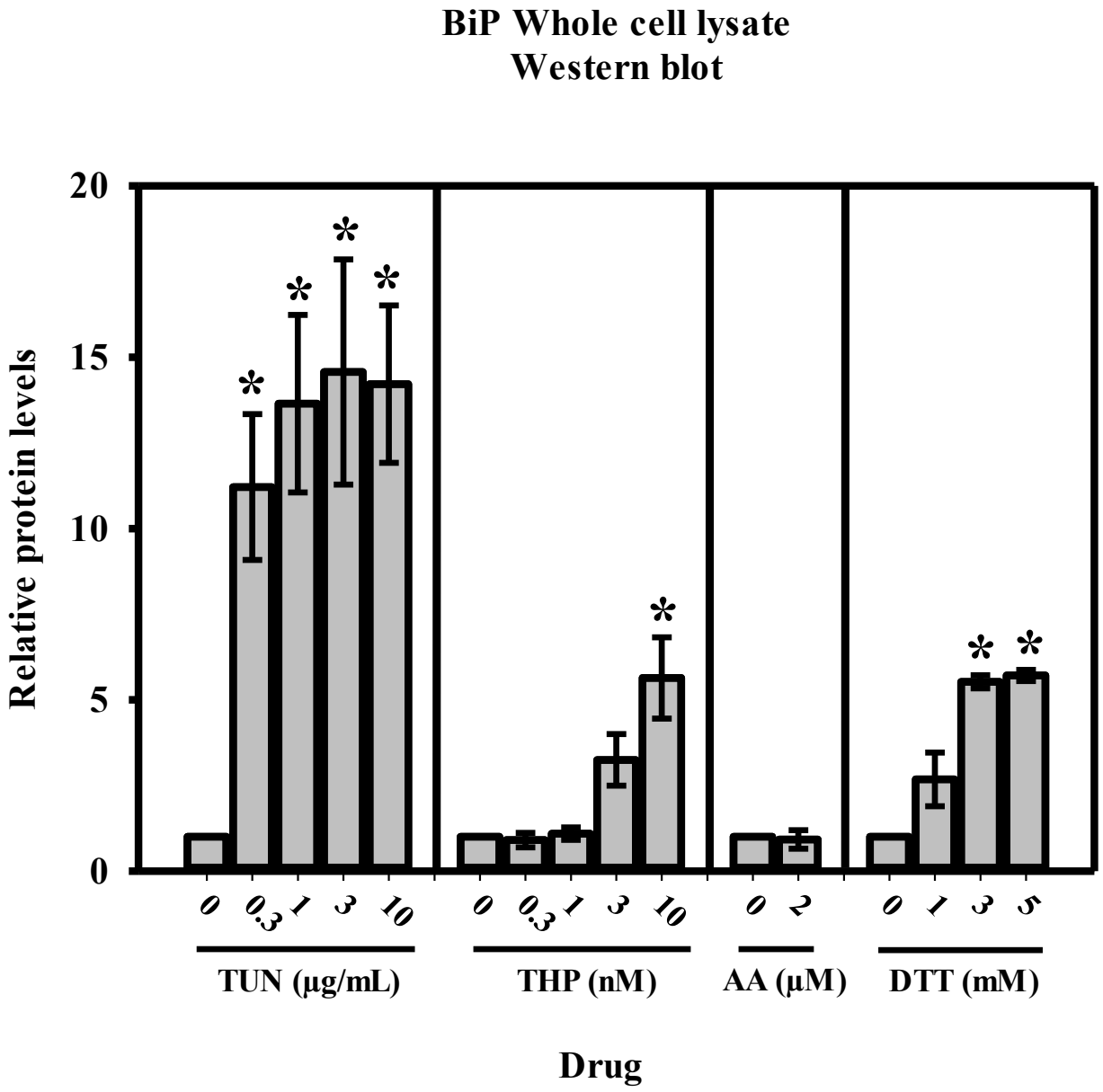
BiP Whole cell lysate
Western blot
Relative protein levels
20
15
10
5
0
0
0.3
1
3
10
0
0.3
1
3
10
0
2
0
1
3
5
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 9. **Western blot analysis of endogenous BiP expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in whole cell lysates of HEK293T cells**. Primary antibody Rabbit anti-BiP (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-rabbit 1in5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1in5000) primary antibody; secondary antibody goat anti-mouse (1in5000). Values shown are the means normalized to the control ±.SEM; (*) indicates significance ($p<0.05$).

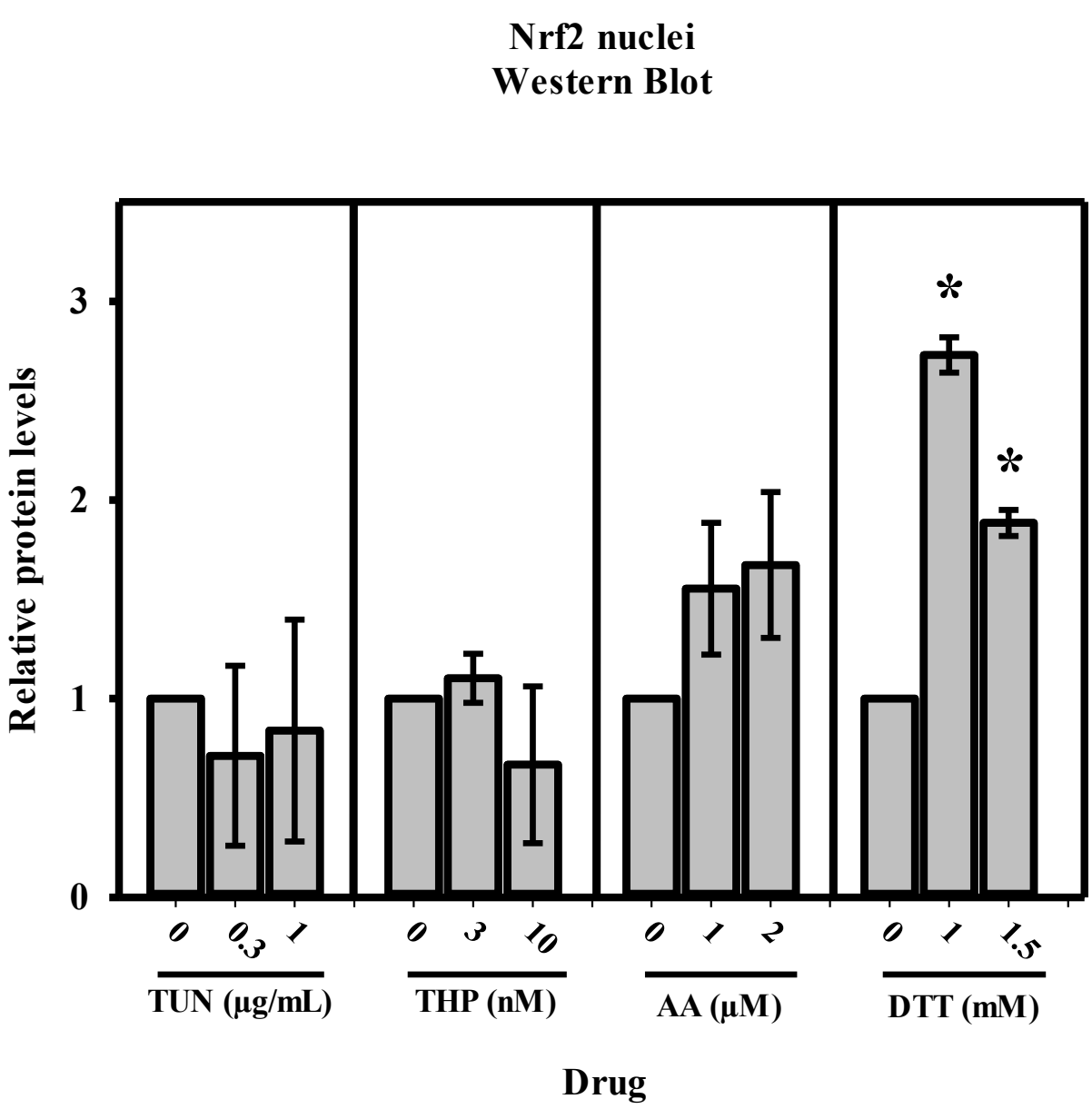
Nrf2 nuclei
Western Blot
Relative protein levels
3
2
1
0
*
*
0
0.3
1
0
3
10
0
1
2
0
1
1.5
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 10. Western blot analysis of endogenous Nrf2 expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei of HEK293T cells.

