MECHANISMS CONTROLLING THE KEAP1-NRF2 SIGNALING PATHWAY IN LUNG CANCER

Bridgid Elizabeth Hast

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell and Molecular Physiology.

Chapel Hill
2013

Approved by:

Michael Ben Major, PhD

Dale Ramsden, PhD

Doug Cyr, PhD

D. Neil Hayes, MD, MPH

Patrick Brennwald, PhD

ABSTRACT

BRIDGID ELIZABETH HAST: Mechanisms controlling the KEAP1-NRF2 signaling pathway in lung cancer
(Under the direction of Michael Benjamin Major)

The ability to effectively regulate intracellular reactive oxygen species is imperative to prevent conditions of oxidative stress, and ultimately aberrant cell death. The primary means by which cells control reactive species is through the KEAP1-NRF2 signaling pathway. NRF2 is a transcription factor that is constitutively degraded by the proteasome in a KEAP1-dependent manner, where KEAP1 acts as the E3 ligase substrate adaptor for the E3 ligase CUL3. The canonical mechanism for NRF2 activation states that when intracellular levels of reactive oxygen species rise, KEAP1 is inactivated, thus inhibiting degradation of NRF2. NRF2 then translocates to the nucleus where it drives transcription of several genes including reactive oxygen species-scavenging genes, drug efflux genes, and cell survival genes.

NRF2 interacts with KEAP1 via two amino acid motifs, the ETGE and DLG, which position NRF2 in a sterically favorable position for ubiquitination. An emerging alternative mechanism for activation of NRF2, referred to as the Competitive Binding Model, proposes that NRF2 activation occurs when KEAP1 interacting proteins containing an amino acid sequence similar to the ETGE motif of NRF2 compete with NRF2 for binding to KEAP1. We have identified several interacting proteins that bind to

KEAP1 in an ETGE-dependent manner, including the dipeptidase DPP3. Identification of these interacting proteins not only validate the Competitive Binding Model, but also introduce DPP3 as a protein relevant for NRF2 activation in cancer.

In addition to competitive binding, somatic mutations in KEAP1 have been shown to activate NRF2. Unlike activating mutations in NRF2, which cluster to the ETGE and DLG motifs, mutations in KEAP1 are present throughout the entirety of the protein. How these somatic mutations affect KEAP1 function is currently not known. We have characterized 18 mutations in KEAP1 derived from The Cancer Genome Atlas lung squamous cell carcinoma cohort. In addition to determining that the majority of KEAP1 mutations are hypomorphic with respect to suppression of NRF2 transcriptional activity, we also identify a novel class of KEAP1 mutations that bind NRF2 and facilitate the ubiquitination, but not the degradation of NRF2.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Redox	Oxidation-reduction
ALS	Amyotrophic lateral sclerosis
ARE	Antioxidant response element
BTB	Bric-a-brac, tramtrack domain
CO	Carbon monoxide
CO_2	Carbon dioxide
CYP	Cytochrome P450 enzyme
DME	Drug metabolizing enzyme
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
H2O2	Hydrogen peroxide
IVR	Intervening region
LDL	Low density lipoprotein
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOX	NADPH oxidase enzyme
·O2-	Superoxide
·OH-	Hydroxyl radical
ONOO-	Peroxynitrite
PIN	Protein Interaction Network
PPP	Pentose phosphate pathway
RLS	Reactive lipid species

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SPR	Surface Plasmon Resonance
TCA cycle	Tricarboxylic acid cycle
W.blot	Western blot

I. CHAPTER ONE: INTRODUCTION

1.A Oxidative Stress and Cellular Coping Mechanisms

Cells are continuously exposed to a host of stressors and toxicants capable of causing intracellular damage. Originating from several sources, such as mitochondrial respiration and environmental exposure, nearly all of these compounds have the potential to participate in oxidation-reduction (redox) reactions. The reactive moieties of these compounds modify intracellular molecules to produce unstable intermediates, which act in a cascade of redox reactions, and when in excess culminate in global cellular damage. Consequently, the ability to maintain redox balance, as well as effectively mitigate harmful redox cascades is essential for cellular fitness and survival.

Uncontrolled oxidative damage resulting from redox reaction cascades produces an intracellular state called oxidative stress. Oxidative stress is characterized by an acute spike in reactive oxygen (ROS) and reactive nitrogen (RNS) species, damage to macromolecules, including DNA adducts, fatty acid oxidation, and protein oxidation and adduct formation [1-2]. This process drives the upregulation of ROS scavenging genes, global cellular stress response genes, detoxifying and drug efflux enzymes, as well as drug metabolizing enzymes. If these cellular responses to oxidative stress are able to quickly alleviate the acute spike in ROS, processes including DNA damage repair and

protein degradation are executed to promote cell survival; otherwise, cellular death pathways such as necrosis and apoptosis will occur [3-4].

1.A.1 Reactive oxygen and reactive nitrogen species

The most common reactive oxygen and reactive nitrogen species include hydrogen peroxide (H_2O_2), superoxide ($\bullet O_2^-$), hydroxyl radical ($\bullet OH^-$), nitric oxide (NO), peroxynitrite ($ONOO^-$), and reactive lipid species (RLS) [4-5]. All of these species are short lived, and they are all capable of reacting non-specifically and rapidly with DNA, lipids, carbohydrates, and proteins [6]. Superoxide is commonly referred to as the primary reactive oxygen species, because it gives rise to other forms of ROS [6], and is directly synthesized by complex I and complex II of the mitochondrial respiration chain [5-7]. Due to the electronegativity of oxygen, superoxide is one of the most stable reactive oxygen species, and can even act as both an oxidant and reductant [6]. Hydrogen peroxide is less stable than superoxide, which makes it both a more potent and more reactive species. The enhanced reactivity of hydrogen peroxide enables the modification of enzyme active sites that include residues susceptible to oxidation, like cysteine and methionine, to alter enzymatic function [8]. Finally, the most reactive—and most short-lived—reactive oxygen molecule is the hydroxyl radical. The high reactivity of this molecule makes for the most non-specific modifications of biological macromolecules, such as lipid oxidation [6]. The hierarchy of reactivity, as well as the molecules modified, is similar with reactive nitrogen species.

Endogenously, most RNS and ROS are produced by reactions in the endoplasmic reticulum, mitochondria, and peroxisomes. Reactive oxygen species, however, are not just produced as byproducts of other enzymatic reactions. Enzymes such as NADPH oxidases (*NOX*) produce superoxides as their sole enzymatic product, and these superoxides are then utilized in a multitude of downstream cellular functions, including proliferation, migration, membrane receptor signaling, and signaling within the extracellular matrix [6, 9]. Additionally, a number of different cell types produce ROS as a means to execute non-autonomous cellular signaling cascades. For example, phagocytes and activated astrocytes utilize ROS to initiate inflammatory responses to microbial invasion and neuronal injury, respectively [6].

In addition to ROS and RNS produced by endogenous pathways, such as in organelles and by NOX enzymes, exposure to environmental toxicants can also contribute to rising intracellular levels of reactive species. Reactive moieties on small molecules and environmental toxicants are capable of undergoing redox reactions with intracellular ROS, such as superoxide, often at the cost of producing a more reactive species like hydroxyl radicals and hydrogen peroxide [1, 10]. Additionally, environmental toxicants can react directly with biological molecules, including forming adducts with DNA and proteins. The various means by which ROS, RNS, and small molecules can participate in intracellular redox reactions underscores the importance of cells maintaining the ability to quickly and efficiently mitigate acute spikes in ROS that may lead to situations of oxidative stress.

1.A.2 NRF2-dependent mechanisms to control oxidative stress

Buffering the intracellular levels of reactive species requires multiple levels of regulation, including enzymes capable of neutralizing endogenously produced RNS and ROS, and exogenously introduced environmental toxins. Detoxifying, or drug metabolizing (DME) enzymes are used to neutralize reactive species, and are divided into two general classes—phase I DMEs, and phase II DMEs. Phase I DMEs include the very large and diverse family of cytochrome P450 genes. These enzymes are receptor mediated, and binding of small molecules to receptors based on structural and biochemical characteristics activates expression of specific phase I enzymes [2]. While these enzymes are capable of modifying small molecules, often via a redox reaction that enhances hydrophilicity to promote excretion via the urine or bile [11], they are primarily used as the first step in modifying a compound for further metabolism by phase II enzymes or other stress response proteins [2].

Phase II enzymes, also known as conjugating enzymes, act to increase hydrophilicity and enhance secretion of toxic compounds. However, whereas phase I DMEs tend to be expressed in response to a more ubiquitous range of stimuli and with limited substrate specificity, phase II DMEs exhibit greater substrate specificity and more direct pathways for induction. Many phase II DMEs have an antioxidant response element (ARE) sequence in the promoter region of the gene [1-2]. In response to oxidative stress, the transcription factor nuclear factor (erythroid-derived)-like 2, *NFE2L2 (NRF2)* binds to the promoter ARE to drive transcription of phase II DMEs [1, 12]. NRF2-dependent phase II enzymes can be further classified into four main

categories: glutathione homeostasis, drug metabolism, stress response and iron metabolism, and drug efflux [13]. The activity of the enzymes contained within some of these categories can be altered by global metabolic changes, such as glutathione homeostasis via pentose phosphate pathway (PPP) flux; however, it should be noted that NRF2 is ultimately required for the gene expression changes that contribute to alterations in flux [13-14]. Regulation of NRF2 will be discussed in a later section, and a brief overview of some NRF2-dependent phase II drug metabolizing enzymes follows.

Glutathione, also known as γ–glutamyl-cysteinyl glycine (GSH), is the primary cytosolic molecule responsible for ROS scavenging [13, 15]. It is synthesized in a two-step reaction involving first glutamate-cysteine ligase *(GCL)*, followed by glutathione synthetase *(GS)*. Ligation of glutamate to cysteine, which is catalyzed by GCL, is the rate-limiting step of glutathione synthesis, and this enzyme is required to maintain adequate intracellular levels of GSH [15-16]. Through a reaction catalyzed by glutathione S-transferase *(GST)*, GSH becomes conjugated to electrophilic molecules to enhance secretion of the modified molecule. Intracellular levels of glutathione must be replenished to maintain a high ratio of reduced (GSH) to oxidized (GSSG) glutathione, which is in part accomplished by the activity of glutathione peroxidase *(GPX)* and glutathione reductase *(GSR)* [15]. Genes involved in GSH synthesis and turnover, including *GCL* and to a lesser extent *GS*, *GPX*, and *GSR*, are regulated by NRF2 transcriptional activity [13]. In addition to regulating the transcription of gluthathione synthesis genes, recent studies suggest that NRF2 also regulates the expression of several pentose phosphate pathway genes [14]. Increased flux through the PPP increases levels of intracellular GSH, as well

as the ribose-5-phosphate, which plays a critical role in purine synthesis. Pentose phosphate pathway flux and intermediates will be discussed in the section on NRF2 function in cancer.

Glutathione homeostasis acts as a ubiquitous regulatory process to control intracellular ROS levels, whereas the other functions of NRF2-dependent phase II DMEs are more specific. NAD(P)H quinone oxidoreductase I (NQO1) is one of the drug metabolizing phase II enzymes that acts to neutralize reactive endogenous and exogenous quinones. Quinones, whether produced via an endogenous reaction, such as the oxidation of certain hormones [13, 17], or present in the chemical structure of an environmental toxin, can participate in reactions that produce superoxide and hydrogen peroxide [8, 12]. A redox reaction wherein NQO1 transfers two electrons to reduce the quinone inhibits formation of superoxide or hydrogen peroxide. Another NRF2-dependent drug metabolizing enzyme is UDP-glucuronosyltransferase *(UGT).* Unlike NQO1, which participates in a redox reaction, UGT conjugates glucuronic acid to reactive moieties of small molecules to inhibit production of ROS and/or adduct formation with DNA and proteins [12].

Like the drug metabolizing enzymes *NQO1* and *UGT*, *ferritin* and heme oxygenase-1 *(HMOX1)* are induced by NRF2-dependent transcription and participate in the iron metabolism-mediated stress response. Ferritin is an iron binding protein that sequesters free iron, which would otherwise produce hydroxyl radicals via redox reactions, in the cytosol [12]. In addition to NRF2-mediated transcription, the expression

of *ferritin* is also positively regulated by the presence of free iron. A portion of the free iron that regulates ferritin expression is formed in the primary reaction catalyzed by HMOX1. Using the cytochrome c and the heme biological precursor Fe-protoporphyrin-IX as a substrate, HMOX1 catalyzes an oxidative cleavage reaction that produces biliverdin, carbon monoxide, and free iron [18]. The free iron goes on to contribute to enhance *ferritin* expression, and the biliverdin is rapidly converted to bilirubin, which also acts an intracellular antioxidant [18-19].

Finally, genes regulating drug efflux are also targets of the NRF2-ARE response pathway. Acquired resistance to chemotherapeutics is a complex process that represents a major clinical hurdle in the treatment of cancer, and drug efflux is thought to be a primary contributing factor [13, 20]. The membrane-bound family of drug efflux genes consists of multidrug resistance proteins *(MDR)* and multidrug resistance-associated proteins *(MRP)*, and act in an ATP-dependent manner to transport small molecules out of the cell [21-22]. While MDR and MRP enzymes also contribute to clearing natural products from cells, members of this gene family are overexpressed in several cancers, including lung, ovarian, and breast cancer [21], and this expression promotes efflux of chemotherapeutics. In accordance with this functional role, in lung cancer moderate to high expression of *MRP* correlates with poor prognosis [23-24]. Knockdown of members of this gene family results in increased sensitivity to chemotherapeutics, further underscoring their importance in chemotherapeutic resistance [25]. Intriguingly, the function of one MRP protein, *MRP1*, requires the presence of GSH to transport unmodified chemotherapeutics through biological membranes [26], suggesting that the

many facets of NRF2-mediated regulation of oxidative stress can may act collectively to enhance the overall fitness of cancer cells.

1.B KEAP1 and NRF2: Structure, Regulation, and Mechanism

Since the early 1950s, it has been known that small quantities of certain xenobiotic agents were capable of reducing the incidence of cancer in rats fed large quantities of carcinogens [27-28]. Nearly forty years elapsed before it was discovered that these compounds mediate this protective effect by inducing phase II DMEs [27, 29-30], and that expression of these phase II enzymes was regulated by antioxidant response element (ARE) sequences in the promoters of the genes [27, 31]. In 1996 a screen to identify transcription factors that bind ARE sequences lead to the discovery of *NRF2* [32]; shortly thereafter, Kelch-like ECH-associated protein 1 *(KEAP1)* was found to be a negative regulator of NRF2 activity [33]. While the discovery of *NRF2*, *KEAP1*, and their role in regulating oxidative stress took over fifty years to appreciate, the field has since made great strides in identifying not only how critical KEAP1-NRF2 signaling is for inducing cell stress responses, but also the importance this signaling pathway holds in multiple diseases.

1.B.1 NRF2 domains and structure

NRF2 belongs to a family of cap'n'collar transcription factors, which share a basic leucine zipper homology. In addition to NRF2, the family contains five other members—nuclear factor (erythroid-derived) 2 (*NF-E2)*, nuclear factor (erythroid-derived) like 1 (*NRF1)*, nuclear factor (erythroid-derived) like 3 (*NRF3)*, and two more

distantly related members, BTB and CNC homology 1, basic leucine zipper transcription factor 1 (*BACH1)* and BTB and CNC homology 1, basic leucine zipper transcription factor 2 (*BACH2)* [27]. *NF-E2* and *NRF3* exhibit expression specific to erythroid cells and the placenta, respectively, while both *NRF1* and *NRF2* are ubiquitously expressed [34-35]. The knockout mouse for *Nrf1* is embryonic lethal [34], and while *Nrf2* knockout mice are viable and survive well into adulthood [35], they exhibit subtle phenotypes that will be discussed later.

The NRF2 protein is made up of six domains, Neh1-6, with each mediating different functional aspects of NRF2 (Fig. 1.1.A). The Neh1 domain is responsible for DNA binding, as well as regulating binding to interacting partners, namely small Maf proteins, on chromatin [36]. Domains Neh4 and Neh5 are the transactivation domains, and Neh3 recruits the helicase coactivator CHD6, making it indispensible for *NRF2* function [36-37]. A functional role for the Neh6 domain is currently not known. Finally, the Neh2 domain contains seven lysine residues (K44, K50, K52, K53, K56, K64, K68), which are targeted for ubiquitination in a KEAP1-dependent manner [38]. In addition to the seven lysine residues, the Neh2 domain also contains two critical regulatory motifs—the ETGE and DLG—which are essential for the recognition and binding of NRF2 to KEAP1 [39].

1.B.2 KEAP1 domains and structure

The discovery of *KEAP1* was made using a yeast two-hybrid screen identifying proteins that would bind to the Neh2 domain of NRF2 [33, 40]. Following its discovery

as the suppressor of *NRF2*, it was noted that KEAP1 is very cysteine-rich protein, containing 27 cysteines within the 624 residue protein. Due to the inherent reactivity of the thiol group of cysteines, it was proposed that one or more of the cysteine residues within KEAP1 act as sensors of oxidative stress, thus mediating NRF2 function [41]. Modification of these residues by ROS and xenobiotic compounds do, in fact, modulate KEAP1 activity, making these cysteines function as intracellular biosensors [30, 38, 40-43].