Primary antibody rabbit anti-Nrf2 (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-rabbit 1 in 5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1 in 5000) primary antibody; secondary antibody goat anti-mouse (1 in 5000). Values shown are the means normalized to the control ± SEM; (*) indicates significance ($p<0.05$).

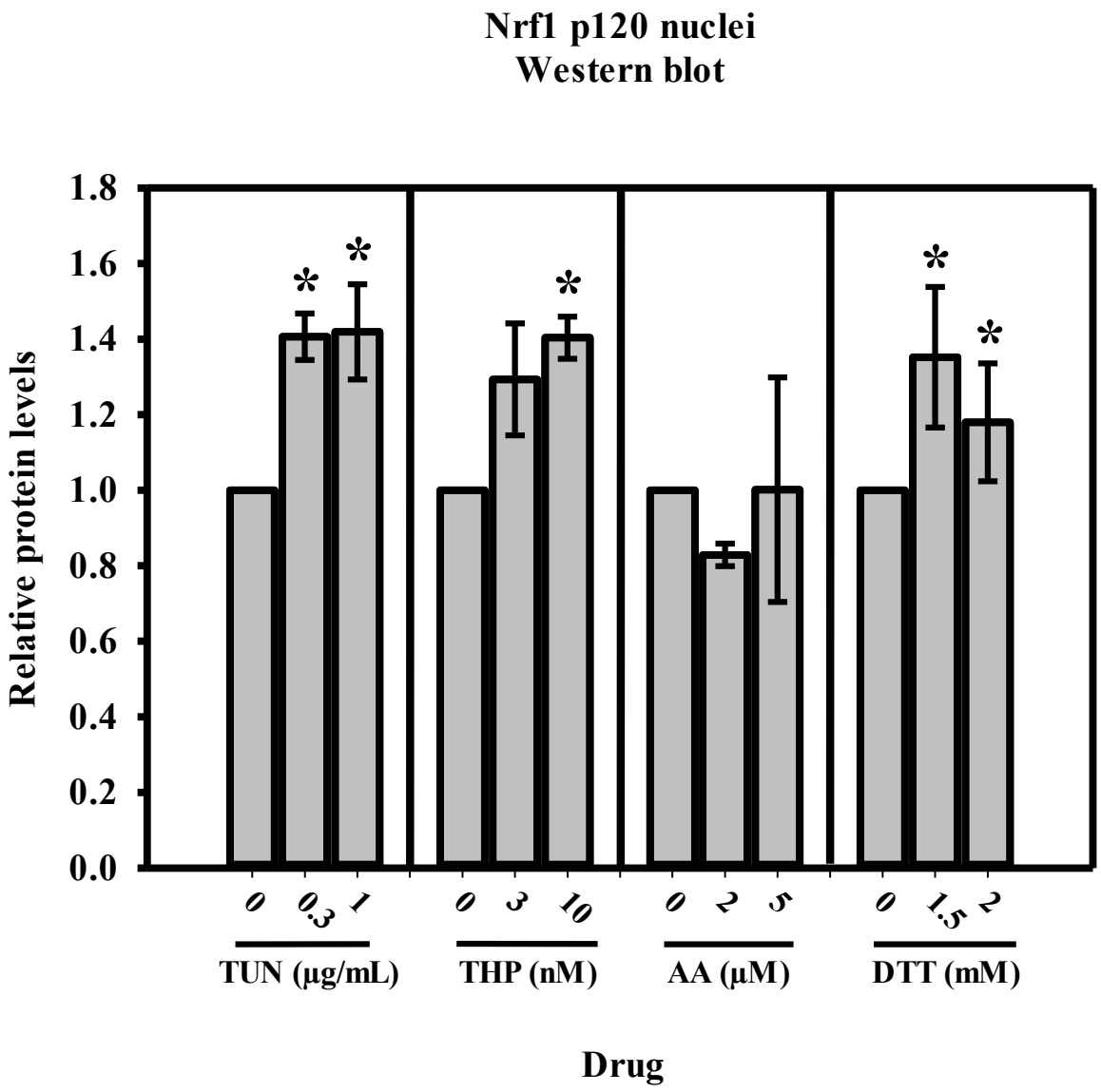
Nrf1 p120 nuclei
Western blot
Relative protein levels
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
*
0
0.3
1
0
3
10
0
2
5
0
1.5
2
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 11. Western blot analysis of endogenous Nrf1 p120 expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei of HEK293T cells.