The KEAP1 protein consists of three domains: the N-terminal BTB (broad complex, tramtrack, and bric-a-brac), an intervening region (IVR), and the C-terminal KELCH domain (Fig. 1.1.B). KEAP1 is thought to act intracellularly as a quaternary homodimer structure [44]. The BTB domain (residues 1-179) is responsible for this homodimerization, and is also the domain that interacts with the E3 ligase cullin-3 (CUL3). The KELCH domain (residues 315-624) is the substrate-binding domain, and forms a six-bladed beta propeller structure, the center of which forms a substrate-binding pocket [27, 45]. Additionally, the KELCH domain is in part responsible for the largely cytosolic localization of KEAP1, as interaction between the actin cytoskeleton and KEAP1 occurs via this domain [46-47]. Finally, the intervening region (residues 180-314), which is indispensible for KEAP1 function [38], contains several of the reactive cysteine residues previously described [38, 41-43]. Single particle electron microscopy and structural modeling confirm biochemical interaction data for each domain of KEAP1, as well as demonstrating that KEAP1 does, indeed, form a forked-stem homodimer (Fig. 1.1.C) [48].

Crystal structure analysis of the KELCH domain of KEAP1 indicates that NRF2, in particular the N-terminal Neh2 domain, binds in the pocket of the KELCH domain [45]. Endogenous interaction between KEAP1 and NRF2 is thought to occur in a 2:1 KEAP1:NRF2 ratio, where the two regulatory ETGE and DLG sequences within NRF2 each bind a KEAP1 molecule to span the KEAP1 homodimer [39, 45, 48-49] (Fig. 1.1.C). The residues within the KEAP1 KELCH domain that interface with the ETGE motif of NRF2 are: R483, S508, R415, S363, R380, N382, Q530, S555, S602, and Y334 [50]. The three arginine residues are referred to as an “arginine triad”, which facilitates NRF2 binding via electrostatic interactions with the aspartic and glutamic residues within the NRF2 regulatory sequences [51]. Binding within the pocket of the KELCH domain via the ETGE positions NRF2 in a sterically favorable position for ubiquitination of the seven lysine residues that mediate degradation [52].

The cysteine reactivity of KEAP1 provides an elegant mechanism for KEAP1 to respond to both oxidative and xenobiotic cellular stimuli. Although cysteine residues are located throughout the entirety of the KEAP1 protein, several independent studies have shown that many of the key cysteines required for modulation of KEAP1-NRF2 function lie within the IVR [38, 41-43]. These residues include C257, C273, C299, C297, and C151 (Fig. 1.1.B). Interestingly, C151 is also required for a post-translational modification of KEAP1, which results in the formation of an SDS-resistant dimer [38, 53]. Given the cysteine enrichment of KEAP1, it was proposed that these residues act as sensors of electrophilic and xenobiotic insult, and that each cysteine residue may have differential reactivity depending on the insult. Consistent with this hypothesis, it was

determined that C151 was primarily reactive with electrophilic compounds, whereas C273 and C288 exhibited more reactivity with heavy metal and alkenal insult, respectively [54]. Furthermore, structural analysis of the amino acid microenvironment surrounding these residues revealed that C151, C273, and C288 are surrounded primarily by strongly basic amino acids. Consequently, the pKa of the thiol group is lowered, making those cysteines more reactive to specific small electrophilic molecules. Further studies are required to elucidate the contribution of the other putatively reactive cysteines to the stress-sensing abilities of KEAP1, as well as whether disease-derived mutations in *KEAP1* may alter cysteine reactivity to impact KEAP1 function.

1.B.3 E3 ligase-mediated degradation of NRF2

E3 ligases exert an effect at the level of protein degradation; consequently, regulation of proteins via an E3 ligase enables a faster cellular response than a transcriptionally mediated process. In situations of oxidative stress, regulation of stress response proteins via an E3 ligase-based mechanism is ideal, because it allows cells to mitigate acute spikes in ROS quickly to avoid execution of the apoptotic cascade. Under homeostatic conditions, NRF2 has a half-life of less than fifteen minutes [55]; however, upon ROS or xenobiotic stimuli, NRF2 is rapidly stabilized to facilitate NRF2-mediation transcription of target genes. Another slower pathway for NRF2 exists in the nucleus [27, 56-57], presumably to facilitate turnover of transcriptionally active NRF2.

Regulation of proteins via the ubiquitin-proteasome system is a multi-step process that begins with a ubiquitin activating enzyme (E1), which forms a thioester bond with

the small protein ubiquitin. The E1 then transfers ubiquitin to a ubiquitin conjugating enzyme (E2), at which point the ubiquitin moiety is activated. Following the E1 to E2 transfer of ubiquitin, a ubiquitin ligase (E3) enzyme then accepts the activated ubiquitin from the E2 to transfer it to lysine residues within the targeted substrate [27, 58]. While specificity of the ubiquitin-proteasome system enzymes occurs at the E3 ligase, these enzymes may require the substrate to be scaffolded with other proteins to provide a sterically favorable environment for ubiquitination [58]. These scaffolding proteins are typically referred to as E3 ligase adaptor proteins. As previously stated, KEAP1 acts as the E3 ligase adaptor subunit in the CUL3 ubiquitin ligase complex.

KEAP1 was identified as the adaptor subunit for CUL3 based on the observation that several BTB-containing proteins were substrate adaptor proteins for the CUL3 E3 ligase complex [33]. Further studies identified the IVR region of KEAP1 as facilitating the KEAP1-CUL3 interaction in addition to the BTB domain [59]. Initially it was predicted that regulation of NRF2 degradation occurred through a direct mechanism in which modification of KEAP1 via ROS or electrophilic agents caused the dissociation of the E3 ligase complex. Indeed, the addition of pathway agonists, such as *tert*-butylhydroquinone (tBHQ) and sulforaphane, demonstrate that these compounds inhibit degradation of NRF2. Site-directed mutagenesis in which cysteine 273 and 288 were mutated to serine indicates that these residues are required for NRF2 suppression under basal cell culture conditions only. Unexpectedly, however, when C151 was mutated to a serine, unlike C273 or C288 NRF2 degradation occurred regardless of the presence of agonist [27, 38, 40, 59]. Additionally, it was also observed that treatment of cells with

pathway agonists did not result in dissociation of NRF2 from KEAP1 despite an intracellular stabilization of NRF2 [27, 42, 60]. Collectively, these results indicate that the interaction between KEAP1 and NRF2, as well as post-translational inactivation of KEAP1 via reactive cysteine residues is more dynamic than a simplistic complex dissociation model of regulation.

1.B.4 KEAP1 regulation of NRF2: The hinge and latch mechanism

The regulatory DLG and ETGE sequences within NRF2 are two evolutionarily conserved sequences that reside within the Neh2 domain, and comprise residues 29-31 and 79-82 of the NRF2 primary protein sequence, respectively [39, 49, 52] (Fig. 1.1.A). While both sequences are required for NRF2 to be degraded in a KEAP1- and CUL3-dependent manner, it is thought that the ETGE motif is required for interaction with KEAP1 [50], whereas the DLG is required for NRF2 ubiquitination and degradation, but not KEAP1 interaction [52, 61-62]. The seven lysine residues that are ubiquitinated by CUL3 reside upstream of the ETGE motif [27, 40] on an alpha helix that spans residues 39-71 [40]. With the exception of the secondary alpha helix structure, the Neh2 domain of NRF2 is intrinsically unstructured and flexible [52], which likely facilitates the binding dynamics that mediate KEAP1 regulation of NRF2.

Mutation of the residues within the DLG and ETGE motifs indicate that the acidic aspartic and glutamic acid residues of the DLG and ETGE, respectively, mediate electrostatic interactions with the KELCH domain of KEAP1 [49]. Furthermore, isothermal calorimetry shows that the binding affinities of these two sequences differ

dramatically: the binding constant of the DLG motif is approximately $1.0X10^{6}M^{-1}$, whereas the binding constant of the ETGE is $1.9X10^{8}M^{-1}$ [49, 52]. Structural studies using both nuclear magnetic resonance (NMR) and X-ray crystallography also demonstrate that while the KEAP1 homodimer is structurally capable of binding two molecules of NRF2, it is more likely that the binding ratio is 2:1 for KEAP1:NRF2 [52]. In addition to the observed electrostatic potentials and affinities of the ETGE and DLG sequences in mediating KEAP1 binding, the notion that conformational changes in KEAP1 may also mediate regulation of NRF2 lead to the proposal of the "hinge-and-latch" mechanism for NRF2 activation and degradation.

The basis of the hinge-and-latch mechanism is the discrepancy in binding constants of the DLG and ETGE sequences. The ETGE motif is required for binding to KEAP1, and has a smaller binding constant, making it act as the "hinge" in the model. While the DLG is required for NRF2 stability and degradation, the lower affinity of the DLG for the binding pocket of KEAP1 allows for dynamic binding and disassociation from the other KEAP1 KELCH domain, making this sequence the "latch". When both the DLG and ETGE sequences are bound to KEAP1, meaning the latch is closed, the alpha helix containing the ubiquitinated lysines is in a steric conformation that favors ubiquitination and subsequent degradation of NRF2 (Fig. 1.2, top). However, when cysteine residues within KEAP1 become oxidized by either ROS or electrophilic species, a conformational change in the KEAP1 homodimer occurs (Fig. 1.2, bottom left) [38-39, 42-43, 52, 54]. Consequently, the lower affinity DLG sequence dissociates from the structure, rendering NRF2 unable to be ubiquitinated due to steric constraints. Newly

translated NRF2, which would otherwise be degraded in a KEAP1-dependent manner, is then available to translocate to the nucleus to drive transcription of target genes. This model, also referred to as the “saturation model” because intracellular levels of KEAP1 become saturated in an inactive complex with NRF2, predicts that turnover of the inactivated KEAP1-NRF2 complex will occur until excessive ROS or xenobiotic agents are cleared [27]. A combination of the hinge-and-latch and saturation models also support the previously discussed observation that treatment of cells with tBHQ and sulforaphane do not disrupt the KEAP1-NRF2 interaction, despite resulting in enhanced NRF2 transcriptional activity and stabilization.

The cysteine residues within KEAP1 act as the biological sensors of the intracellular redox state. A hinge-and-latch model of regulation, however, also helps define another function of KEAP1—acting as a molecular switch following oxidative stress sensing. The saturation model coupled with the hinge-and-latch mechanism predicts that inactive KEAP1-NRF2 complex will be turned over via the proteasome. Once a period of oxidative stress passes, newly synthesized KEAP1 will bind and repress free NRF2. Consequently, signaling through KEAP1 is ultimately self-limiting, and follows a sinusoidal pattern. More specifically, KEAP1 senses oxidative stress via oxidation and modification of cysteine residues to “switch on” *NRF2* signaling. When reactive species are no longer present in excess, unmodified KEAP1 “switches off” NRF2 signaling by reinitiating KEAP1-dependent NRF2 degradation. Dysregulation of this pattern of signaling is implicated in many diseases, and will be discussed in a later section.

The hinge-and-latch model of KEAP1-NRF2 signaling enables a rapid and effective response to oxidative stress; however, the protein interaction dynamics of this model are also the basis of an emerging alternative means of regulation, referred to as the competitive binding model. The competitive binding model suggests that proteins containing motifs and sequences with affinities for KEAP1 or NRF2 can activate NRF2 independently of oxidative or xenobiotic stress by disrupting binding of the DLG "latch" to KEAP1 (Fig. 1.2, bottom right). The first indication of the competitive binding model came with the observation that the mitochondrial protein phosphoglycerate mutase family member *5 (PGAM5)* was not only a KEAP1 binding partner, but also that this interaction was mediated via an ESGE sequence in PGAM5 [63-64]. Under homeostatic conditions KEAP1 targeted PGAM5 for proteasome-mediated degradation. Interestingly, however, both sulforaphane and quinone-induced oxidative stress inhibited KEAP1-mediated PGAM5 degradation [64]. Additionally, siRNA-mediated knockdown of *PGAM5* was found to activate NRF2 in a manner similar to knockdown of KEAP1 [63-64], suggesting that the PGAM5-KEAP1 interaction may be more significant than merely targeting PGAM5 for degradation.

It was later determined that the while PGAM5 was degraded in a KEAP1-dependent manner, the KEAP1-PGAM5 interaction also served to sequester a pool of intracellular KEAP1 at the mitochondrial membrane [64]. Furthermore, NRF2 was part of this complex, indicating that a ternary complex of KEAP1, PGAM5, and NRF2 was localized to the mitochondria. As mitochondria are one of the main intracellular producers of ROS, it was hypothesized that the KEAP1-PGAM5-NRF2 ternary complex

facilitated ROS sensing at the mitochondrial membrane [64]. In addition to a pool of KEAP1 that localizes to the mitochondria with NRF2, cytosolic puncta containing KEAP1 are also present intracellularly, suggesting that multiple pools of KEAP1 are primed to respond to different oxidative and xenobiotic stimuli.

While PGAM5 does not activate NRF2 via a competitive binding mechanism—in fact, it facilitates its degradation under homeostatic conditions—PGAM5 set the precedent for protein interactions with KEAP1 or NRF2 to alter pathway activity independently of oxidative stress. The first protein to be identified to activate NRF2 independently of oxidative stress was the cyclin kinase inhibitor, p21 *(p21/WAF1/CIP1)* [65], although it should be noted that p21 expression is increased in response to oxidative stress [66-69]. p21 function is linked to multiple cellular processes, including DNA damage, cell cycle regulation, apoptosis, and even oxidative stress [67, 70-72]. The p21 protein contains a KRR domain, which is biochemically similar to the arginine triad (K380, R415, and R483), and mediates interaction of the acidic residues of the DLG and ETGE of NRF2 with the KELCH domain of KEAP1 [65]. Consequently, both the DLG and ETGE exhibit binding to the KRR sequence of p21, although endogenous interaction with the DLG appears to be more significant. Overexpression of p21 results in activation of NRF2, because it facilitates dissociation of the DLG from the KELCH domain.

Currently, proteins that bind to KEAP1 in a competitive manner with NRF2 are more common than those that directly bind to *NRF2*, and several have been identified. The first was p62/sequestosome-1 (*p62/SQSTM*), an autophagy-related protein, which

binds to the arginine triad via a KIR motif (DPSTGE) in the KELCH domain in a manner similar to the acidic ETGE and DLG sequences in NRF2 [73]. Like p21, this interaction facilitates dissociation of the DLG of NRF2 from KEAP1, thus promoting NRF2 transcriptional activity. In addition to p62, several other proteins have been found to bind the KELCH domain of KEAP1, via an ETGE or ESGE motif [74-76]. Importantly, accumulation of these ETGE-containing proteins predicts that the self-limiting mechanism of KEAP1-NRF2 signaling will be perturbed, resulting in constitutive NRF2 activation. Intriguingly, we found one of these proteins, DPP3, activates NRF2 signaling via the competitive binding model [74], and that expression of this protein correlates with a NRF2 gene signature in squamous cell lung cancer [74, 77]. Proteins such as DPP3 underscore the need to further characterize how protein-protein interactions modulate KEAP1-NRF2 activity in disease. Additionally, we have identified other proteins that interact with KEAP1 in an ETGE- or ESGE-dependent manner (Fig. 2.1.B) [74], although the majority of these proteins do not appear to activate or suppress NRF2 mediated transcription (Fig. 2.2.F). However, we cannot rule out the possibility that these proteins function similarly to PGAM5 to sequester KEAP1 and/or the KEAP1-NRF2 complex via competitive binding. Furthermore, these alternative ETGE-containing proteins may function in regulating NRF2-independent functions of KEAP1.

1.B.5 Alternative substrates of KEAP1

In addition to identifying novel protein interactions that alter KEAP1-NRF2 signaling, some KEAP1 interacting proteins have been found to be alternative substrates. The first substrate of KEAP1 to be found outside of NRF2 was IκB kinase *(IKKβ)*. IKKβ

targets IκBα, the inhibitory protein of nuclear factor κB *(NFκB)*, for degradation [78]. Similar to regulation of NRF2, NFκB is a transcription factor that is constitutively degraded, but NFκB is degraded in an IκBα-dependent manner. Signaling through NFκB is largely considered to be tumorigenic because of the myriad of downstream targets of NFκB related to cell growth, proliferation, and survival [78-82]. When IKKβ is targeted for degradation in a KEAP1-dependent manner, IκBα is stabilized, thus keeping intracellular levels of NFκB low. Consequently, if *KEAP1* expression is lost, or if KEAP1 function is compromised, IκBα is targeted for degradation in an IKKβ-dependent manner, and *NFκB* signaling is activated.

Two other substrates of KEAP1 are the mitochondrial pro-survival proteins B-cell CLL/lymphoma 2 (*BCL-2*) and BCL-2 like 1 (*BCL-xL*) [83-85]. When the apoptotic cascade is executed, the pro-apoptotic protein BCL-2-associated X protein (*BAX*) translocates to the mitochondrial membrane, forming pores that allow the release of cytochrome c [86]. Both BCL-2 and BCL-xL exert their anti-apoptotic effects by complexing with and sequestering BAX, to inhibit mitochondrial pore formation [83-84]. Under homeostatic conditions KEAP1 degrades BCL-2 and BCL-xL, which promotes the dissociation of BCL-2-BAX and BCL-xL-BAX complexes. This dissociation increases the levels of free intracellular BAX, which promotes apoptosis. Degradation of both BCL-2 and BCL-xL depends on the PGAM5-KEAP1 interaction to tether KEAP1 to the mitochondrial membrane [63]. Likewise, xenobiotics that adduct to KEAP1 antagonize the KEAP1-BCL-xL and KEAP1-BCL-2 interaction, stabilizing both anti-apoptotic proteins [63, 83-84], which may play a role in cancer cell survival.