Primary antibody Mouse anti-Nrf1 (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1 in 5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1 in 5000) primary antibody; secondary antibody goat anti-mouse (1 in 5000). Values shown are the means normalized to the control ± SEM; (*) indicates significance ($p<0.05$).

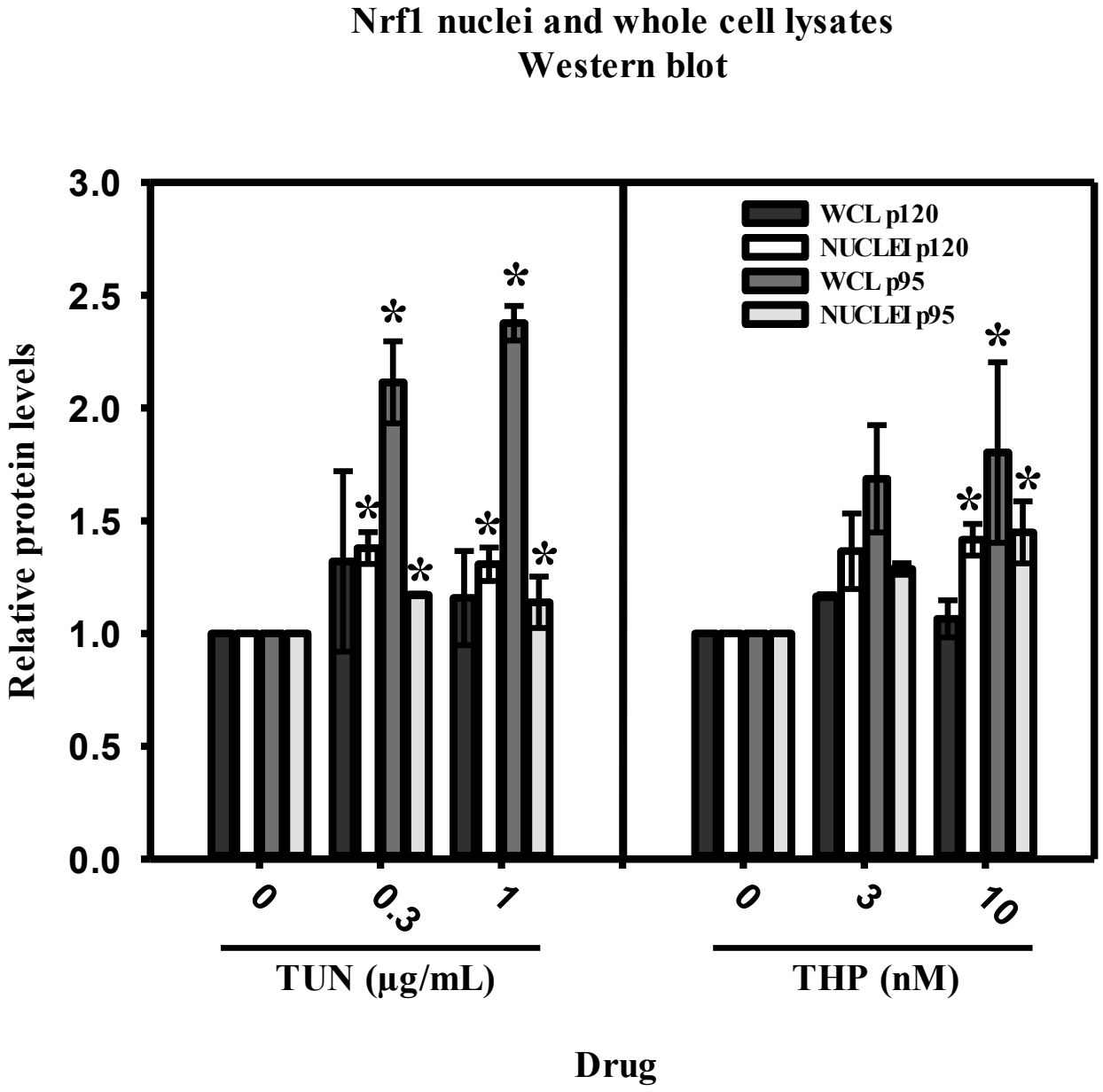
Nrf1 nuclei and whole cell lysates
Western blot
Relative protein levels
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WCL p120
NUCLEI p120
WCL p95
NUCLEI p95
0
0.3
1
0
3
10
TUN (µg/mL)
THP (nM)
Drug

Figure 12. Western blot analysis of Nrf1 forms after treatment with ER stressors.

Western blot analysis of active forms of Nrf1, p120 and p95, endogenously expressed in HEK293T cells following 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei (white and light grey) and cytosol (black and dark grey) respectively. Primary antibody Mouse anti-Nrf1 (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1 in 5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1 in 5000) primary antibody; secondary antibody goat anti-mouse (1in5000). Values shown are the means normalized to the control ± SEM; (*) indicates significance ($p<0.05$).

Nrf1 p110 nuclei
Western blot

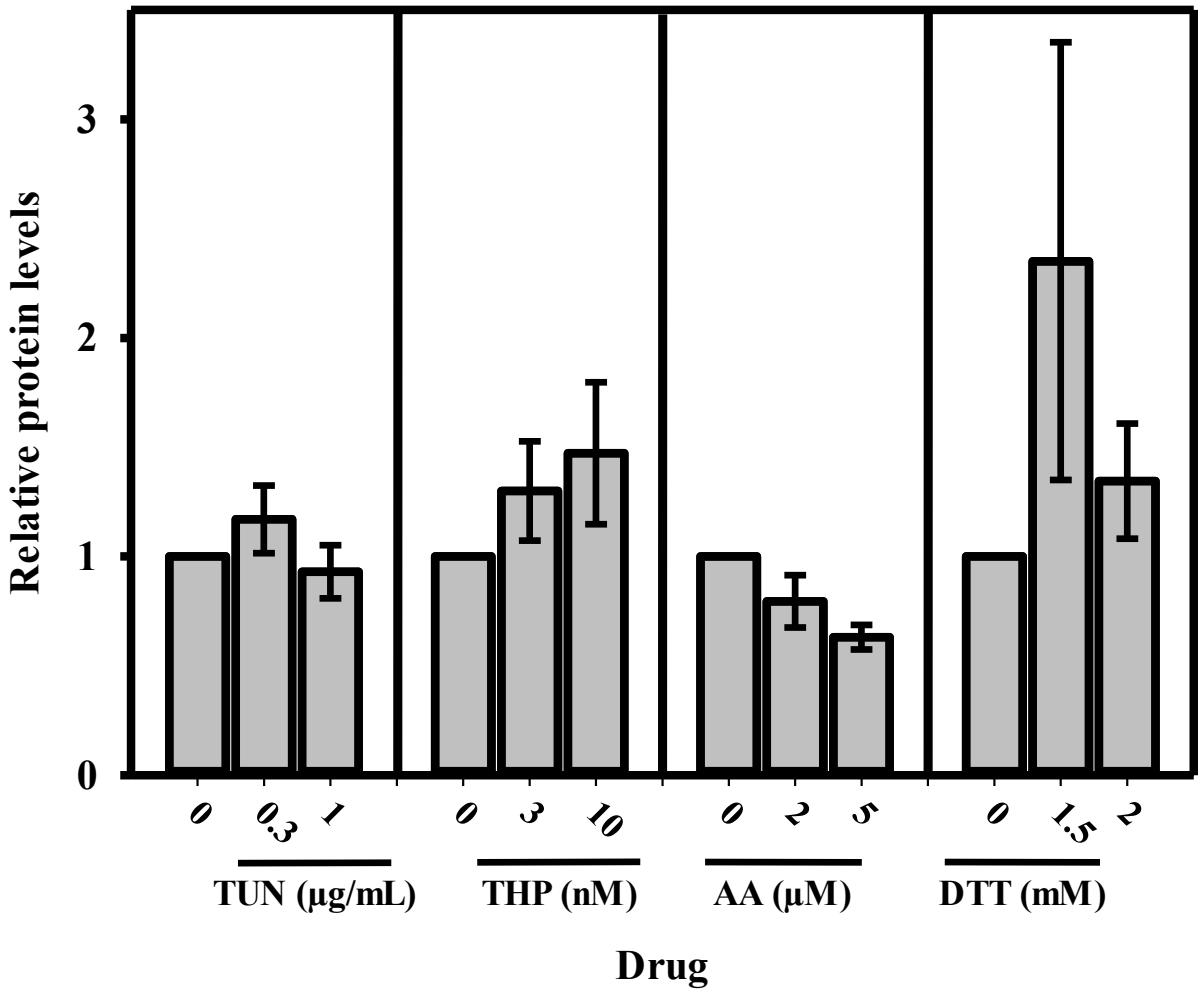

Figure 13. Western blot analysis of endogenous Nrf1 p110 expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei of HEK293T cells.

Primary antibody mouse anti-Nrf1 (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1 in 5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1 in 5000) primary antibody; secondary antibody goat anti-mouse (1 in 5000). Values shown are the means normalized to the control ± SEM; (*) indicates significance ($p<0.05$).

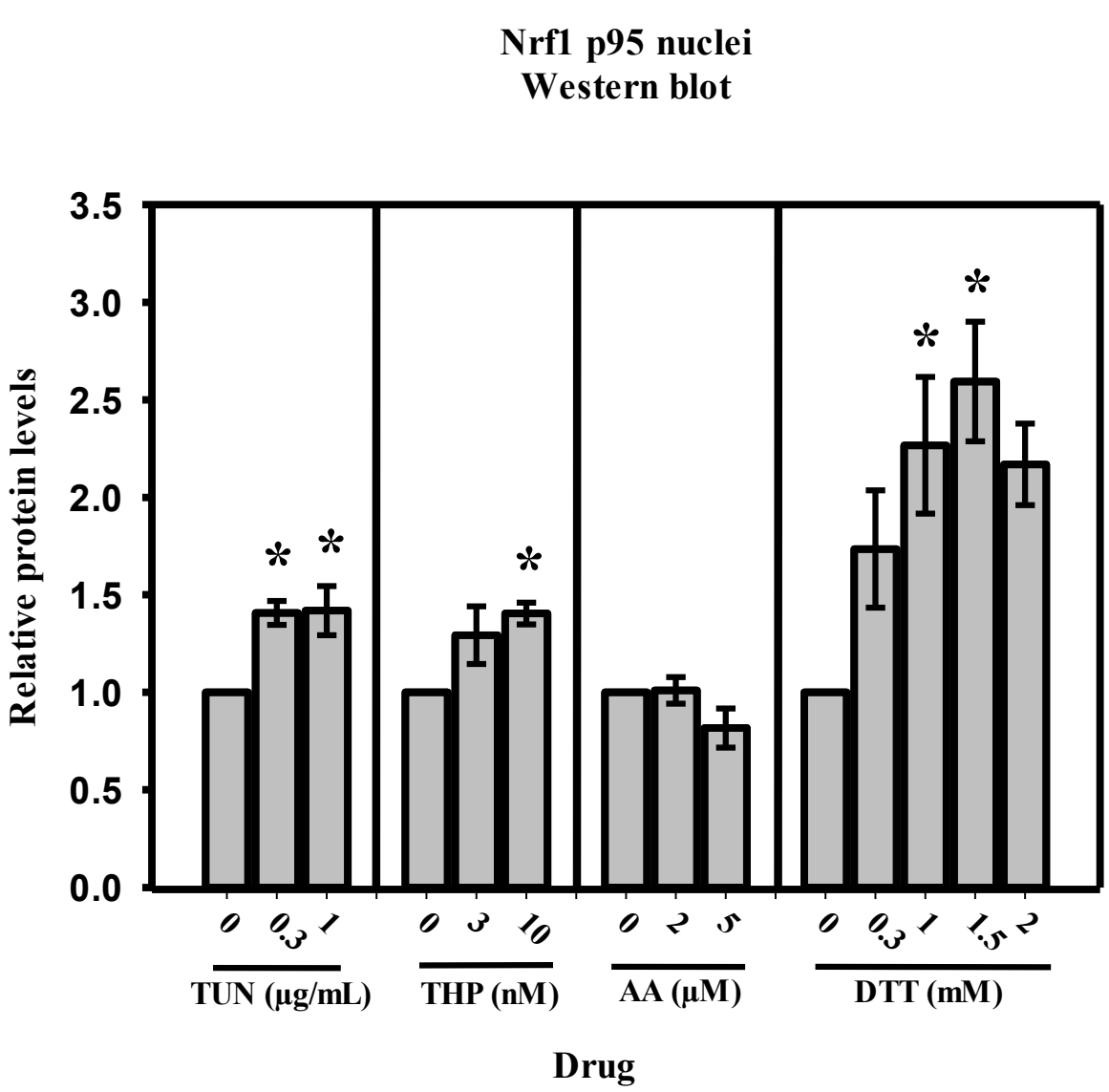
Nrf1 p95 nuclei
Western blot
Relative protein levels
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
0
0.3
1
0
3
10
0
2
5
0
0.3
1
1.5
2
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 14. Western blot analysis of endogenous Nrf1 p95 expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei of HEK293T cells.