Whether through activation of NRF2 via the competitive binding model, or through modulation of alternative KEAP1 substrates, it is clear that protein interactions play a critical role in the importance of KEAP1-NRF2 signaling. Further studies are required to identify new proteins that act as either competitive binding activators or novel KEAP1 substrates. These proteins, like those documented above, may hold clinical significance for the role of KEAP1-NRF2 in disease.

1.C The Role of KEAP1 and NRF2 in Disease

Maintaining an appropriate balance between the activity of KEAP1 and NRF2 is of paramount importance for homeostasis within an organism. Signaling via the KEAP1-NRF2 pathway is often referred to as a "double edged sword", because both hyperactivity and hypoactivity of the pathway is a contributing factor to several disease pathologies. Diseases in which KEAP1-NRF2 dysregulation occurs share common ground in that reactive oxygen and nitrogen species cannot be properly regulated, with most diseases skewing toward conditions of oxidative stress. A notable exception is cancer, which will be reviewed at the end of the section. Perhaps unsurprisingly, much of the observed oxidative stress can be linked to mitochondrial malfunction and dysregulation of other endogenous ROS- and RNS-producing enzymes. More recently, however, KEAP1-NRF2 signaling has been found to respond to a growing list of noncanonical sources of oxidative stress, including aberrant proteomic and autophagic regulation, inflammatory responses, and necrosis and apoptosis. Collectively, these observations suggest that the extent to which the KEAP1-NRF2 pathway is utilized by organisms to control oxidative stress and prevent disease is only beginning to be fully appreciated.

1.C.1 KEAP1 and NRF2 knockout mouse phenotypes

The *Keap1* knockout mouse exhibits a post-natal lethal phenotype caused by hyperkeratosis of the upper gastrointestinal system, which leads to obstructive lesions and ultimately malnutrition [1, 87]. In mice containing a floxed *Keap1* allele, reduced expression of *Keap1* was observed, particularly in heterozygous *Keap1* $^{lox/-}$ animals [88]. These knockdown *Keap1* mice are viable, and display increased nuclear Nrf2 accumulation and *Nqo1* expression. Additionally, they exhibit hyperkeratosis of the esophagus and upper stomach; however, neither the *Nrf2* accumulation nor esophageal hyperkeratosis is as exacerbated as in *Keap1* knockout mice. No other gross phenotypes are noted in *Keap1* knockout or knockdown mice. Interestingly, the *Keap1* knockout phenotype can be completely reversed by crossing animals with *Nrf2* deficient animals [1, 87].

Unlike *Keap1* knockout mice, *Nrf2* null animals are viable and display no gross phenotype associated with loss of *Nrf2* expression [35]. Phenotypes associated with *Nrf2* knockdown are typically only observed when mice are exposed to stressors. Accordingly, *Nrf2* knockout mice are more susceptible to hepatotoxicity [89-90], DNA adduct formation [91], cigarette smoke-induced emphysema [92-93], pulmonary fibrosis [94], and lung injury [92, 94-95]. Only a relatively mild inflammatory phenotype is noted in *Nrf2* knockout mice in the absence of insult. Knockout animals are more likely than wild type littermates to develop spontaneous chronic inflammatory disorders including glomerulonephritis, immune-related hemolytic anemia, and multi-organ autoimmune inflammation [1, 96-97].

1.C.2 Finding an ideal balance of KEAP1 and NRF2

One of the hallmarks of diseases associated with perturbations in KEAP1-NRF2 signaling is aberrant cell death, such as in neurodegenerative disorders, or lack thereof, as in cancer. Intracellular levels of ROS are one metric used to determine whether a cell executes the apoptotic cascade. If ROS levels overcome endogenous ROS-scavenging mechanisms, such as glutathione, apoptosis occurs; however, cell stress response and survival pathways may be executed if ROS levels are not prohibitively high. Under circumstances where an acute spike in ROS initiates the apoptotic cascade, late apoptotic stage accumulation of ROS can result in necrotic death of surrounding cells [98]. These cell death processes contribute to the pathologies associated with neurodegenerative disorders, as well as is cancers exhibiting intratumoral necrosis.

As depicted in Figure 1.3, disease prevention offered by KEAP1-NRF2 signaling is optimal when neither KEAP1 nor NRF2 is present in excess. For example, in a NRF2-null situation, ROS levels will be elevated due to decreased expression of ROS-scavenging and pro-survival NRF2-target genes, enhancing the risk of ROS-induced apoptosis and necrosis. Under these conditions, an organism is more susceptible to neurodegenerative disorders and some cardiovascular diseases. Conversely, in a KEAP1-null setting, NRF2-target genes will be present in excess, producing an intracellular environment that is particularly adept at neutralizing ROS and clearing xenobiotics and other toxicants. Consequently, cells are at a greater risk to “miss” ROS-dependent apoptotic cues, resulting in enhanced susceptibility to cancer. Interestingly, augmented NRF2 activity, particularly activation through intermittent exposure to antioxidants and

chemopreventive agents, has been correlated with reduced incidence of cancer [99]. While this observation seemingly confounds reports that NRF2 is overactive in many cancers, these conflicting ideas may be reconciled by understanding the importance of temporal regulation and context of NRF2 activation.

1.C.3 Neurodegenerative disorders

Neurodegenerative diseases, such Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS), share many pathological features, including protein aggregation from proteasomal and autophagic dysregulation, inflammation, and mitochondrial dysfunction [100]. Most of the pathological hallmarks of neurodegeneration either contribute to, or result from oxidative stress. For example, high levels of ROS can result in the formation of oxidized proteins, which exhibit reduced folding and produce intracellular aggregates. Additionally, deficiencies in autophagic processes result in damaged mitochondria that accumulate because of poor autophagosome formation, which can become a source of toxic reactive oxygen species.

Given the host of *NRF2* target genes associated with ROS-scavenging, redox homeostasis, and cell survival, it is unsurprising that adequate NRF2 function has been implicated as having a role in the prevention of neurodegenerative disorders. Most evidence stems from observations in primary neuronal cultures from both wild-type and *Nrf2* deficient mice. First, it has been shown that glutathione metabolism via shunting through the pentose phosphate pathway in primary neurons contributes a cytosolic reducing environment that inhibits apoptosis [101]. Although flux through the PPP in

neurons has not yet been conclusively linked to NRF2, recently it was found that NRF2 directs metabolic reprogramming of the pentose phosphate pathway in cancer [14]. Modulation of metabolic pathways, including the pentose phosphate pathway, is a baseline defense to prevent acute spikes in ROS to hinder oxidative stress. Likewise, treatment of neurons with oxidative stressors and mitochondrial toxins that selectively inhibit components of the electron transport chain result in upregulation of NRF2 expression [102-105]. Collectively these data suggest NRF2 activity is a first-line defense for mitigating neuronal oxidative stress. Furthermore, *Nrf2* null neurons are more susceptible to models of oxidative stress than wild-type neurons [100, 102, 106]. Together these studies demonstrate that NRF2 plays a critical role in maintaining a proper neuronal redox environment, which may prevent intracellular events leading to enhanced susceptibility to neurodegenerative disease.

In addition to direct neuronal cell-autonomous functions for NRF2, the emerging importance of the interaction between glial cells and neurons in maintaining neuronal integrity also appears to rely on NRF2 function. Co-culture experiments in which *Nrf2*$^{-/-}$ neurons were grown with astrocytes from wild-type mice demonstrated that astrocytes expressing *Nrf2* have a protective effect on *Nrf2*$^{-/-}$ neurons exposed to oxidative stress [107]. Similarly, even astrocytes derived from an ALS mouse model were able to provide a protective effect in a co-culture model when NRF2 was activated [107-109], suggesting that *NRF2* may have potential as a therapeutic target in neurodegeneration. The mechanism of action behind this observation is thought to involve shunting of metabolites between astrocytes and neighboring neurons. Astrocytes secrete glutathione,

which is used by neurons to help maintain a high intracellular reducing environment [100, 105, 107]. Enhanced NRF2-dependent glutathione secretion from astrocytes to neurons would certainly contribute to the protective effects of astrocyte in co-culture experiments. However, recent studies in astrocytes also indicate that NRF2 activation causes global changes in astrocyte gene expression, supporting the idea that NRF2 has a role in glial cell biology that extends beyond glutathione secretion and metabolite shunting.

1.C.4 Cardiovascular disease

Like neurodegenerative disorders, diseases of the cardiovascular system have several commonalities, in particular mitochondrial deficiency and inflammation. The cardiovascular system is unique, however, in that it is an organ system that relies heavily on the production of reactive oxygen and reactive nitrogen species for homeostatic signaling. Consequently, regulation of NRF2 within this system must not only function to mitigate oxidative stress caused by toxicants and inflammatory processes, but it must also function in equilibrium with enzymes producing ROS and RNS to maintain physiological homeostasis.

Atherosclerosis is a thickening of the walls of arteries and vasculature most commonly associated with the abnormal accumulation of lipids in the endothelium. In addition to lipid accumulation, particularly accumulation of low-density lipoprotein (LDL), atherosclerosis is an inflammatory condition, and biological processes such as viral infection, physical damage, and toxicant exposure can lead to atherosclerotic

plaques [110-112]. The inflammatory response mounted against these stressors to the cardiovasculature is a major contributing factor to the oxidative stress associated with atherosclerosis [111], and it is for this reason that a significant role for NRF2 signaling in this disease has come to light.

Early accumulations of atherosclerotic plaques begin to alter blood flow within vessels from that of laminar (unidirectional) to oscillatory, or turbulent, flow [113-114]. The direction and force of blood flow through the vasculature stimulates the endothelium to produce nitric oxide, NO, via NADPH oxidases (*NOX* enzymes) in the cell membrane [6]. Protective NO release during laminar flow helps regulate blood pressure through vasodilation; however, when flow is oscillatory, the release of protective NO release is replaced by secretion of superoxides [115-117]. Production of superoxides from oscillatory flow results in the oxidation of LDL, which infiltrates the endothelium, and stimulates the migration of inflammatory cells to the site, further compounding oxidative stress [111-112]. Cellular coping mechanisms during turbulent blood flow depend on NRF2 in primarily two ways: first, NRF2 is upregulated in the endothelium of vasculature during shear stress [118], and second, macrophages that infiltrate sites of atherosclerotic plaques upregulate expression of NRF2 [119-121]. Upregulation of NRF2 in macrophages helps, in part, to maintain macrophage integrity in such a ROS-rich environment [110, 121]. The endothelium, however, also uses *NRF2* to combat atherosclerosis, primarily through the *NRF2* target gene *HMOX1* [122-126]. In addition to the production of previously described ROS-scavenging byproducts, expression of HMOX1 is associated with downregulation of matrix metalloproteinase 9 *(MMP9)* [127-

128]. MMP9 works to degrade collagens within the extracellular matrix, and is associated with plaque destabilization [110, 127-128]. Consequently, reduced expression of MMP9 in an HMOX1-dependent manner helps prevent disruption of plaque sites, which could lead to a stroke event. In addition to *HMOX1*, the other *NRF2* target genes *GCL* and *NQO1* are associated with protection against atherosclerosis [129-131]. Furthermore, low serum levels of glutathione and the NRF2 target gene *GPX* are considered risk factors for heart disease [129-130]. These data suggest that NRF2 not only plays a critical role in the cellular response to atherosclerosis, but also that NRF2 could be a therapeutic target for the prevention of atherosclerotic disease.

Like atherosclerosis, hypertension is associated with increased levels of oxidative stress, mostly through the dysregulation of NOX enzymes. Activation of some NOX enzymes, such as NOX1, results in the upregulation of NRF2, particularly during intermittent hypoxia [132-133]. This data is suggestive of a relationship wherein feedback regulation between NOX enzymes and NRF2 expression promote normal blood pressure while supplying ROS-scavenging mechanisms to reduce the potential for oxidative stress that may facilitate hypertension. More significantly, however, is the role that NRF2 plays in regulating blood pressure through expression of HMOX1. HMOX1 exhibits hypotensive effects, primarily through the production of carbon monoxide (CO) [134-136]. In addition to possessing vasodilatory effects that act independently of NO, carbon monoxide is a potent inhibitor of endothelin, a vasoconstrictor [110, 134-136]. The role of HMOX1 in maintaining normal blood pressure was confirmed in hypertensive rats, where HMOX1 activity correlated with reduced blood pressure [134].

1.C.5 Inflammation

Inflammation is a common pathology of multiple diseases, including neurodegenerative and cardiovascular disease previously discussed, and is undoubtedly a unifying factor in diseases associated with KEAP1-NRF2 dysregulation. There are two general types of inflammation: 1) acute inflammation, which is associated with innate immune processes and is typically a beneficial process, and 2) chronic inflammation, which is now widely accepted to predispose organisms to chronic illness, including cancer [137-138]. Inflammatory processes are characterized by the migration of a host of immune cells to sites of injury or stress, secretion of cytokines, chemokines, and metabolites, and importantly, oxidative stress. Much of the oxidative stress that helps define an immune response is due to a "respiratory burst" when mast cells and leukocytes release ROS upon migration to a site of damage [137-139]. In addition to having a physiological role during an immune response, including the destruction of invading microorganisms, the respiratory burst also serves as a signaling mechanism to recruit other immune cells to the site of inflammation.

Changes in signal transduction pathway activity are also a hallmark of inflammatory responses, and include alterations in many pathways, including *NFκB*, *STAT3*, *AP-1*, T cell activation, *TNF*, *TGFβ*, and *NRF2* signaling [137-141]. Importantly, alterations of signaling within these pathways are not cell autonomous; neighboring cells and tissues are also affected, which contributes to damage created during chronic inflammatory disorders. The host of inflammatory disorders that *Nrf2* knockout mice spontaneously develop further underscores the importance of KEAP1-NRF2 in

inflammation. These conditions include glomerulonephritis, immune-related hemolytic anemia, and multi-organ autoimmune disorders [140, 142-146].

Similar to the need to balance KEAP1 and NRF2 activity to achieve optimal disease prevention, balance between pro- and anti-inflammatory signals aid in ensuring acute inflammatory responses do not escalate to a chronic condition. This balance is achieved through the production of another set of cytokines and chemokines, which act as antagonists to their pro-inflammatory counterparts [137-139, 147]. Thus, homeostatic immune responses follow a cyclical pattern similar to KEAP1-NRF2 signaling: just as KEAP1 acts as a switch to turn off NRF2 signaling following activation, anti-inflammatory cytokines counteract pro-inflammatory signals to ensure an acute immune response remains self-limiting. When constitutive activation of some inflammatory pathways, such as NFκB, occurs, the balance between pro- and anti-inflammatory cytokine signaling skews to favor pro-inflammatory signals [138]. Several factors contribute to constitutive activation of pro-inflammatory pathways, many of which are outside the scope of this review; however, crosstalk between NFκB and KEAP1-NRF2 signaling is particularly noteworthy.

Functions of both KEAP1 and NRF2 act in the repression of NFκB activity. As previously stated, the E3 ligase activity of KEAP1 suppresses NFκB through the degradation of IKKβ, which targets the NFκB repressor IκBα for destruction [78, 85]. Consequently, when KEAP1 function is intact, NFκB activity is low. Similarly, NRF2 activity suppresses NFκB signaling through activation of ROS scavenging genes.

Reactive oxygen species activate NFκB signaling, therefore, when NRF2 activity is intact, both ROS levels and NFκB activity remain low [78-79, 81-82, 138, 143, 145]. When either KEAP1 or NRF2 function is compromised, as is frequently observed in cancer, the sinusoidal patterns of both KEAP1-NRF2 signaling and the inflammatory response are perturbed. In this situation, NRF2 and NFκB both exhibit constitutive activation, resulting prolonged NRF2 availability to mitigate ROS, while the downstream effects of NFκB signaling promote cell proliferation, growth, and survival.

1.D KEAP1 and NRF2 in Cancer

The role of KEAP1 and NRF2 in cancer is not only the most defined and studied subject regarding KEAP1-NRF2 redox biology, but seemingly also the most dynamic. Nearly every biological process that KEAP1 and NRF2 function in, including ROS scavenging, metabolism, and inflammation, can be linked to an attribute of cancer that affects survival, proliferation, or tumorigenicity. Additionally, multiple genetic alterations to *KEAP1* and *NRF2*, such as somatic mutation, copy number amplification, and epigenetic modification, are not only prevalent in cancer, but also often correlate with poor prognosis and survival. Finally, emerging evidence suggests that the cellular context in which KEAP1-NRF2 is deregulated is likely the key to understanding the "double-edged sword" nature of this pathway.

1.D.1 NRF2-mediated metabolic reprogramming in cancer

Since the mid-1950s it has been known that cancer cells display metabolic alterations that contribute to their growth and survival [27, 148]. This observation, most

commonly known as The Warburg Effect, has been continuously evolving since its discovery. Today it is appreciated that cancer metabolism, in particular mitochondrial metabolism, is reprogrammed to specifically meet the needs of macromolecular synthesis required by cancer cells [148]. Non-proliferating cells, such as terminally differentiated and quiescent cells, utilize glycolysis and the tricarboxylic acid (TCA) cycle to completely oxidize glucose to carbon dioxide (CO_2). Metabolizing glucose in this manner ensures that cells have an adequate amount of ATP to meet the requirements of cellular homeostasis.