Primary antibody Mouse anti-Nrf1 (1 in 500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1 in 5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1 in 5000) primary antibody; secondary antibody goat anti-mouse (1 in 5000). Values shown are the means normalized to the control ±.SEM; (*) indicates significance ($p<0.05$).

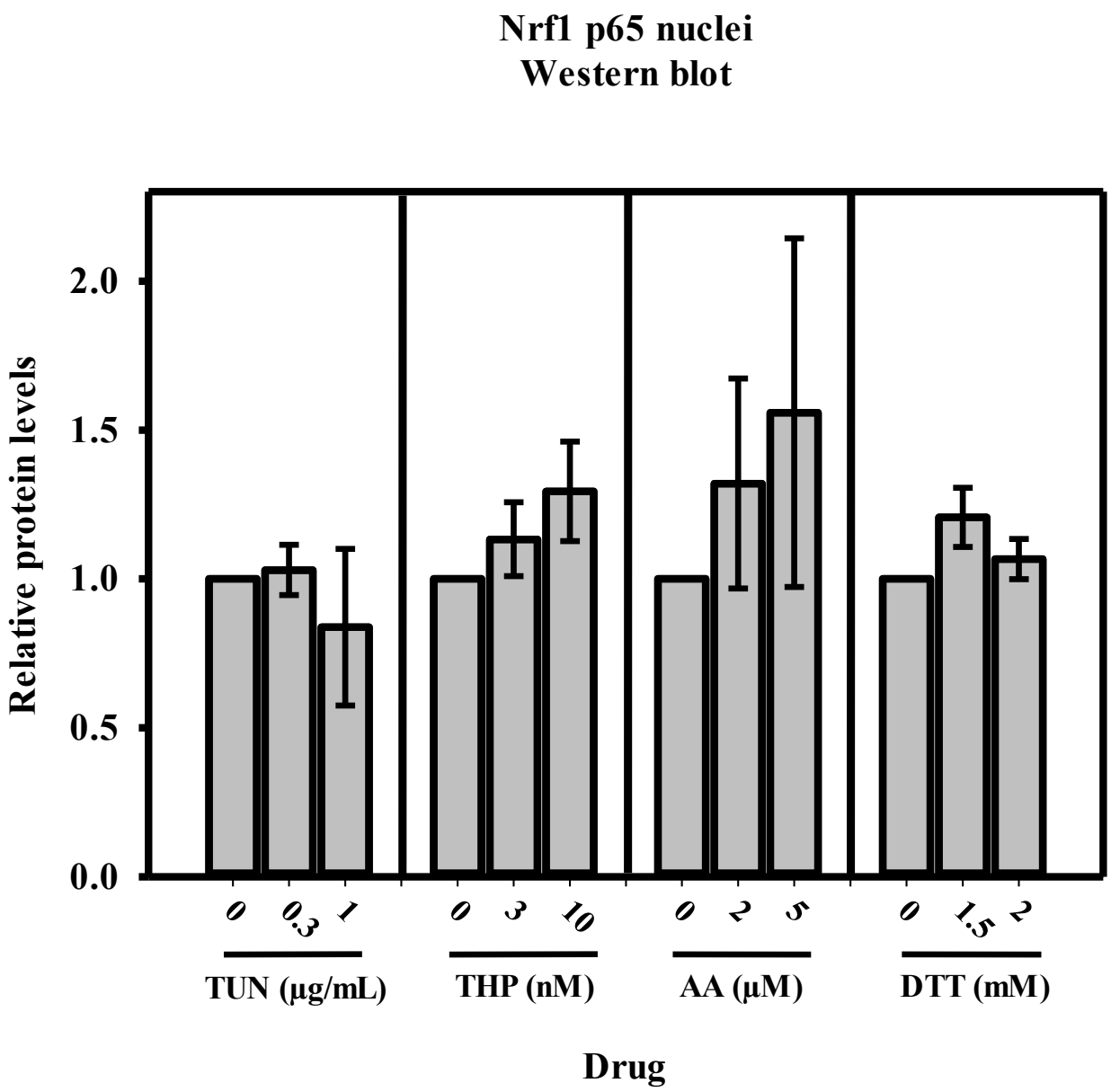
Nrf1 p65 nuclei
Western blot
Relative protein levels
2.0
1.5
1.0
0.5
0.0
0
0.3
1
0
3
10
0
2
5
0
1.5
2
TUN (µg/mL)
THP (nM)
AA (µM)
DTT (mM)
Drug

Figure 15. Western blot analysis of endogenous Nrf1 p65 inhibitory form expression in response to 24 hour treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and 1 hour treatment with antimycin A (AA) in the nuclei of HEK293T cells. Primary antibody Mouse anti-Nrf1 (1in500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1in5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1in5000) primary antibody; secondary antibody goat anti-mouse (1in5000). Values shown are the means normalized to the control ±.SEM; (*) indicates significance ($p<0.05$).

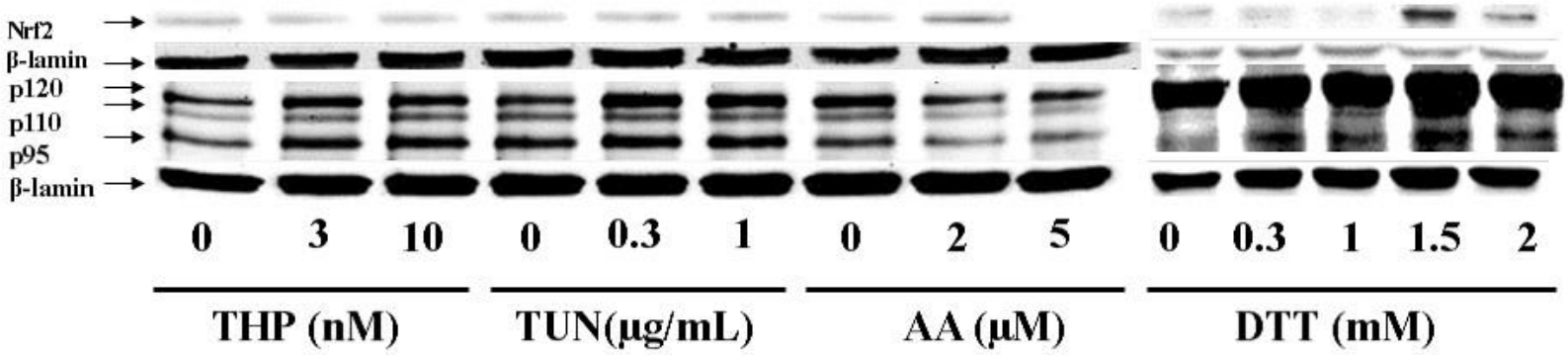
Nrf2
β-lamin
p120
p110
p95
β-lamin
0 3 10 0 0.3 1 0 2 5 0 0.3 1 1.5 2
THP (nM)
TUN(μg/mL)
AA (μM)
DTT (mM)

Figure 16. Western blot representative bands for Nrf nuclear isolations.

DTT induces both p95 Nrf1 and Nrf2 detected just below 100kDA molecular weight markers. Only active Nrf1 forms are shown in HEK293T cells.

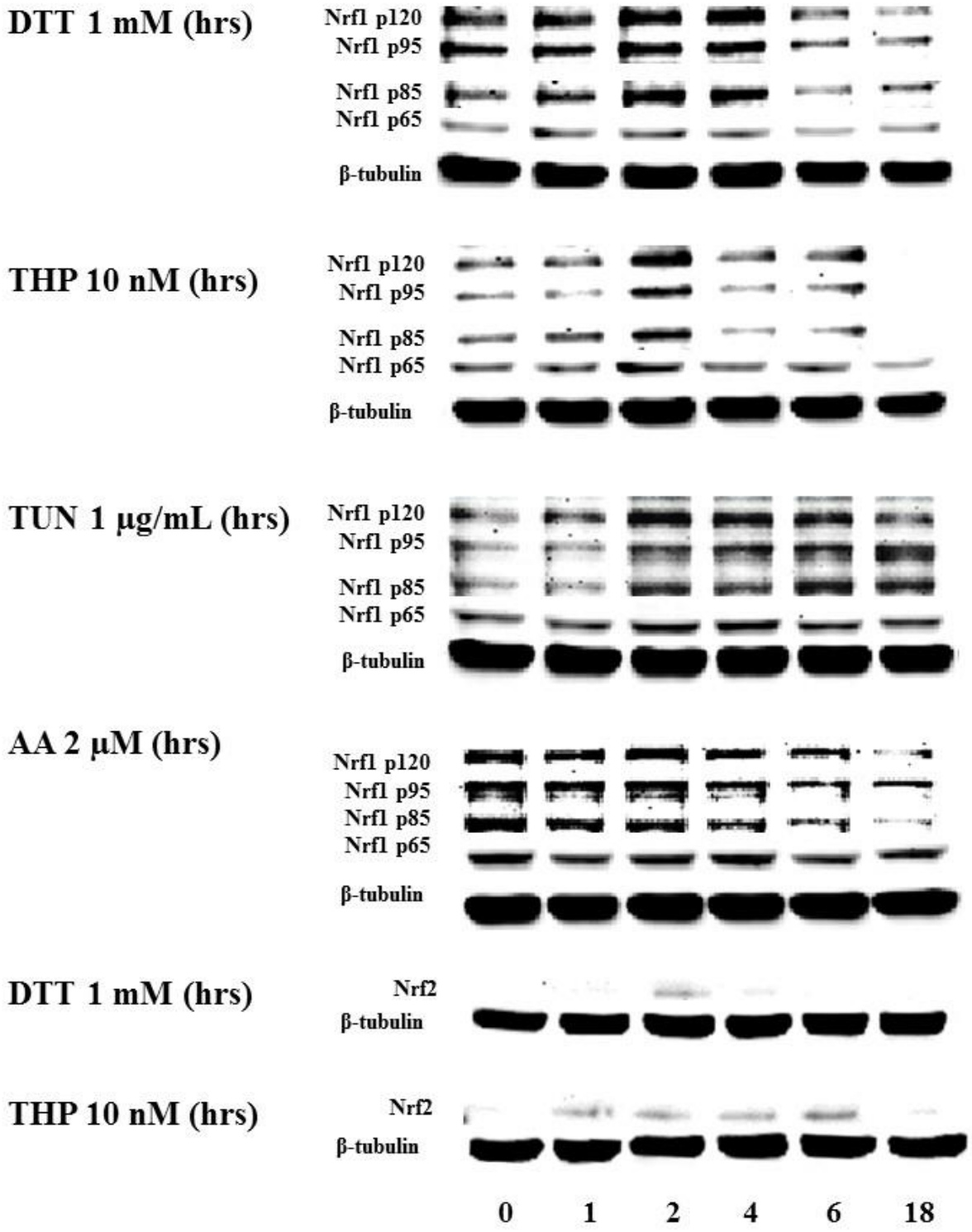
DTT 1 mM (hrs)
Nrf1 p120
Nrf1 p95
Nrf1 p85
Nrf1 p65
β-tubulin
THP 10 nM (hrs)
Nrf1 p120
Nrf1 p95
Nrf1 p85
Nrf1 p65
β-tubulin
TUN 1 µg/mL (hrs)
Nrf1 p120
Nrf1 p95
Nrf1 p85
Nrf1 p65
β-tubulin
AA 2 µM (hrs)
Nrf1 p120
Nrf1 p95
Nrf1 p85
Nrf1 p65
β-tubulin
DTT 1 mM (hrs)
Nrf2
β-tubulin
THP 10 nM (hrs)
Nrf2
β-tubulin
0 1 2 4 6 18

Figure 17. Western blot showing Nrf response in time to treatment with UPR drugs tunicamycin (TUN), thapsigargin (THP), and dithiothreitol (DTT) and antimycin A (AA) in whole cell lysates of HEK293T cells.

Primary antibody Mouse anti-Nrf1 or Rabbit anti-Nrf2 (1in500) was incubated overnight in TBST; secondary antibody was goat anti-mouse 1in5000, in 5% TBST milk for 1 hour. Bands were normalized to anti-β-tubulin (1in5000) primary antibody; secondary antibody goat anti-mouse or anti-rabbit (1in5000).

CHAPTER 4: DISCUSSION AND CONCLUSION

It was established that alterations of glutathione homeostasis affect the redox status such that adaptations of subcellular compartments might critically determine survival and cell death fates (Zhang et al. 2012). While Nrf2 is predominant regulator of inducible and baseline levels of GSH, Nrf1 predominantly regulates inducible and baseline levels of proteasomes, which in turn, promote ROS homeostasis (reviewed in Bugno et al. 2015). The significance of Nrf1 negative regulation, by its N-terminus, and the unfolded protein response, may be to mediate the expression of proteins more specific to redox regulation within the oxidized environment of the ER (Wang W and Chang, 2006; Zhang et al. 2009; Ho et al. 2015). We therefore sought out whether there is any association with Nrf1 induction, distinctly with ER stressors TUN, THP, and DTT and Nrf2 with signaling levels of ROS mediated by AA in HEK293T cells.