One requirement of cells fully oxidizing glucose to CO_2, however, is a relatively insignificant need for macromolecular synthesis of molecules such as DNA, RNA, proteins, and lipids [149-151]. Although hydrolysis of ATP is sufficient for providing energy to catalyze multiple cellular reactions, it is not particularly useful in anabolic processes. Synthetic reactions require molecules containing reduced carbon and nitrogen, which is gained via the reductive potential of molecules such as NADPH. Increased levels of TCA cycle intermediates are also of paramount importance, as they act as common precursors for many macromolecules [148, 152]. Additionally, flux through the pentose phosphate pathway is the source of intermediate molecules for purine production used in synthesis of nucleic acid for DNA and RNA. The anabolic and energetic demands of cancer cells culminate in global metabolic reprogramming, in which mitochondria are used primarily as synthetic organelles [14, 148]. Much of the metabolic reprogramming is growth factor dependent, as growth factors act as liaisons between the availability of nutrients in the extracellular environments and downstream signaling events such as re-

entry into the cell cycle. Perhaps unsurprisingly, activating mutations in growth factor receptors common to many cancers contribute to the initiation of cancer-specific global metabolic changes. Recent findings, however, have elucidated a striking and important role for NRF2 in affecting cancer cell metabolic reprogramming.

The primary axis of where NRF2 functions in metabolic reprogramming is by driving transcription of multiple genes in the pentose phosphate pathway (Fig. 1.4). Enhanced flux through the PPP produces an excess of several molecules that are beneficial to cancer cell survival (Fig. 1.4) [14, 148]. As previously stated, glutathione-mediated ROS scavenging is required to maintain cytosolic redox homeostasis. In addition to *de novo* synthesis of glutathione, the regeneration of the reduced form (GSH) occurs through the catalytic activity of glucose-6-phosphate dehydrogenase *(G6PD)*, which produces the reducing equivalent NADPH as a byproduct. Expression of *G6PD* is also NRF2-dependent, suggesting that in addition to targeting expression of genes involved in glutathione synthesis, NRF2 modulates the availability of reduced glutathione through transcription of *G6PD*. NRF2 regulates the expression of three other enzymes that produce NADPH—malic enzyme 1 *(ME1)*, isocitrate dehydrogenase 1 *(IDH1)*, and phosphogluconate dehydrogenase *(PGD)* [14]. Both ME-1 and IDH1 act within the TCA cycle, creating pyruvate precursor for fatty acid synthesis, and α-ketoglutarate, respectively. Thus, in addition to increased levels of NADPH, NRF2-mediated expression of *ME1* and *IDH1* support anabolic processes via production of metabolic intermediates [151].

Phosphogluconate dehydrogenase is another PPP gene, and along with expression of two other NRF2 target genes, tansaldolase *(TALDO1)* and transketolase *(TKT),* critically enhance flux through the PPP [99]. Regeneration of GSH occurs in the early steps of this pathway; however, the final enzymatic reactions of the PPP culminate into the production of ribose-5-phosphate, a main precursor for nucleotide synthesis. Enhanced production of ribose-5-phosphate occurs via expression of phosphoribosyl pyrophosphate amidotransferase *(PPAT)* and methylenetetrahydrofolate dehydrogenase 2 *(MTHFD2)*. It should be noted that expression of these two genes correlates with expression of NRF2, but NRF2 is not found to be associated with the promoters of *PPAT* or *MTHFD2*, as it is with *G6PD, PGD, ME-1, IDH1, TKT,* and *TALDO1* [107]. Regardless, increased availability of nucleotide precursors, such as ribose-5-phosphate, allows proliferating cancer cells to meet the demands of rapid cell division through synthesis of purines and pyrimidines for DNA and RNA.

While the mechanistic downstream effects of NRF2-dependent metabolic reprogramming clearly augment cancer cell fitness, a cancer cell must first upregulate expression of NRF2. Genetic alterations in *KEAP1* or *NRF2*, which will be discussed in the next section, are the primary source of elevated levels of NRF2 in cancer; however, KEAP1- and NRF2-independent changes also impact expression. For example, mutational lesions and epigenetic modification in v-akt murine thymoma viral oncogene homolog 1 (*AKT)* and phosphatidylinositol-4,5-bisphosphate 3-kinase (*PIK3CA)* that alter signaling have been shown to upregulate *NRF2* expression (Fig. 1.4) [148, 153-154]. In addition to somatic mutations in both *AKT* and *PIK3CA*, these two proteins are also

downstream of many growth factor receptors. Activating mutations in growth factor receptors are one of the first steps toward cancer-specific metabolic reprogramming, as mentioned earlier [148-151, 153, 155-160]. Furthermore, it was recently discovered that activating mutations in *KRAS*, *BRAF*, and *MYC* are also capable of activating NRF2, and that this activation assists in ROS detoxification in murine cancer models [161-162]. Consequently, increase in NRF2 expression is likely to occur to some degree in most cancers irrespective of activating NRF2 or inactivating KEAP1 mutations due to amplified signaling through growth factors and their downstream effectors.

1.D.2 Genetic alterations in KEAP1 and NRF2 in cancer

Several genetic changes in *KEAP1* and *NRF2* have been noted in cancer (Fig. 1.5) [163-166], and more recently, it has been proposed that *KEAP1* and *NRF2* exhibit a tumor suppressor-oncogene relationship, respectively [167-168]. Much of the speculation surrounding this relationship is due to the distribution of somatic mutations throughout each gene. Mutations within *NRF2* cluster specifically within the DLG and ETGE motifs (Fig. 1.5.A), a pattern similar to clustered mutations seen in most oncogenes [21, 33, 163-164]. When mutations occur within the ETGE motif, NRF2 association with KEAP1 is disrupted, allowing NRF2 to evade degradation and accumulate in the nucleus to drive transcription of target genes. Mutations within the DLG also function to activate NRF2, but this activation occurs through a slightly different mechanism. NRF2 maintains an association with KEAP1 with mutations in the DLG; however, the KEAP1-NRF2 interaction in this case produces a protein complex in which KEAP1 is unable to degrade

NRF2. Consequently, newly synthesized NRF2 is able to accumulate without subsequent degradation, resulting in increased NRF2 transcriptional activity.

Somatic mutations in *KEAP1* are distributed throughout the entirety of the gene (Fig. 1.5.A and Table S3.1), similar to the distribution of mutations found in tumor suppressor genes [169]. While some mutations resulting in a truncated protein product have been observed, most mutations are missense. Studies have determined that missense mutations in *KEAP1* result in both hypomorphic and dominant negative phenotypes [170]. Perhaps more significant than functional outcomes of *KEAP1* mutations, however, is a recent observation suggesting that at least a 75% reduction in wild type *KEAP1* activity is required for stabilization of NRF2 [88]. This hypothesis becomes clinically relevant due to the observation that in cancer, specifically lung cancer, mutations in *KEAP1* are often heterozygous. Since KEAP1 acts as a homodimer, three permutations are possible when there is a heterozygous somatic mutation in *KEAP1*: a wild type homodimer, a wild type:mutant heterodimer, and a mutant:mutant homodimer. Both a wild type:mutant homodimer and mutant:mutant heterodimer will result in a hypomorphic or inactive KEAP1 complex, therefore a 75% reduction in KEAP1 activity is easily achieved, resulting in NRF2 stabilization. Interestingly, recent studies from The Cancer Genome Atlas (TCGA) Research Network determined that in squamous cell lung carcinoma, expression of KEAP1 alleles in not 50:50 (Table 1.1). While this finding does not alter the need for a 75% reduction in KEAP1 activity to stabilize NRF2, it underscores the importance that relative allelic expression in cancer may not be easily predicted. Accordingly, predicting whether somatic mutations in *KEAP1* will support

enhanced expression of *NRF2* will likely require further study to determine the mechanisms driving relative expression of *KEAP1* alleles, as well as the combined functional outcome of the mutation and relative allelic expression.

Epigenetic modifications, such as *KEAP1* promoter hypermethylation, also result in NRF2 stabilization (Fig. 1.5.B). Hypermethylation of the *KEAP1* promoter region is observed in several cancers, including lung, prostate, and colorectal cancers, as well as gliomas [165, 171-174]. Additionally, some cell lines exhibit *KEAP1* promoter hypermethylation, including the lung adenocarcinoma line A549 [173]. Alterations in promoter methylation result in reduced *KEAP1* expression, which allows for NRF2 stabilization. Copy number amplification of *NRF2* was also recently found in lung squamous cell adenocarcinoma [74, 77]. Copy number amplification correlated with an enhanced *NRF2* gene signature, suggesting that increased expression of *NRF2* is sufficient to overcome *KEAP1* suppression, even in a wild type *KEAP1* background [74].

1.D.3 Alternative mechanisms of NRF2 stabilization

Recent findings have elucidated novel pathways of KEAP1 inactivation and NRF2 stabilization that act independently of genetic alterations. One of these pathways involves post-translational modification of KEAP1 and appears to be cancer specific. The other pathway involves alternative KEAP1 and NRF2 interaction partners and the competitive binding mechanism mentioned during discussion of the hinge-and-latch mechanism of regulation. Although this second pathway is not necessarily engaged only

in situations of carcinogenesis, emerging evidence suggests that several cancers do take advantage of this mechanism of activation.

Modulation of TCA cycle activity for use in producing anabolic precursors frequently occurs in cancer; however, in addition to altered flux, mutations in several metabolic enzymes have been discovered to potentially play a role in cancer cell fitness. Most mutations in metabolic enzymes result in the buildup of oncometabolites, or metabolic intermediates that are found specifically in cancerous cells. One of these oncometabolites is fumarate, the endogenous substrate for fumarate hydratase *(FH)*. Some cancers, including papillary renal carcinoma, exhibit inactivating somatic mutations in *FH*, which result in a buildup of fumarate [175-176]. Fumarate possesses low electrophilic activity; however, when fumarate levels are excessive it modifies KEAP1 in a redox reaction that results in KEAP1 succinylation at reactive cysteine residues (Fig. 1.4 and 1.5.C) [177]. Intriguingly, HMOX1 activity is required for cell viability in FH deficient cells, and siRNA-mediated knockdown of *HMOX1* is sufficient to induce synthetic lethality [178]. Collectively these findings suggest that metabolite-mediated post-translational modification of KEAP1 enhances cancer cell fitness, and further, FH is now being examined as a therapeutic candidate because of this observed synthetic lethality phenotype. Several other oncometabolites have been identified, some of which do not yet have defined functions in the cell, such as the mutant IDH1 and IDH2 product 2-hydroxyglutarate [148, 151, 179]. Further studies to determine whether these oncometabolites may also create cysteine modifications of KEAP1 may yield novel therapeutic targets, such as in the case of fumarate hydratase.

In addition to post-translational modification of KEAP1, protein-protein interactions of KEAP1 and NRF2 have also been shown to indirectly activate NRF2 through a competitive binding mechanism (Fig. 1.5.E). Intriguingly, several of the proteins that are known to stabilize NRF2 through binding competition are overexpressed in neoplastic lesions and cancer, or in response to spikes in ROS. The autophagic protein p62/SQSTM, which binds to the KELCH domain of KEAP1 in a competitive manner with NRF2 [73], is overexpressed in hepatocellular carcinoma [180]. Moreover, overexpression of p62/SQSTM has also been observed in neoplastic prostate tissue, suggesting that NRF2 stabilization through competitive binding could facilitate progression to malignant neoplasia [181]. We have also shown that the dipeptidyl peptidase *DPP3* contributes to *NRF2* activation through competitive binding (Fig. 1.5.E). Furthermore, analysis of the TCGA squamous cell lung carcinoma dataset revealed that *DPP3* overexpression correlates with the NRF2 gene signature [74]. These data suggest that *DPP3* overexpression could contribute to NRF2 activation in lung cancer. In addition to lung squamous cell carcinoma, studies have shown that *DPP3* is also overexpressed in ovarian and endometrial carcinoma [182-183]. Importantly, enhanced NRF2 activity is also observed in epithelial ovarian carcinoma, even in the absence of *KEAP1* or *NRF2* mutation [184]. Further studies are required to determine if *DPP3* overexpression correlates with the observed NRF2 activity in ovarian cancer.

1.D.4 KEAP1-NRF2 pathway activity in cancer: the importance of timing and context

The KEAP1-NRF2 signaling pathway, more specifically activation of the pathway, is intriguing because of the importance that temporal regulation and the context

in which activation occurs appear to have in disease. Transcriptional activity of NRF2 is required for the global cellular health of an organism—it is the main defense against oxidative stress that would otherwise result in DNA damage, protein and lipid oxidation, and ultimately aberrant cell death. In diseases where reduced NRF2 activity is observed, such as cardiovascular disease and neurodegeneration, pathway agonists may hold the key to inhibiting disease progression, and even reversing tissue and cellular damage. Sustained NRF2 activation, as seen in chronic inflammation and cancer however, is now being seen as an indicator of disease progression and poor prognosis [94, 185-186]. Reconciling these seemingly confounding aspects of KEAP1-NRF2 signaling underscores the importance of understanding the context in which NRF2 activation occurs.

Any deviation from homeostasis elicits an intracellular response, typically in the form of signaling pathway modulation resulting in gene expression changes. Healthy cells constantly receive information from the microenvironment, meaning they are subject to both activating and inhibitory cues, thus enabling cell responses to stimuli to be self-limiting. When a cell sustains DNA damage that produces cancer-driving mutations, however, the cell may become cell-autonomous and no longer responsive to extracellular and intracellular signaling that regulate proliferation and survival. Thus constitutive activation of pathways that are otherwise beneficial in a non-transformed cell becomes the driving mechanism behind progression toward malignancy. This concept is perhaps best illustrated by the observation that chronic inflammation is a risk factor for cancer.

Acute inflammatory responses are absolutely required to maintain organismal homeostasis. Release of cytokines and migration of inflammatory cells to sites of damage ensure invading microorganisms are destroyed, terminally damaged cells undergo apoptosis and subsequent phagocytosis, and cells in acute stress initiate cytokine-dependent survival pathways. As release of reactive oxygen species is a hallmark of inflammation, it is of paramount importance that NRF2 is activated to prevent excessive ROS-mediated damage. While acute inflammatory responses are self-limiting due to anti-inflammatory cytokine release, misregulation of the pathway results in continued pro-inflammatory responses. The long-term presence of inflammatory cells results in increased levels of ROS and DNA damage, which contribute to neoplastic progression [185]. NRF2 pathway activity also remains constitutively high, and when coupled with mutations from excessive DNA damage creates a situation in which these now cell-autonomous neoplastic cells are acutely adept at coping with cellular stress.

The number of mutations found within a tumor type is highly variable and tissue specific. Mutation rates range from approximately 9 in some forms of leukemia and pediatric tumors, to more than 200 in melanoma and lung cancer [187-191]. Determining which of these mutations actually contribute to tumor progression, as opposed to those that do not impart any advantageous phenotype is one of the most important goals currently emerging in cancer research. Carcinogenesis is thought to begin with a “gate keeping” mutation, which provides a selective growth advantage to the cell. More mutations within that cell can occur, but only those that further facilitate growth and proliferation are considered “driver” mutations. All other mutations are labeled as

"passenger" mutations [189]. Importantly, NRF2 itself is not sufficient to induce carcinogenesis, and is even associated with chemoprevention [21, 164, 192-193]. Together these findings imply the need for at least one other independent gate keeping mutation in NRF2-dependent cancers. However, observations that some cancers and many cell lines appear to require NRF2 for viability certainly imply that *NRF2* is a driver mutation. Mutations in *KEAP1* that promote NRF2 activation are also likely to be driver mutations, and the fact that *KEAP1* and *NRF2* mutations are mutually exclusive supports this hypothesis [74, 77]. As previously stated, many of the KEAP1 mutations in cancer are hypomorphic with respect to suppression of NRF2. While these mutations likely contribute to NRF2 activity within a tumor, it is intriguing to think about NRF2-independent functions of KEAP1 with respect to KEAP1 mutations acting as driver mutations. It is feasible that hypomorphic KEAP1 mutations also promote tumorigenesis through inefficient degradation of other KEAP1 substrates, such as cell cycle components. The putative NRF2-independent functions of KEAP1 underscore the need to further elucidate the importance of *KEAP1* as a driver gene in cancer.

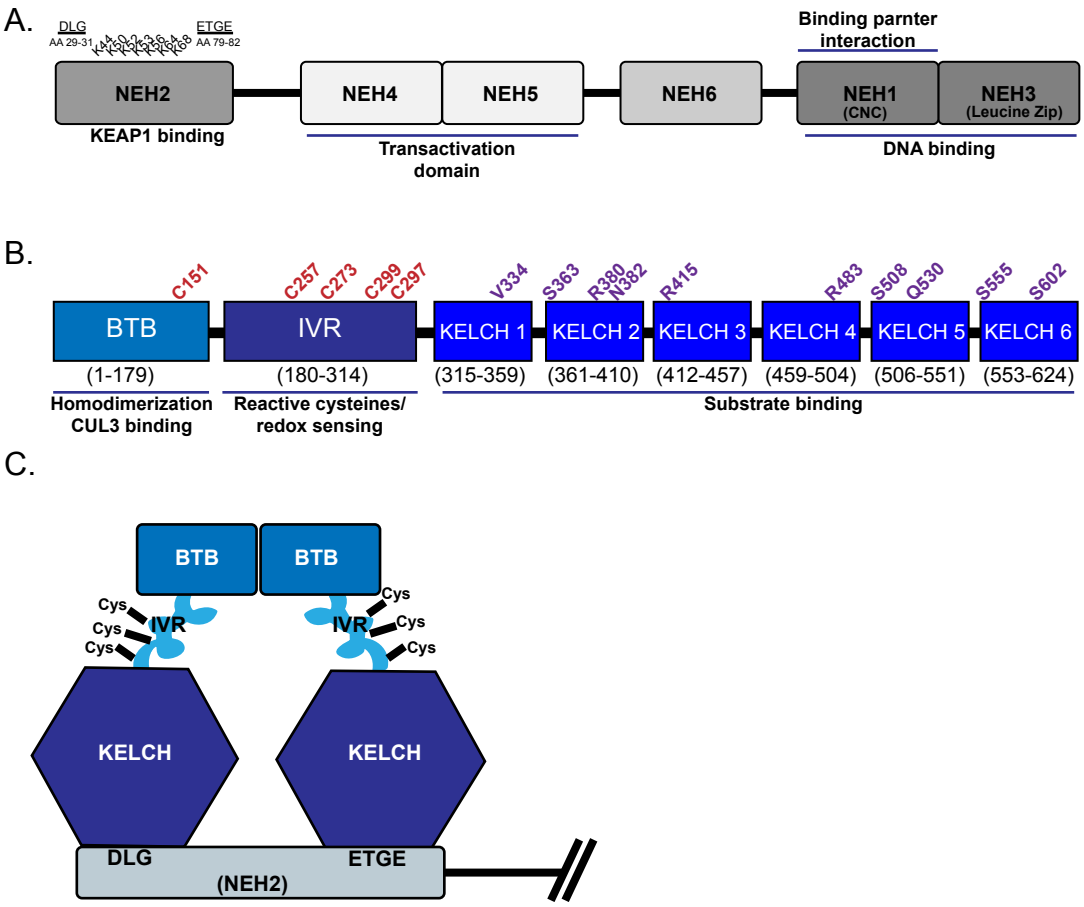

Figure 1.1. NRF2 is a transcription factor that binds to the E3 ubiquitin ligase adaptor KEAP1 via two regulatory motifs. (A) NRF2 has six Neh domains. The Neh2 binds to KEAP1 via the ETGE and DLG amino acid motifs; the seven lysines targeted for ubiquitination reside between the regulatory motifs. (B) KEAP1 has three main domains: BTB, IVR, and KELCH. The KELCH domain has six KELCH repeats. Reactive cysteines (red) and residues that interface with NRF2 (purple) are annotated above each domain. (C) KEAP1 forms a homodimer through binding of the BTB domain. The ETGE and DLG of one NRF2 molecule each bind a KELCH domain of the KEAP1 homodimer.

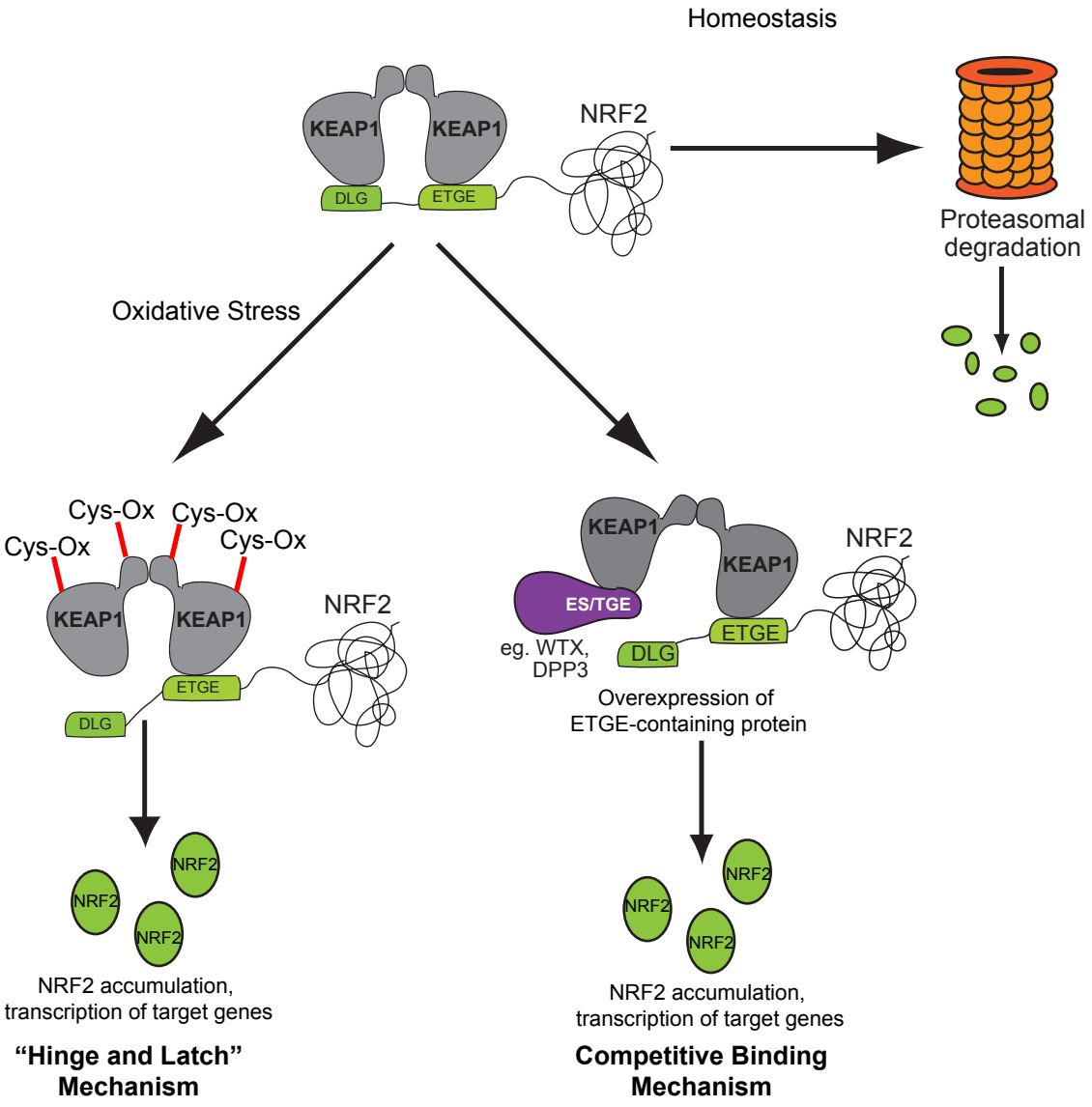

Figure 1.2. NRF2 is subject to proteasome-mediated degradation in a KEAP1-dependent manner under homeostatic conditions, but can be activated via two distinct mechanisms. Under homeostatic conditions NRF2 is ubiquitinated in a KEAP1-dependent manner, after which it is degraded via the proteasome (top). The "Hinge and Latch" mechanism occurs when oxidative stress modifies cysteines within KEAP1, causing dissociation of the DLG to allow NRF2 to evade degradation and drive transcription of target genes (left). The "Competitive Binding" mechanism proposes that proteins may interact with either KEAP1 or NRF2 in a manner that disrupts the KEAP1-NRF2 association. NRF2 is no longer degraded and can drive transcription of target genes (right).

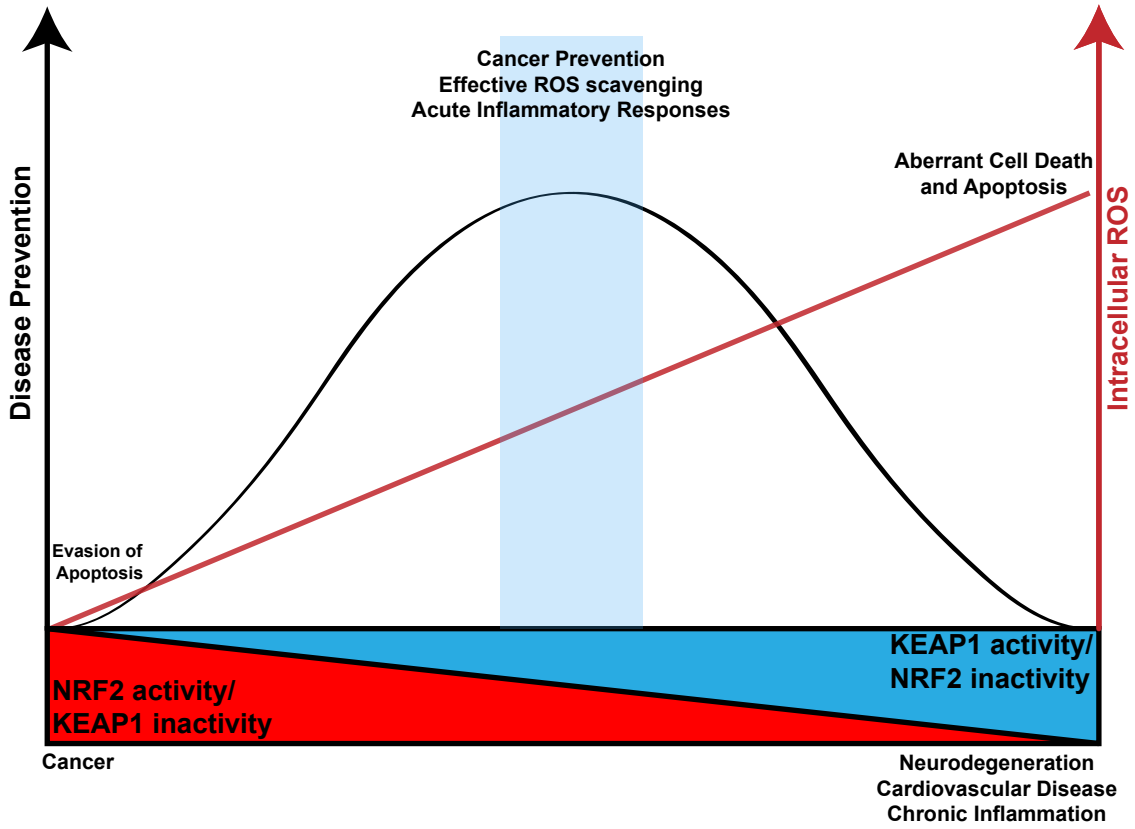

Figure 1.3. A balance of KEAP1 and NRF2 activity is optimal for disease prevention. Acute spikes in intracellular ROS occur in a NRF2-null setting, resulting in aberrant cell death and predisposing an organism to degenerative disorders, chronic inflammation, and cardiovascular disease (right). In situations of unregulated NRF2 activity, cellular stress coping mechanisms result in evasion of apoptosis and an increased risk of cancer (left).

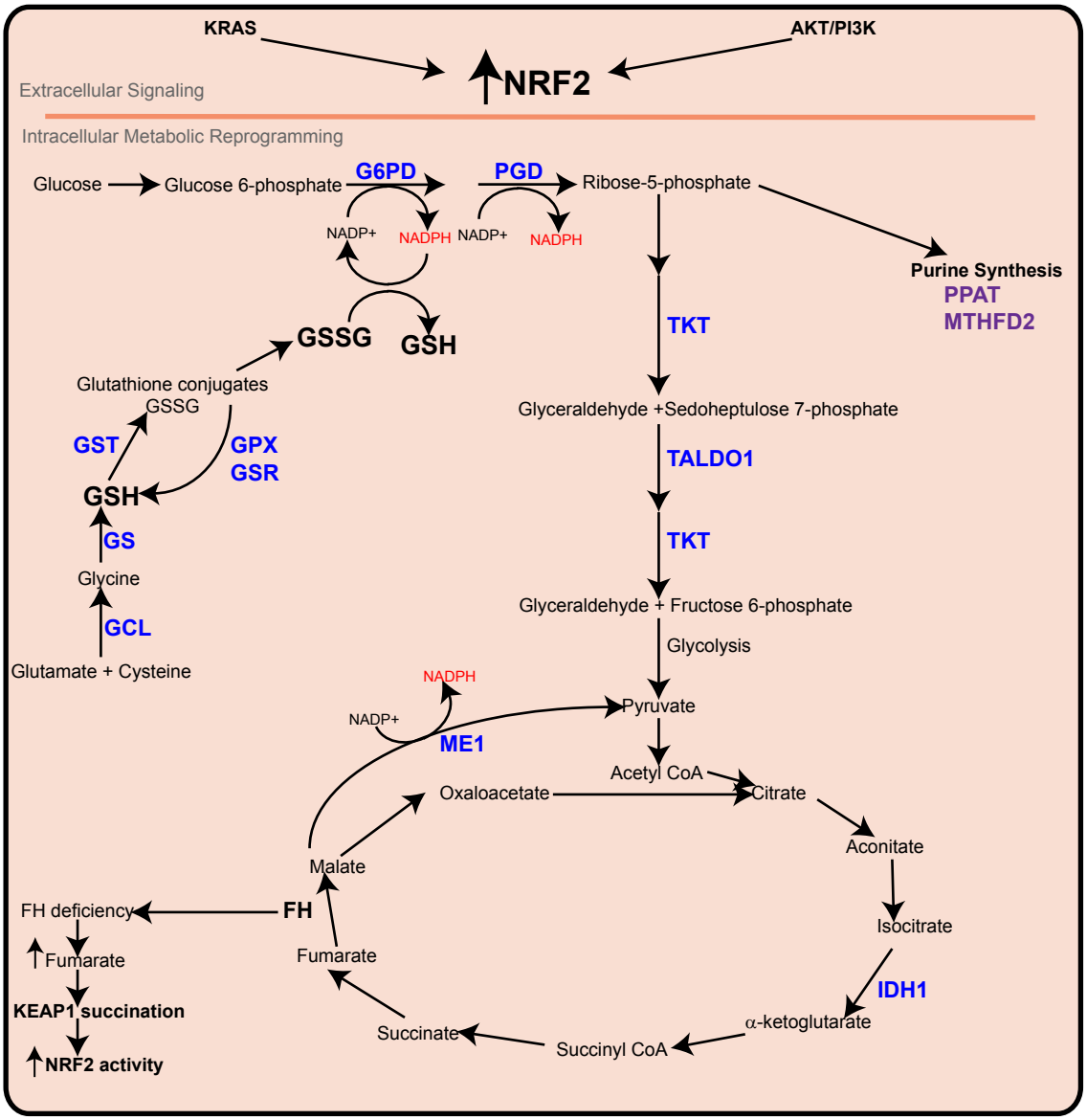

Figure 1.4. NRF2 activity can reprogram cellular metabolism to favor ROS-scavenging and anabolic processes. Enzymes in blue are directly regulated by NRF2-mediated transcription, enzymes in purple are indirectly activated by NRF2, and NADPH annotated in red can contribute to anabolic reactions.

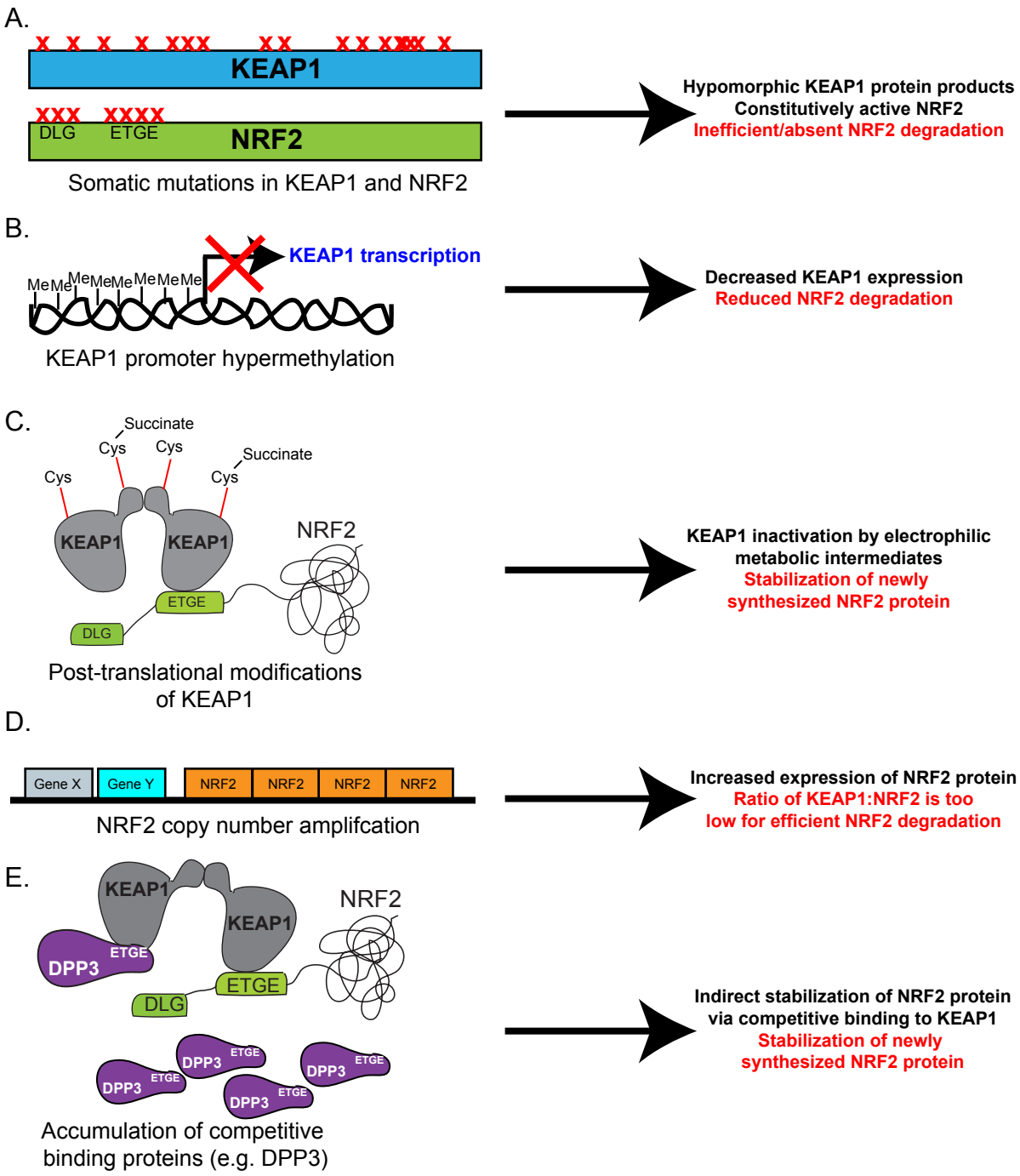

Figure 1.5. Constitutive NRF2 activation in cancer arises from several mechanisms. A) Somatic mutations in KEAP1 are distributed throughout the entire protein sequence, whereas mutations in NRF2 localize to the ETGE or DLG motifs. B) KEAP1 promoter hypermethylation reduces endogenous KEAP1 expression. C) Post-translational modification of KEAP1 by electrophilic byproducts of metabolic enzymes inactive KEAP1. D) Epigenetic changes in NRF2, including copy number amplification, increase intracellular levels of NRF2. E) Overexpression of KEAP1-interacting proteins indirectly activate NRF2 by binding to KEAP1 and competing off NRF2.