Nrf1 is activated following retrotranslocation from the ER to the cytosol via the TLC channel. Since BiP levels in the ER proportionally regulate Ca^{2+} leak during stress through the TLC channel (Hammadi et al. 2013), BiP is an appropriate control for Nrf1 activation during ER stress.

The H_2DCFDA assay was used to indicate a change in oxidative stress, since it cannot be used to reliably measure ROS levels. The source of oxidative stress must be interpreted with caution due to the photosensitivity of DCFH and side reactions with peroxidases, hematin and cytochrome c that can all highly increase the formation of flourescent DCF (Gomes et al. 2005). For instance in the presence of reducing agent, such as GSH or NADH, or through photoreduction, a semiquinone of DCF is formed (DCF*) which generates superoxide (Rota et al. 1999; Gomes et al. 2005). Superoxide may then through dismutation generate hydrogen peroxide which artificially increases the oxidation of DCFH and amplifies the signal observed (Gomes et al. 2005). Numerous

reports indicate superoxide dismutases controversially inhibit oxidative stress as measured by H_2DCFDA (Rota et al. 1999).

4.1 Nrf1 is activated by ER stress

UPR drugs used generate ER stress in slightly different temporal manners, as they generate slightly different trends for BiP timecourses (Shinjo et al. 2013). The drugs which are used in this study, THP, TUN and DTT all belong to one cluster of drugs regulating the same set of ER stress response genes (Shinjo et al. 2013). Concurrently with BiP induction (Figure 7), Nrf1 active forms p95 and p120 were significantly induced in the nuclei of cells hit with all ER stress drugs (Figures 10, and 11) indicating Nrf1 activation by the UPR. Another drug belonging to the same group of ER stress gene regulators that inhibits N-linked glycosylation, 2-deoxyglucose, is not used here (Shinjo et al. 2014). However Nrf1 is reported to be activated under low glucose conditions via deglycosylation, that 2-deoxyglucose mimics (Zhang et al. 2014c; Shinjo et al. 2013).

Artero-Castro et al. (2015) recently shown that ROS activates autophagy rapidly and efficiently by UPR activation. In contrast, AA was shown to inhibit autophagy through its inhibition of complex III (Ma et al. 2011). It was also shown antioxidant treatment prevents UPR activation and autophagy while restoring the proliferative capacity of these cells (Artero-Castro et al. 2015). THP induction of specifically Nrf1 and AA-mediated reduction of Nrf1 support the role of Nrf1 in autophagy under opposing oxidative stress trends (Figures 8, 11, and 14). Furthermore, given Nrf2 activation by mitochondrial stress, autophagy should decline following Nrf2-mediated induction of GCLC and other genes regulating GSH biosynthesis in a ROS dependent manner (Artero-Castro et al. 2015). Indeed, oxidative stress returns to non-significant levels at the highest dose of AA (Figure 8).

The role of Nrf1 in autophagy induction following ROS induction is further supported by the greater overall ROS deregulations (Figure 8) from treatments yielding greater relative inductions of Nrf1 p120 (Figure 11) and p95 (Figure 14) active forms in TUN and DTT compared to THP where the ROS trend was negative (Figure 8). Since Nrf1 is significantly induced at the 24 hour time point of ER stresses, this strongly suggests it is the primary ER stress regulator compared to Nrf2 (Figure 8 and 10).

4.2 Nrf1 is distinctly regulated by ER stress compared to Nrf2

The significant ROS rises (Figure 6) are in agreement with Nrf2 trends albeit these are not significant for TUN THP and AA at n2 sample size (Figure 8); Nrf2 bands were not detected in most blots where Nrf1 was, despite similar half-lives of the proteins (Bugno et al. 2015). On the other hand, Nrf2 was significantly and strongly induced in response to high oxidative stress with DTT treatment at n2 (Figure 8) suggesting that antibodies are not to blame for lack of Nrf2 trends in 24 hour treatments with UPR drugs (see Appendix B). Nrf1 and Nrf2 were strongly induced by DTT (Figure 8 and 11), a chemical which likely auto-oxidized its thiols (Halleck et al. 1997) concurrently with cell death (Figures 4 and 6). This highly suggests the inability of Nrf1 and Nrf2 to mitigate redox stress in DTT treatments and thus serves as a positive control for the proteins.

Song et al. (2014) *in vitro* luciferase and qPCR data suggested that Nrf2 functions in acute or strong ROS stress while Nrf1 functions in chronic or weaker ER stress. Significantly decreased H_2DCFDA fluorescence is indicative of reductive stress and evidence that HDCF is not auto-oxidized (Zhang et al. 2012) in THP treatment. Nrf2 appears to decline concurrently with significant induction of p120 and p95- Nrf1 in THP treatment (Figure 8 and 10) suggesting that Nrf1 may also function to counter Nrf2 in response to prolonged stress; trends at 24 hours may be

in fact showing Nrf2 declining. Too much GSH, whether from prolonged Nrf2 signaling or not, is known to deregulate mitochondria redox coupling, oxidation, toxicity and death (Zhang et al. 2012; Wakabayashi et al. 2003; Kannan et al. 2013).

To test this hypothesis, a time course was performed using UPR drugs, and in fact, Nrf1 and Nrf2 appeared to be co-regulated, peaking at 4-6 hours post-treatments (Figure 15) in agreement with BiP time-courses from Shinjo et al. (2013) and biphasic activation response of Nrf1 to proteasomal inhibition (Zhang et al. 2015). Furthermore, Nrf2 seemed more pronounced in THP compared to DTT stress, but still declined to control levels at 18 hours.

Therefore it seems that autophagy and GSH homeostasis counter one another directly, in part through the actions of Nrf1 and Nrf2 and their organelle-distinct redox-response. When autophagy increases in response to ROS-crosstalk, ROS diminishes substantially, possibly through a decline in lipid peroxidation and radical propagation. When the levels reach a reductive threshold, Nrf2 declines in a ROS damage-dependent manner. This sort of signaling could possibly enhance the range of redox signaling in eukaryotes during hypoxic development. Nrf ARE promoter cross-talk may occur for fine-tuning this redox mechanism within development to regulate differentiation.