Mutation	Allelic Fraction
P318L	0.2
E493D	0.377778
R260Q	0.391753
D422N	0.16129
V155F	0.30303
L310P	0.373016
S243C	0.652174
G480W	0.88
R15L	0.036145
G423V	0.555556
V167F	0.411765
I506V	0.880952
Q75*	0.535211
V418L	0.3
R470C	0.35
G480W	0.653061
L231V	0.395062
W544C	0.891892
V369L	0.75
R470C	0.236364
R320Q	0.194175
S224Y	0.304878
N469fs	0.408602
V155F	0.337838

Table 1.1. Lung squamous cell carcinoma with KEAP1 mutations exhibit varied expression of the mutant allele. The allelic fraction is defined to be (t_alt_count)/(t_alt_count + t_ref_count), where t_alt_count is the read count for the mutant allele, and t_ref_count is the read count for the reference allele.

REFERENCES

1. Kensler, T.W., N. Wakabayashi, and S. Biswal, *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 89-116.

2. Rushmore, T.H. and A.N. Kong, *Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes.* Curr Drug Metab, 2002. **3**(5): p. 481-90.

3. Buttke, T.M. and P.A. Sandstrom, *Oxidative stress as a mediator of apoptosis.* Immunol Today, 1994. **15**(1): p. 7-10.

4. Yu, B.P., *Cellular defenses against damage from reactive oxygen species.* Physiol Rev, 1994. **74**(1): p. 139-62.

5. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. **552**(Pt 2): p. 335-44.

6. Lambeth, J.D., *NOX enzymes and the biology of reactive oxygen.* Nat Rev Immunol, 2004. **4**(3): p. 181-9.

7. Fleury, C., B. Mignotte, and J.L. Vayssiere, *Mitochondrial reactive oxygen species in cell death signaling.* Biochimie, 2002. **84**(2-3): p. 131-41.

8. Hancock, J.T., *The role of redox mechanisms in cell signalling.* Mol Biotechnol, 2009. **43**(2): p. 162-6.

9. Carnesecchi, S., J.C. Pache, and C. Barazzone-Argiroffo, *NOX enzymes: potential target for the treatment of acute lung injury.* Cell Mol Life Sci, 2012. **69**(14): p. 2373-85.

10. Ziech, D., et al., *The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development.* Chem Biol Interact, 2010. **188**(2): p. 334-9.

11. Glatt, H., *Sulfation and sulfotransferases 4: bioactivation of mutagens via sulfation.* FASEB J, 1997. **11**(5): p. 314-21.

12. Nguyen, T., P. Nioi, and C.B. Pickett, *The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress.* J Biol Chem, 2009. **284**(20): p. 13291-5.

13. Singh, S., et al., *Nrf2-ARE stress response mechanism: a control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases.* Free Radic Res, 2010. **44**(11): p. 1267-88.

14. Mitsuishi, Y., et al., *Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming.* Cancer Cell, 2012. **22**(1): p. 66-79.

15. Forman, H.J., H. Zhang, and A. Rinna, *Glutathione: overview of its protective roles, measurement, and biosynthesis.* Mol Aspects Med, 2009. **30**(1-2): p. 1-12.

16. Huang, Z.Z., et al., *Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration.* FASEB J, 2001. **15**(1): p. 19-21.

17. Li, Y. and A.K. Jaiswal, *Regulation of human NAD(P)H:quinone oxidoreductase gene. Role of AP1 binding site contained within human antioxidant response element.* J Biol Chem, 1993. **268**(28): p. 21454.

18. Orino, K., et al., *Ferritin and the response to oxidative stress.* Biochem J, 2001. **357**(Pt 1): p. 241-7.

19. McDonagh, A.F., *The biliverdin-bilirubin antioxidant cycle of cellular protection: Missing a wheel?* Free Radic Biol Med, 2010. **49**(5): p. 814-20.

20. Liu, F.S., *Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review.* Taiwan J Obstet Gynecol, 2009. **48**(3): p. 239-44.

21. Hayes, J.D., et al., *Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway.* Antioxid Redox Signal, 2010. **13**(11): p. 1713-48.

22. Chen, Z.S. and A.K. Tiwari, *Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases.* FEBS J, 2011. **278**(18): p. 3226-45.

23. Campa, D., et al., *A comprehensive study of polymorphisms in ABCB1, ABCC2 and ABCG2 and lung cancer chemotherapy response and prognosis.* Int J Cancer, 2012. **131**(12): p. 2920-8.

24. Merk, J., et al., *Chemoresistance in non-small-cell lung cancer: can multidrug resistance markers predict the response of xenograft lung cancer models to chemotherapy?* Eur J Cardiothorac Surg, 2011. **40**(1): p. e29-33.

25. Sanchez, C., et al., *Chemotherapy sensitivity recovery of prostate cancer cells by functional inhibition and knock down of multidrug resistance proteins.* Prostate, 2011. **71**(16): p. 1810-7.

26. Sau, A., et al., *Glutathione transferases and development of new principles to overcome drug resistance.* Arch Biochem Biophys, 2010. **500**(2): p. 116-22.

27. Zhang, D.D., *Mechanistic studies of the Nrf2-Keap1 signaling pathway.* Drug Metab Rev, 2006. **38**(4): p. 769-89.

28. Richardson, H.L., Cunningham, L., *The inhibitory action of methylcholanthrene on rats fed the azo dye 3'-methyl-4-dimethylaminobenzene.* Cancer Res, 1951. **11**(274).

29. Kensler, T.W., et al., *Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins.* Drug Metabol Drug Interact, 2000. **17**(1-4): p. 3-22.

30. Talalay, P. and J.W. Fahey, *Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism.* J Nutr, 2001. **131**(11 Suppl): p. 3027S-33S.

31. Jeyapaul, J. and A.K. Jaiswal, *Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene.* Biochem Pharmacol, 2000. **59**(11): p. 1433-9.

32. Venugopal, R. and A.K. Jaiswal, *Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene.* Proc Natl Acad Sci U S A, 1996. **93**(25): p. 14960-5.

33. Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes Dev, 1999. **13**(1): p. 76-86.

34. Chan, J.Y., et al., *Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice.* EMBO J, 1998. **17**(6): p. 1779-87.

35. Chan, K., et al., *NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13943-8.

36. Nioi, P., et al., *The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation.* Mol Cell Biol, 2005. **25**(24): p. 10895-906.

37. Katoh, Y., et al., *Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription.* Genes Cells, 2001. **6**(10): p. 857-68.

38. Zhang, D.D. and M. Hannink, *Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by*

chemopreventive agents and oxidative stress. Mol Cell Biol, 2003. **23**(22): p. 8137-51.

39. Tong, K.I., et al., *Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model.* Mol Cell Biol, 2006. **26**(8): p. 2887-900.

40. Zhang, D.D., et al., *Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex.* Mol Cell Biol, 2004. **24**(24): p. 10941-53.

41. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11908-13.

42. Eggler, A.L., et al., *Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2.* Proc Natl Acad Sci U S A, 2005. **102**(29): p. 10070-5.

43. Hong, F., et al., *Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation.* J Biol Chem, 2005. **280**(36): p. 31768-75.

44. Zipper, L.M. and R.T. Mulcahy, *The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm.* J Biol Chem, 2002. **277**(39): p. 36544-52.

45. Li, X., et al., *Crystal structure of the Kelch domain of human Keap1.* J Biol Chem, 2004. **279**(52): p. 54750-8.

46. Albagli, O., et al., *The BTB/POZ domain: a new protein-protein interaction motif common to DNA- and actin-binding proteins.* Cell Growth Differ, 1995. **6**(9): p. 1193-8.

47. Kaspar, J.W., S.K. Niture, and A.K. Jaiswal, *Nrf2:INrf2 (Keap1) signaling in oxidative stress.* Free Radic Biol Med, 2009. **47**(9): p. 1304-9.

48. Ogura, T., et al., *Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening, double glycine repeat, and C-terminal domains.* Proc Natl Acad Sci U S A, 2010. **107**(7): p. 2842-7.

49. Tong, K.I., et al., *Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response.* Mol Cell Biol, 2007. **27**(21): p. 7511-21.

50. Kobayashi, M., et al., *Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system.* Genes Cells, 2002. **7**(8): p. 807-20.

51. McMahon, M., et al., *Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex.* J Biol Chem, 2006. **281**(34): p. 24756-68.

52. Tong, K.I., et al., *Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism.* Biol Chem, 2006. **387**(10-11): p. 1311-20.

53. Fourquet, S., et al., *Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation.* J Biol Chem, 2010. **285**(11): p. 8463-71.

54. McMahon, M., et al., *Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18838-43.

55. Kobayashi, A., et al., *Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2.* Mol Cell Biol, 2004. **24**(16): p. 7130-9.

56. Nguyen, T., et al., *Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1.* J Biol Chem, 2005. **280**(37): p. 32485-92.

57. Salazar, M., et al., *Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2.* J Biol Chem, 2006. **281**(21): p. 14841-51.

58. Deshaies, R.J. and C.A. Joazeiro, *RING domain E3 ubiquitin ligases.* Annu Rev Biochem, 2009. **78**: p. 399-434.

59. Taguchi, K., H. Motohashi, and M. Yamamoto, *Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution.* Genes Cells, 2011. **16**(2): p. 123-40.

60. Kobayashi, A., et al., *Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1.* Mol Cell Biol, 2006. **26**(1): p. 221-9.

61. Katoh, Y., et al., *Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome.* Arch Biochem Biophys, 2005. **433**(2): p. 342-50.

62. McMahon, M., et al., *Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron.* J Biol Chem, 2004. **279**(30): p. 31556-67.

63. Lo, S.C. and M. Hannink, *PGAM5, a Bcl-XL-interacting protein, is a novel substrate for the redox-regulated Keap1-dependent ubiquitin ligase complex.* J Biol Chem, 2006. **281**(49): p. 37893-903.

64. Lo, S.C. and M. Hannink, *PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria.* Exp Cell Res, 2008. **314**(8): p. 1789-803.

65. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response.* Mol Cell, 2009. **34**(6): p. 663-73.

66. Esposito, F., et al., *A new p21waf1/cip1 isoform is an early event of cell response to oxidative stress.* Cell Death Differ, 1998. **5**(11): p. 940-5.

67. Gartel, A.L. and A.L. Tyner, *The role of the cyclin-dependent kinase inhibitor p21 in apoptosis.* Mol Cancer Ther, 2002. **1**(8): p. 639-49.

68. O'Reilly, M.A., et al., *The cyclin-dependent kinase inhibitor p21 protects the lung from oxidative stress.* Am J Respir Cell Mol Biol, 2001. **24**(6): p. 703-10.

69. Poon, R.Y. and T. Hunter, *Expression of a novel form of p21Cip1/Waf1 in UV-irradiated and transformed cells.* Oncogene, 1998. **16**(10): p. 1333-43.

70. Dotto, G.P., *p21(WAF1/Cip1): more than a break to the cell cycle?* Biochim Biophys Acta, 2000. **1471**(1): p. M43-56.

71. Li, R., et al., *Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair.* Nature, 1994. **371**(6497): p. 534-7.

72. O'Reilly, M.A., *Redox activation of p21Cip1/WAF1/Sdi1: a multifunctional regulator of cell survival and death.* Antioxid Redox Signal, 2005. **7**(1-2): p. 108-18.

73. Komatsu, M., et al., *The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1.* Nat Cell Biol, 2010. **12**(3): p. 213-23.

74. Hast, B.E., et al., *Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination.* Cancer Res, 2013. **73**(7): p. 2199-210.

75. Karapetian, R.N., et al., *Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes.* Mol Cell Biol, 2005. **25**(3): p. 1089-99.

76. Ma, J., et al., *PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function.* Mol Cell Biol, 2012. **32**(8): p. 1506-17.

77. Cancer Genome Atlas Research, N., *Comprehensive genomic characterization of squamous cell lung cancers.* Nature, 2012. **489**(7417): p. 519-25.

78. Lee, D.F., et al., *KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta.* Mol Cell, 2009. **36**(1): p. 131-40.

79. Antunes, F. and D. Han, *Redox regulation of NF-kappaB: from basic to clinical research.* Antioxid Redox Signal, 2009. **11**(9): p. 2055-6.

80. Hagemann, T., et al., *Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB.* Blood, 2009. **113**(14): p. 3139-46.

81. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008. **132**(3): p. 344-62.

82. Mauro, C., et al., *The NF-kappaB transcription factor pathway as a therapeutic target in cancer: methods for detection of NF-kappaB activity.* Methods Mol Biol, 2009. **512**: p. 169-207.

83. Niture, S.K. and A.K. Jaiswal, *Inhibitor of Nrf2 (INrf2 or Keap1) protein degrades Bcl-xL via phosphoglycerate mutase 5 and controls cellular apoptosis.* J Biol Chem, 2011. **286**(52): p. 44542-56.

84. Niture, S.K. and A.K. Jaiswal, *INrf2 (Keap1) targets Bcl-2 degradation and controls cellular apoptosis.* Cell Death Differ, 2011. **18**(3): p. 439-51.

85. Tian, H., et al., *Keap1: one stone kills three birds Nrf2, IKKbeta and Bcl-2/Bcl-xL.* Cancer Lett, 2012. **325**(1): p. 26-34.

86. Jurgensmeier, J.M., et al., *Bax directly induces release of cytochrome c from isolated mitochondria.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 4997-5002.

87. Wakabayashi, N., et al., *Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation.* Nat Genet, 2003. **35**(3): p. 238-45.

88. Taguchi, K., et al., *Genetic analysis of cytoprotective functions supported by graded expression of Keap1.* Mol Cell Biol, 2010. **30**(12): p. 3016-26.

89. Chan, K., X.D. Han, and Y.W. Kan, *An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4611-6.

90. Enomoto, A., et al., *High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes.* Toxicol Sci, 2001. **59**(1): p. 169-77.

91. Aoki, Y., et al., *Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust.* Toxicol Appl Pharmacol, 2001. **173**(3): p. 154-60.

92. Ishii, Y., et al., *Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema.* J Immunol, 2005. **175**(10): p. 6968-75.

93. Rangasamy, T., et al., *Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice.* J Clin Invest, 2004. **114**(9): p. 1248-59.

94. Cho, H.Y., et al., *The transcription factor NRF2 protects against pulmonary fibrosis.* FASEB J, 2004. **18**(11): p. 1258-60.

95. Iizuka, T., et al., *Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema.* Genes Cells, 2005. **10**(12): p. 1113-25.

96. Kim, J., Y.N. Cha, and Y.J. Surh, *A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders.* Mutat Res, 2010. **690**(1-2): p. 12-23.

97. Thimmulappa, R.K., et al., *Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis.* J Clin Invest, 2006. **116**(4): p. 984-95.

98. Fiers, W., et al., *More than one way to die: apoptosis, necrosis and reactive oxygen damage.* Oncogene, 1999. **18**(54): p. 7719-30.

99. Kensler, T.W. and N. Wakabayashi, *Nrf2: friend or foe for chemoprevention?* Carcinogenesis, 2010. **31**(1): p. 90-9.

100. Calkins, M.J., et al., *The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease.* Antioxid Redox Signal, 2009. **11**(3): p. 497-508.

101. Vaughn, A.E. and M. Deshmukh, *Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c.* Nat Cell Biol, 2008. **10**(12): p. 1477-83.

102. Lee, J.M., et al., *NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons.* J Biol Chem, 2003. **278**(39): p. 37948-56.

103. Calkins, M.J., et al., *Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 244-9.

104. Jakel, R.J., et al., *Induction of the protective antioxidant response element pathway by 6-hydroxydopamine in vivo and in vitro.* Toxicol Sci, 2005. **87**(1): p. 176-86.

105. Shih, A.Y., et al., *Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress.* J Neurosci, 2003. **23**(8): p. 3394-406.

106. Lee, J.M., et al., *Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis.* J Biol Chem, 2003. **278**(14): p. 12029-38.

107. Vargas, M.R., et al., *Increased glutathione biosynthesis by Nrf2 activation in astrocytes prevents p75NTR-dependent motor neuron apoptosis.* J Neurochem, 2006. **97**(3): p. 687-96.

108. Shih, A.Y., et al., *Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo.* J Biol Chem, 2005. **280**(24): p. 22925-36.

109. Vargas, M.R., et al., *Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival.* J Biol Chem, 2005. **280**(27): p. 25571-9.

110. Howden, R., *Nrf2 and cardiovascular defense.* Oxid Med Cell Longev, 2013. **2013**: p. 104308.

111. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.

112. Harrison, D.G., et al., *Endothelial mechanotransduction, nitric oxide and vascular inflammation.* J Intern Med, 2006. **259**(4): p. 351-63.

113. Asakura, T. and T. Karino, *Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries.* Circ Res, 1990. **66**(4): p. 1045-66.

114. Gibson, C.M., et al., *Relation of vessel wall shear stress to atherosclerosis progression in human coronary arteries.* Arterioscler Thromb, 1993. **13**(2): p. 310-5.

115. Delles, C., W.H. Miller, and A.F. Dominiczak, *Targeting reactive oxygen species in hypertension.* Antioxid Redox Signal, 2008. **10**(6): p. 1061-77.

116. Brandes, R.P., N. Weissmann, and K. Schroder, *NADPH oxidases in cardiovascular disease.* Free Radic Biol Med, 2010. **49**(5): p. 687-706.

117. Jung, O., et al., *gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension.* Circulation, 2004. **109**(14): p. 1795-801.

118. Hosoya, T., et al., *Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells.* J Biol Chem, 2005. **280**(29): p. 27244-50.

119. Collins, A.R., et al., *Myeloid deletion of nuclear factor erythroid 2-related factor 2 increases atherosclerosis and liver injury.* Arterioscler Thromb Vasc Biol, 2012. **32**(12): p. 2839-46.

120. Ruotsalainen, A.K., et al., *The absence of macrophage Nrf2 promotes early atherogenesis.* Cardiovasc Res, 2013. **98**(1): p. 107-15.

121. Zhu, H., et al., *Antioxidants and phase 2 enzymes in macrophages: regulation by Nrf2 signaling and protection against oxidative and electrophilic stress.* Exp Biol Med (Maywood), 2008. **233**(4): p. 463-74.

122. Ishikawa, K. and Y. Maruyama, *Heme oxygenase as an intrinsic defense system in vascular wall: implication against atherogenesis.* J Atheroscler Thromb, 2001. **8**(3): p. 63-70.

123. Ishikawa, K., et al., *Heme oxygenase-1 inhibits atherosclerotic lesion formation in ldl-receptor knockout mice.* Circ Res, 2001. **88**(5): p. 506-12.

124. Juan, S.H., et al., *Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2001. **104**(13): p. 1519-25.

125. Jyrkkanen, H.K., et al., *Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo.* Circ Res, 2008. **103**(1): p. e1-9.

126. Orozco, L.D., et al., *Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis.* Circ Res, 2007. **100**(12): p. 1703-11.

127. Gough, P.J., et al., *Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice.* J Clin Invest, 2006. **116**(1): p. 59-69.

128. Konstantino, Y., et al., *Potential implications of matrix metalloproteinase-9 in assessment and treatment of coronary artery disease.* Biomarkers, 2009. **14**(2): p. 118-29.

129. Buijsse, B., et al., *Low serum glutathione peroxidase activity is associated with increased cardiovascular mortality in individuals with low HDLc's.* PLoS One, 2012. **7**(6): p. e38901.

130. Morrison, J.A., et al., *Serum glutathione in adolescent males predicts parental coronary heart disease.* Circulation, 1999. **100**(22): p. 2244-7.

131. Singh, A., et al., *Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs, is regulated by Nrf2.* Am J Respir Cell Mol Biol, 2006. **35**(6): p. 639-50.

132. Malec, V., et al., *HIF-1 alpha signaling is augmented during intermittent hypoxia by induction of the Nrf2 pathway in NOX1-expressing adenocarcinoma A549 cells.* Free Radic Biol Med, 2010. **48**(12): p. 1626-35.

133. Matsuno, K., et al., *Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice.* Circulation, 2005. **112**(17): p. 2677-85.

134. Chen, T.M., et al., *Effects of heme oxygenase-1 upregulation on blood pressure and cardiac function in an animal model of hypertensive myocardial infarction.* Int J Mol Sci, 2013. **14**(2): p. 2684-706.

135. Escalante, B., et al., *Chronic treatment with tin normalizes blood pressure in spontaneously hypertensive rats.* Hypertension, 1991. **17**(6 Pt 1): p. 776-9.

136. Sacerdoti, D., et al., *Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats.* Science, 1989. **243**(4889): p. 388-90.

137. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.

138. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: how are they linked?* Free Radic Biol Med, 2010. **49**(11): p. 1603-16.

139. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-85.

140. Lee, J.M., et al., *Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9751-6.

141. Lee, J.M. and J.A. Johnson, *An important role of Nrf2-ARE pathway in the cellular defense mechanism.* J Biochem Mol Biol, 2004. **37**(2): p. 139-43.

142. Asghar, M., L. George, and M.F. Lokhandwala, *Exercise decreases oxidative stress and inflammation and restores renal dopamine D1 receptor function in old rats.* Am J Physiol Renal Physiol, 2007. **293**(3): p. F914-9.

143. Chen, X.L., et al., *Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression.* Am J Physiol Heart Circ Physiol, 2006. **290**(5): p. H1862-70.

144. Johnson, D.A., et al., *The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis.* Toxicol Sci, 2010. **114**(2): p. 237-46.

145. Ma, Q., L. Battelli, and A.F. Hubbs, *Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2.* Am J Pathol, 2006. **168**(6): p. 1960-74.

146. Yoh, K., et al., *Nrf2-deficient female mice develop lupus-like autoimmune nephritis.* Kidney Int, 2001. **60**(4): p. 1343-53.

147. Schreck, R., P. Rieber, and P.A. Baeuerle, *Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1.* EMBO J, 1991. **10**(8): p. 2247-58.

148. Ward, P.S. and C.B. Thompson, *Metabolic reprogramming: a cancer hallmark even warburg did not anticipate.* Cancer Cell, 2012. **21**(3): p. 297-308.

149. Deberardinis, R.J., J.J. Lum, and C.B. Thompson, *Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth.* J Biol Chem, 2006. **281**(49): p. 37372-80.

150. Lum, J.J., et al., *Growth factor regulation of autophagy and cell survival in the absence of apoptosis.* Cell, 2005. **120**(2): p. 237-48.

151. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.

152. Bauer, D.E., et al., *Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand.* FASEB J, 2004. **18**(11): p. 1303-5.

153. Bryan, H.K., et al., *The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation.* Biochem Pharmacol, 2013. **85**(6): p. 705-17.

154. Yamadori, T., et al., *Molecular mechanisms for the regulation of Nrf2-mediated cell proliferation in non-small-cell lung cancers.* Oncogene, 2012. **31**(45): p. 4768-77.

155. Rosenzweig, S.A., *Acquired resistance to drugs targeting receptor tyrosine kinases.* Biochem Pharmacol, 2012. **83**(8): p. 1041-8.

156. Elstrom, R.L., et al., *Akt stimulates aerobic glycolysis in cancer cells.* Cancer Res, 2004. **64**(11): p. 3892-9.

157. Soria, J.C., et al., *EGFR-mutated oncogene-addicted non-small cell lung cancer: current trends and future prospects.* Cancer Treat Rev, 2012. **38**(5): p. 416-30.

158. Ohashi, K., et al., *Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease.* J Clin Oncol, 2013. **31**(8): p. 1070-80.

159. De Roock, W., et al., *KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer.* Lancet Oncol, 2011. **12**(6): p. 594-603.

160. Tognon, C.E. and P.H. Sorensen, *Targeting the insulin-like growth factor 1 receptor (IGF1R) signaling pathway for cancer therapy.* Expert Opin Ther Targets, 2012. **16**(1): p. 33-48.

161. Satoh, H., et al., *Nrf2 prevents initiation but accelerates progression through the Kras signaling pathway during lung carcinogenesis.* Cancer Res, 2013.

162. DeNicola, G.M., et al., *Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis.* Nature, 2011. **475**(7354): p. 106-9.

163. Shibata, T., et al., *Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy.* Proc Natl Acad Sci U S A, 2008. **105**(36): p. 13568-73.

164. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends Biochem Sci, 2009. **34**(4): p. 176-88.

165. Muscarella, L.A., et al., *Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer.* Epigenetics, 2011. **6**(6): p. 710-9.

166. Sasaki, H., et al., *Increased NRF2 gene (NFE2L2) copy number correlates with mutations in lung squamous cell carcinomas.* Mol Med Rep, 2012. **6**(2): p. 391-4.

167. Shelton, P. and A.K. Jaiswal, *The transcription factor NF-E2-related factor 2 (Nrf2): a protooncogene?* FASEB J, 2013. **27**(2): p. 414-23.

168. Shen, H., S. Zhou, and J. Wang, *The paradoxical role of Nrf2 in tumor biology.* Crit Rev Eukaryot Gene Expr, 2013. **23**(1): p. 37-47.

169. Levine, A.J., *The tumor suppressor genes.* Annu Rev Biochem, 1993. **62**: p. 623-51.

170. Suzuki, T., J. Maher, and M. Yamamoto, *Select heterozygous Keap1 mutations have a dominant-negative effect on wild-type Keap1 in vivo.* Cancer Res, 2011. **71**(5): p. 1700-9.

171. Hanada, N., et al., *Methylation of the KEAP1 gene promoter region in human colorectal cancer.* BMC Cancer, 2012. **12**: p. 66.

172. Muscarella, L.A., et al., *Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome.* Epigenetics, 2011. **6**(3): p. 317-25.

173. Wang, R., et al., *Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues.* Biochem Biophys Res Commun, 2008. **373**(1): p. 151-4.

174. Zhang, P., et al., *Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth.* Mol Cancer Ther, 2010. **9**(2): p. 336-46.

175. Isaacs, J.S., et al., *HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability.* Cancer Cell, 2005. **8**(2): p. 143-53.

176. Toro, J.R., et al., *Mutations in the fumarate hydratase gene cause hereditary leiomyomatosis and renal cell cancer in families in North America.* Am J Hum Genet, 2003. **73**(1): p. 95-106.

177. Kinch, L., N.V. Grishin, and J. Brugarolas, *Succination of Keap1 and activation of Nrf2-dependent antioxidant pathways in FH-deficient papillary renal cell carcinoma type 2.* Cancer Cell, 2011. **20**(4): p. 418-20.

178. Frezza, C., et al., *Haem oxygenase is synthetically lethal with the tumour suppressor fumarate hydratase.* Nature, 2011. **477**(7363): p. 225-8.

179. Losman, J.A. and W.G. Kaelin, Jr., *What a difference a hydroxyl makes: mutant IDH, (R)-2-hydroxyglutarate, and cancer.* Genes Dev, 2013. **27**(8): p. 836-52.

180. Inami, Y., et al., *Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells.* J Cell Biol, 2011. **193**(2): p. 275-84.

181. Kitamura, H., et al., *Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue.* Histopathology, 2006. **48**(2): p. 157-61.

182. Simaga, S., et al., *Dipeptidyl peptidase III in malignant and non-malignant gynaecological tissue.* Eur J Cancer, 1998. **34**(3): p. 399-405.

183. Simaga, S., et al., *Tumor cytosol dipeptidyl peptidase III activity is increased with histological aggressiveness of ovarian primary carcinomas.* Gynecol Oncol, 2003. **91**(1): p. 194-200.

184. Konstantinopoulos, P.A., et al., *Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer.* Cancer Res, 2011. **71**(15): p. 5081-9.

185. Lu, H., W. Ouyang, and C. Huang, *Inflammation, a key event in cancer development.* Mol Cancer Res, 2006. **4**(4): p. 221-33.

186. Braun, S., et al., *Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound.* Mol Cell Biol, 2002. **22**(15): p. 5492-505.

187. Govindan, R., et al., *Genomic landscape of non-small cell lung cancer in smokers and never-smokers.* Cell, 2012. **150**(6): p. 1121-34.

188. Rudin, C.M., et al., *Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer.* Nat Genet, 2012. **44**(10): p. 1111-6.

189. Vogelstein, B., et al., *Cancer genome landscapes.* Science, 2013. **339**(6127): p. 1546-58.

190. Wei, X., et al., *Exome sequencing identifies GRIN2A as frequently mutated in melanoma.* Nat Genet, 2011. **43**(5): p. 442-6.

191. Zhang, J., et al., *The genetic basis of early T-cell precursor acute lymphoblastic leukaemia.* Nature, 2012. **481**(7380): p. 157-63.

192. Hu, R., et al., *Regulation of NF-E2-related factor 2 signaling for cancer chemoprevention: antioxidant coupled with antiinflammatory.* Antioxid Redox Signal, 2010. **13**(11): p. 1679-98.

193. Lau, A., et al., *Dual roles of Nrf2 in cancer.* Pharmacol Res, 2008. **58**(5-6): p. 262-70.

II. CHAPTER TWO: PROTEOMIC ANALYSIS OF UBIQUITIN LIGASE KEAP1 REVEALS ASSOCIATED PROTEINS THAT INHIBIT NRF2 UBIQUITINATION

[1]2.A. OVERVIEW

Somatic mutations in the KEAP1 ubiquitin ligase or its substrate NRF2 (NFE2L2) commonly occur in human cancer, resulting in constitutive NRF2-mediated transcription of cytoprotective genes. However, many tumors display high NRF2 activity in the absence of mutation, supporting the hypothesis that alternative mechanisms of pathway activation exist. Previously, we and others discovered that via a competitive binding mechanism, the proteins WTX (AMER1), PALB2 and SQSTM1 bind KEAP1 to activate NRF2. Proteomic analysis of the KEAP1 protein interaction network revealed a significant enrichment of associated proteins containing an ETGE amino acid motif, which matches the KEAP1 interaction motif found in NRF2. Like WTX, PALB2, and SQSTM1, we found that the dipeptidyl peptidase 3 (DPP3) protein binds KEAP1 via an 'ETGE' motif to displace NRF2, thus inhibiting NRF2 ubiquitination and driving NRF2-dependent transcription. Comparing the spectrum of KEAP1 interacting proteins with the genomic profile of 178 squamous cell lung carcinomas characterized by The Cancer Genome Atlas revealed amplification and mRNA over-expression of the DPP3 gene in tumors with high NRF2 activity but lacking NRF2 stabilizing mutations. We further show that tumor-derived mutations in KEAP1 are hypomorphic with

[1] The following manuscript has been previously published in the journal Cancer Research. The manuscript, figures, and tables contained within this chapter can be found using the reference: Hast, B.E., et al., *Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination.* Cancer Res, 2013. **73**(7): p. 2199-210.

respect to NRF2 inhibition and that DPP3 over-expression in the presence of these mutants further promotes NRF2 activation. Collectively, our findings further support the competition model of NRF2 activation and suggest that 'ETGE'-containing proteins like DPP3 contribute to NRF2 activity in cancer.

2.B. INTRODUCTION

Constitutive activation of the NF-E2-related factor 2 (NRF2) cap-n-collar transcription factor is emerging as a prominent molecular feature of many tumors. When active, NRF2 controls the expression of ~200 genes that collectively function to maintain a healthy intracellular reduction-oxidation (redox) balance, clear electrophilic xenobiotics, and degrade damaged and misfolded proteins [1-2]. The leading hypothesis posits that whereas short-term NRF2 activation antagonizes oncogenesis by curtailing oxidative damage, constitutive activation promotes the survival of metabolically stressed cancer cells, as well as cancer cells under chemotherapeutic insult. Indeed, depletion of NRF2 from cancer-derived cell lines results in apoptosis and increased sensitivity to chemotherapeutic agents [3]. In human non-small cell lung cancer, tumors showing high levels of NRF2 protein are associated with a poor outcome and increased resistance to therapy [4-6].

At basal state, NRF2 protein level and activity is maintained at low levels through ubiquitin-dependent proteasomal degradation [7-9]. The mechanics of this ubiquitination, which is conceptualized in the 'hinge-and-latch' model, involves a homodimeric E3 ubiquitin ligase complex comprising the KEAP1 substrate recognition module and a cullin-3 scaffold [10-11] (Fig. 2.1.A). An amino-terminal DLG and ETGE motif within NRF2 independently

binds two KEAP1 monomers within the complex, yielding a 2:1 stoichiometry of KEAP1:NRF2. The intermolecular protein dynamics governing ubiquitination of NRF2 relies on the differential affinities between the ETGE and DLG motifs for KEAP1; the ETGE motif binds KEAP1 with approximately 100-fold greater affinity than the DLG [10]. In response to oxidative stress, modification of reactive cysteines within KEAP1 induces a conformational change within the homodimer. This architectural re-structuring releases the low affinity DLG motif from KEAP1, thus re-positioning NRF2 in a conformation unfavorable for ubiquitination [10-13].

Recent cancer genomic studies reported somatic mutation of NRF2 or KEAP1 in 34% of squamous cell lung carcinoma and 12% of lung adenocarcinoma [5, 14]. Consistent with the direct inhibition of NRF2 by KEAP1, mutations striking both KEAP1 and NRF2 within the same tumor are typically not observed [15]. Moreover, whereas activating mutations within NRF2 almost invariably target the DLG or ETGE motifs, mutations within KEAP1 span the entire length of the protein [15-16]. Genomic alterations in KEAP1 or NRF2 have also been reported in a variety of other cancers, including gastric carcinoma, colorectal carcinoma, hepatocellular carcinoma, and ovarian cancer [17-21]. In addition to mutation, hypermethylation of the KEAP1 promoter and NRF2 copy number amplifications promote NRF2 activity in lung, colon and prostate cancer [21-23].