Wang et al. (2007) show p65-Nrf1 displaces Nrf2 mediated induction of GCLC and NQO1 following electrophilic stress *in vitr*o, in association with cell death in Hepa1c1c7 cells. While we do not measure significant differences in p65 levels among treatments, we do observe nonetheless a lack of change in p65 especially with cell death and pronounced viability changes in DTT treatments (Figures 5, 6 and 13). Since p65 is also regulated by internal translation sites, it is possible that lack of translation may affect Nrf1/ Nrf2 crosstalk specifically during cell death, when translation is most inhibited in ER stress. This mechanism would potentiate Nrf1 and Nrf2

signaling when radical propagation becomes too damaging, as seen with highest doses of DTT treatment (Figure 4, 5, 8 and 11). Together the data support a general ER stress/ Ca^{2+} leak/protein folding mechanism in the control of the degree of topological repartitioning of Nrf1 from the ER lumen into the cytosol for cleavage regulation and bidirectional feedback on protein folding (Zhang et al. 2015) in contrast to Nrf2.

The redox environment that triggers UPR is believed to regulate the differentiation of osteoblasts as they migrate from a hypoxic niche in the bone marrow via the bloodstream (Ufer et al. 2010; Jang et al. 2007). UPR sensors with bZIP domains are known to mediate specialized, lineage-dependent responses (Asada et al. 2011). Quite a few studies are pointing to Nrf1 regulation of bone differentiation, which is mediated by ER stress and antioxidants (Chai et al. 2015, Inoue et al. 2014; Kim et al. 2010; Xing et al. 2007; Narayanan et al. 2004).

Nrf1 is activated under hypoxia (Chepelev et al. 2011) which was associated with the PERK-ATF4 pathway HeLa cells (Blais et al. 2004). ER stress activates the PERK-eIF2α-ATF4 signaling pathway followed by the promotion of gene expression essential for osteogenesis (Saito et al. 2011). Furthermore, Nrf1 was shown to bind to ATF4 in a yeast-two hybrid screen while ATF4-deficient mice, like LCR-F1 mutant mice, are severely anemic during fetal development due to an impairment in definitive hematopoiesis (Chan et al. 1998; Masuoka and Townes, 2002). Therefore it remains to be determined if ER stress induction of Nrf1 involves ATF4.

Nrf2 can induce ATF4 (Afonyushkin et al. 2010), also potentially downstream of PERK activation (Blais et al. 2004). It remains to be determined how p65 can regulate Nrf1 and Nrf2 crosstalk, and whether Nrf1 is involved in a Nrf2 negative feedback loop involving Nrf1 p65; i.e. to diminish GSH induction under hypoxia-mediated reductive stress during the development of bone cells.

While proteotoxicity is already known to promote Nrf1 stabilization (Sha et al. 2014), TUN and THP were previously reported not to induce Nrf1 nuclear localization nor transcription (Zhang et al. 2009; 2014a). A key problem with Zhang et al. 2009 paper may be that they normalized their reference gene expression to 18S proteasome expression, which is now a target of Nrf1 and is therefore no longer a valid reference gene (Bustin et al. 2009), whereas the latter paper used a different cell line in their study, MCF10A cancer cells. Different cell types have different metabolic profiles, stress activations and cell death regulations. This is even evidenced for example, with the different Nrf1/ Nrf2 ratios among cell types (McMahon et al. 2001).

This study looks at the relative contribution of Nrf1 and Nrf2 to ER stressors in order to provide a more complete picture of ARE gene regulation, keeping in mind the opposing roles of Nrfs in metabolism. We provided evidence for Nrf1 nuclear localization in response to ER stressors concurrently with BiP induction. Finally, the most compelling evidence for Nrf1 regulation of distinctly ER-redox stress comes from Nrf1's-distinct regulation of the proteasome, which in itself functions to regulate ROS homeostasis and consequently, liver steatosis and cancer *in vivo* (Lee et al. 2013; Xu et al. 2005). Together, we propose Nrf1 functions as an ER redox-stress response factor while Nrf2 mediates mitochondrial ROS production.

The Nrf transcription factors are associated with many metabolic diseases including diabetes, cancer and aging. Since Nrf1 is activated via deglycosylation under low glucose conditions, it would be most insightful to see if Nrf1 glycosylation status or post-translational modifications play a role in autophagy initiation following nutrient deprivation, as a means for cells to obtain energy. The use of organelle-targeted roGFP probes can give insight into the redox status of the organelles, and determine how organelle ROS crosstalk plays a role in diseases including diabetes and inflammation. It would also be good to screen for activating proteases

which can play roles in bone development; although caspase 3 regulates bone differentiation, it was reported not to activate Nrf1 (Chen et al. 2003). Lastly, a future application for Nrf1-mediated autophagy control can be in tissue-repair, since Nrf1 has recently been shown to modulate oxidative stress response and regeneration after trauma to skeletal muscles (Zhang et al. 2013).

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APPENDIX A: BUFFERS, CHEMICALS AND SOLUTIONS

Ponceau S stain: 0.1% Ponceau S (w/v); 5.0% acetic Acid.

Resolving buffer: 3g/L Trizma base; 14.4g/L glycine; 1g SDS.

Transfer buffer: 18% methanol; 5.27 g/L Trizma base; 2.63 g/L glycine; 0.91g SDS.

Stripping buffer:

TBST, pH 7.6: Tris-HCl 20mM, NaCl 137mM, 0.1% Tween 20.

TBST-MILK: 5% Carnation Skim Milk (local grocery store) in TBST.

Phosphate-Buffered Saline, pH 7.4 (PBS): NaCl 137 mM; KCl 2.7mM; Na_2HPO_4 10mM; KH_2PO_4 1.8mM.

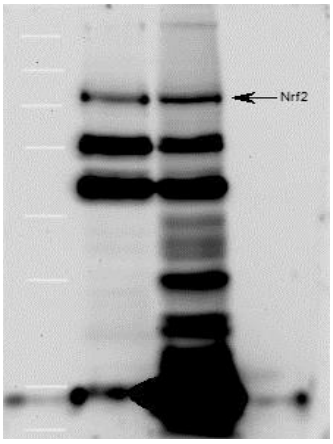
(DTT) Dithiothreitol (Sigma, D9779, stock 500mM) in DMSO

(THP) Thapsigargin (Sigma, T9033, stock 3000nM) in DMSO

(TUN) Tunicamycin (Sigma, T7765, stock 5mg/mL) in DMSO

APPENDIX B: Nrf2 ANTIBODY VALIDATION WESTERN BLOT

Nrf2+ is overexpressed Nrf2 (arrow), compared to wildtype (WT) HEK293T lysate. Ladder (left lane), All-Blue (BioRad, 1610373).



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