Although we have an understanding of how oxidative stress and genetic mutation activate NRF2 signaling, the identity and function of proteins that physically interact with KEAP1 and NRF2 has been comparatively understudied. A growing body of evidence

suggests that cancer-associated increases in NRF2 transcript and protein can occur in the absence of genomic alteration [15, 17, 24], underscoring the importance of identifying the full complement of regulatory mechanisms governing NRF2 activity. We recently reported that the WTX tumor suppressor protein physically binds KEAP1 to competitively inhibit NRF2 ubiquitination [25]. Similarly, p62/SQSTM1, PALB2, and p21 bind KEAP1 or NRF2 to sterically inhibit NRF2 ubiquitination [26-28]. For WTX and PALB2, the association with KEAP1 is achieved through an ETGE motif, which mimics the NRF2 binding interface. As expected, these proteins activate NRF2-mediated transcription in the absence of oxidative stress, through ETGE-dependent competition with NRF2 for KEAP1 binding. Here, we sought to comprehensively define all ETGE or ESGE containing proteins within the KEAP1 protein interaction network, determine whether they functionally control NRF2 and evaluate their expression within human tumors, particularly in relation to NRF2 activity.

2.C. MATERIALS AND METHODS

Tissue Culture, Transfections, and Small Interfering RNAs

HEK293T and H2228 cells were obtained from the American Tissue and Culture Collection, which authenticates cells line using short tandem repeat analysis. Cell lines were not passaged for more than 6 months after resuscitation. HEK293T cells were grown in DMEM supplemented with 10% FBS and 1% GlutaMAX (Life Technologies) in a 37°C humidified incubator with 5% CO2. H2228 cells were grown in RPMI supplemented with 10% FBS. KEAP1 -/- mouse embryo fibroblasts (MEFs) were cultured in IMDM supplemented with 10% FBS. The KEAP1 -/- MEFs were kindly provided by Thomas Kensler and Nobunao Wakabayshi. Expression constructs were transfected in HEK293T

cells with Lipofectamine 2000 (Life Technologies) and KEAP1 -/- MEFs as with Fugene HD (Roche). Transfection of siRNA was performed with Lipofectamine RNAiMAX (Life Technologies).

siRNA sequences: DPP3#1 (CAC CAA AUC CAA UGC UCC UCA CAU A), DPP3#2 (GCU UAC CAU CCU GUC UAC CAG AUG A), DPP3#3 (CCC UCC AUU CGU GUG UGU AUU UAG G), NRF2 (GUA AGA AGC CAG AUG UUA A), KEAP1 (GGG CGU GGC UGU CCU CAA U). Control siRNAs were obtained from Life Technologies; sequences are as follows: CGU ACG CGG AAU ACU UCG ATT and UCG AAG UAU UCC GCG UAC GTT.

Cell lysis buffers: 0.1% NP40 (10% glycerol, 50mM HEPES, 150 mM NaCl, 2mM EDTA, 0.1% NP-40) containing protease inhibitor mixture (Thermo Scientific) and phosphatase inhibitor (Thermo Scientific). RIPA buffer (1% NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 150mM NaCl, 10% glycerol, 25mM Tris, 2mM EDTA). Additionally, NRF2 ubiquitination experiments were performed in the presence of 2mM N-ethylmaleimide.

Cell Staining Buffers: cytoskeletal buffer (5 mM PIPES, pH 6, 137 mM NaCl, 5 mM KCl, 1.1 mM Na2HPO4, 0.4 mM KH2PO4, 0.4 mM MgCl2, 0.4 mM NaHCO3, 2 mM EGTA, 50mM glucose).

Antibodies employed for W. blot analysis: anti-FLAG M2 monoclonal (Sigma), anti-HA monoclonal (Roche), anti-FAM117b (ProteinTech, 21768), anti-MAD2L1 (Bethyl, Montgomery TX, A300-301A), anti-MCM3 (Bethyl, A300-192A), anti-SLK (Bethyl, A300-499A), anti-βactin polyclonal (Sigma, A2066), anti-βtubulin monoclonal (Sigma, T7816), anti-KEAP1 polyclonal (ProteinTech, Chicago IL), anti-DPP3 polyclonal (abcam,

Cambridge MA, 97437), anti-GFP (abcam, ab290), anti-NRF2 H300 polyclonal (Santa Cruz, Santa Cruz CA), and anti-VSV polyclonal (Bethyl, A190-131A).

Quantitative PCR primers: GCLM (F: ACAGCGAGGAGCTTCATGATTG, R: CTCCCTGACCAAATCTGGGTTG), HMOX1 (F: GGCCAGCAACAAAGTGCAAGATTC, R: AGCAACTGTCGCCACCAGAAAG), GAPDH (F: ATGGGGAAGGTGAAGGT, R: AAGCTTCCCGTTCTCAG).

Affinity Pulldowns and Western Blotting

For Streptavidin and FLAG affinity purification, cells were lysed in 0.1% NP-40 lysis buffer. Cell lysates were cleared by centrifugation and incubated with streptavidin resin (GE Healthcare) or FLAG resin (Sigma) before washing with lysis buffer and eluting with NuPAGE loading buffer (Life Technologies). For siRNA, HEK293T cells were transiently transfected and lysed in RIPA buffer 60 h post-transfection. All antibodies and buffers used for Western analysis are listed in Supplemental Methods.

Plasmids, Expression Vectors, and Site-directed Mutagenesis

Expression constructs in the SBPHA backbone were generated with standard PCR techniques. Constructs for DPP3 and DPP3^{Y318F} were a generous gift from Maja Abramić. The reporter gene fusion construct for human hNQO1-ARE-luciferase was a kind gift from Jeffrey Johnson. The SLK-HA construct was a generous gift from Dr. Andrey Cybulsky. Expression constructs for ETGE-containing proteins were obtained from Open Biosystems and cloned into a custom lentiviral vector (pHAGE-CMV-FLAG-DEST). ETGE deletion mutants were generated by PCR-based mutagenesis and sequence verified prior to use (GENEWIZ).

ARE-luciferase Quantification

For DNA, cells were transfected with expression constructs, FLAG-KEAP1, FLAG-NRF2, hNQO1-ARE luciferase, and a control plasmid containing *Renilla* luciferase driven by a constitutive cytomegalovirus (CMV) promoter. Approximately 24 h post-transfection, NRF2-mediated transcription was measured as the ratio of Firefly to *Renilla* luciferase activity (Promega Dual-Luciferase Reporter Assay System). For siRNA, HEK293T cells stably expressing the ARE-luciferase and *Renilla* control reporters were transfected with siRNA. Approximately 60 h post-transfection, activation was measured. For the assay depicted in Figure 6E, treatment with 50 μM tBHQ was performed 48 h post-transfection, and activation was measured 60 h post-transfection.

Cell-based NRF2 ubiquitination experiments

HEK293T stably expressing SBPHA-KEAP1 cells were transfected with VSV-UB1, FLAG-NRF2, and SBPHA-DPP3. Venus-NPM1 was used such that each condition received the same mass of DNA. Cells were lysed in 0.1% NP-40 lysis buffer.

RNA Isolation, Reverse Transcription, and Semi-quantitative Real Time-PCR

Total RNA from cells was harvested in TRIzol (Life Technologies) reagent according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry, and cDNA was created using the RevertAid First Strand cDNA synthesis Kit (Fermentas). PCR was performed in triplicate with 30 cycles of amplification with 1 s denaturation at 95 °C and

5 s annealing at 60 °C, on an ABI 7900HT Fast Realtime PCR machine. Quantitative light cycler PCR primers are listed in the Supplemental Methods.

Crystallographic Modeling

The coordinates for the KEAP1-NRF2 peptide complex and DPP3 were downloaded from the RCSB Protein Data Bank (PDB IDs 1X2R and 3FVY, respectively). The superposition of the ETGE motifs of NRF2 and DPP3 was done in PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). PyMOL was used to prepare the images used in Figure 4D-G.

Affinity Purification and Mass Spectrometry

For Streptavidin and FLAG affinity purification, cells were lysed in 0.1% NP-40 lysis. Cell lysates were incubated with Streptavidin or FLAG resin and washed 5X with lysis buffer. The precipitated proteins were trypsinized directly off beads using the FASP Protein Digestion Kit (Protein Discovery). For tandem purification of the FLAG-KEAP1 and SBPHA-DPP3 complex (Figure 3B), protein complexes were eluted after the first affinity purification with either 150 μg/μl FLAG peptide or 50 mM biotin.

Protein Identification, Filtering and Bioinformatics

Filtering of false interactions from non-tandem, wild-type experiments was accomplished using SPOTLITE, with an internal lab dataset of 158 Streptavidin experiments on 60 different baits, and using a 10% FDR for the entire dataset. FLAG-based APMS data were not scored with SPOTLITE because our FLAG-specific reference dataset is

prohibitively small. Proteins identified in tandem or mutant experiments were accepted if they passed the SPOTLITE filtering on the non-tandem, wild-type experiments. Unfiltered data and associated SPOTLITE results are provided as Table S1.

Protein Identification, Filtering and Bioinformatics: All raw data were converted to mzXML format before a search of the resultant spectra using Sorcerer™-SEQUEST® (build 4.0.4, Sage N Research) and the Transproteomic Pipeline (TPP v4.3.1). Data were searched against either the human UniProtKB/Swiss-Prot sequence database (Release 2011_08) or the human IPI database (Version 3.87), both supplemented with common contaminants, i.e. porcine (Swiss-Prot P00761) and bovine (P00760) trypsin, and further concatenated with its reversed copy as a decoy (40,494 total sequences). Search parameters used were a precursor mass between 400 and 4500 amu, up to 2 missed cleavages, precursor-ion tolerance of 3 amu, accurate mass binning within PeptideProphet, semi-tryptic digestion, a static carbamidomethyl cysteine modification, and variable methionine oxidation. False discovery rates (FDR) were determined by ProteinProphet and minimum protein probability cutoffs resulting in a 1% FDR were selected individually for each experiment. PeptideProphet/ProteinProphet results for each AP-MS experiment were stored in a local Prohits database. Prohits performed the mapping of UniProtKB/IPI accession identifiers to Entrez Gene IDs. These results were then imported into Cytoscape v2.8.2 for network visualization and SPOTLITE for interaction prediction. Gene Ontology annotations were imported from NCBI Entrez Gene through Cytoscape. Known protein-protein interactions were extracted from the BioGRID database (Release 3.1.89).

Motif Analysis

Identification of enriched 4-mer amino acid sequences was performed using a 1-tail Fisher's exact test (Table S2). We individually tested each of the 13265 4-mer sequences present among the KEAP1 interactors, taking into account the number of interacting proteins, interacting proteins having the motif, total proteins in the UniProtKB/SwissProt database, and the total number of proteins having the motif within UniProtKB/SwissProt. Bonferroni correction was applied due to multiple hypothesis testing.

Immunostaining

For subcellular localization of exogenously expressed proteins, cells were co-transfected with the indicated plasmids and plated on 10ug/ml fibronectin-coated coverslips. Cells were fixed in 4% paraformaldehyde in cytoskeletal buffer for 15 minutes, and coverslips were mounted to slides using the Prolong Gold antifade reagent (Molecular Probes). Images were acquired using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope equipped with a 63X/1.42 Oil PlanApo objective lenses. Localization of endogenous KEAP1 was determined by immunostaining cells as described above, except: 1) cells were fixed in 4% PFA in cytoskeletal buffer for 15 minutes and permeabilized with 0.1% Triton in PBS for 5 minutes, 2) after blocking in 1% BSA/PBS for 1h, cells were double stained for KEAP1 (Proteintech) and flag (Sigma) at 4^{o}C, overnight, followed by incubation with FITC-conjugated-donkey anti-rabbit IgG and RRX-conjugated-donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) at room temperature for 2h and 3), images were acquired using a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope.

2.D. RESULTS

2.D.1 Proteomic analysis of the KEAP1 protein interaction network.

We defined the KEAP1 protein interaction network by affinity purification and shotgun mass spectrometry (APMS) (Fig. 2.1.B and S2.1). In total, the KEAP1 complex was analyzed 13 times, where variations in affinity purification, detergent solubilization and cell treatment helped to maximize comprehensive network mapping. True interactions were identified from false positives using SPOTLITE, a novel probabilistic scoring algorithm that couples direct and indirect data to identify false positive interactions within APMS data (Fig. 2.1.B) (manuscript under review). Of 42 high confidence KEAP1-interacting proteins identified, 17 contain an ETGE, ESGE or both. To determine if this motif is enriched within the KEAP1 protein interaction network (PIN) beyond chance observation, we performed a Fisher's exact test. The ETGE motif was identified as the only significant 4 amino acid sequence within the KEAP1 PIN (Table S.2.2). Together, these data support and expand the 'ETGE' competition model of KEAP1 regulation.

2.D.2 The ETGE motif is required for binding to KEAP1.

We selected eight ETGE-containing proteins and validated their association with KEAP1 (Fig. 2.2.A-B). Western blot analysis of affinity purified KEAP1 protein complexes revealed the presence of endogenously expressed DPP3, FAM117B, MCM3, SLK and MAD2L1 (Fig. 2.2.B). Expression of exogenous TSC22D4 and WDR1 also showed interaction with KEAP1 (Fig. 2.2.C). To map the domain within KEAP1 responsible for binding the ETGE proteins, full-length KEAP1, the KEAP1 KELCH domain, or the KEAP1 BTB domain were purified and endogenous associated proteins were detected by Western

blot. With the exception of WDR1 and TSC22D4 which bound only full length KEAP1, DPP3, FAM117B, MCM3, SLK and MAD2L1 bound the KELCH domain of KEAP1 (Fig. 2.2.B-C). To directly evaluate a role for the ETGE motif in binding KEAP1, we generated ETGE-deletion mutants for FAM117B, MCM3, TSC22D4, WDR1, DPP3 and SLK. Like WTX and PALB2, deletion of the ETGE (ΔETGE) motif within these proteins abrogated KEAP1 binding (Fig. 2.2.D and E). Finally, functional impact of the ETGE-containing proteins on NRF2-mediated transcription was evaluated. Of the proteins tested, DPP3 and TSC22D4 strongly activated NRF2-mediated transcription in an ETGE-dependent manner, the former of which was previously identified as an activator of NRF2-dependent transcription in a gain-of-function screen [29] (Fig. 2.2.F). Over-expression of SLK also activated NRF2-mediated transcription, although this activation was independent of the ETGE motif (Fig. S2.1).

2.D.3 DPP3 is a KEAP1 interacting protein.

The protein dipeptidyl peptidase III (DPP3) had the greatest impact on NRF2-dependent transcription and was the most abundant protein within the KEAP1 PIN (Fig. 2.2.F) [30]. To further explore DPP3, we defined and compared the DPP3 PIN to the KEAP1 PIN (Fig. 2.3.A). With the exception of the observed interaction between KEAP1 and DPP3, the integrated PIN revealed no common interacting proteins. We also defined the PIN for DPP3$^{\Delta ETGE}$; as expected KEAP1 was not observed. To more rigorously characterize the KEAP1-DPP3 protein complex, we performed sequential affinity purifications for FLAG-KEAP1 and SBPHA-DPP3. Using HEK293T cells stably expressing both proteins, we purified FLAG-KEAP1 complexes and then from the resulting eluate, purified DPP3 with

Streptavidin (Fig. 2.3.B, top). The reciprocal sequential purification was done and analyzed by APMS (Fig. 2.3.B, bottom). Despite observing over 1500 spectral counts representing each bait protein, the only protein identified in both APMS experiments was SQSTM1, represented by 15 and 1 total spectra, respectively (Fig. 2.3.B). Together these data argue that the KEAP1-DPP3 complex is largely exclusive from other interacting proteins.

We next tested whether endogenously expressed DPP3 and KEAP1 associate. First, endogenous DPP3 was detected within FLAG-KEAP1 affinity purified protein complexes from HEK293T cells (Fig. 2.3.C). Second, endogenous KEAP1 affinity purified with SBPHA-DPP3 (Fig. 2.3.D). Finally, we detected KEAP1 within immunopurified endogenous DPP3 protein complexes (Fig. 2.3.E). In addition to these studies in HEK293T cells, DPP3 was also detected within KEAP1 protein complexes isolated from H2228 lung cancer cells (Fig. 2.3.F). As protein complex purification is subject to post-lysis interactions, we determined if DPP3 and KEAP1 co-localized within cells. Although discrete subcellular localizations were not observed, exogenously expressed DPP3 co-localized with both exogenous and endogenous KEAP1 protein; $DPP3^{\Delta ETGE}$ also co-localized with KEAP1 (Fig. 2.3.G and 2.3.H). Comparing transfected versus untransfected cells, the expression of DPP3 did not affect KEAP1 subcellular localization (Fig. 2.3.G). Finally, we tested whether the catalytic activity of DPP3 affected its association with KEAP1. Stably expressed wild-type (WT) and the catalytically inactive (Y318F) mutant [31] of DPP3 bound endogenous KEAP1 (Fig. 2.3.D), indicating that the catalytic activity of DPP3 is not required for KEAP1 binding. Consistent with this, wild-type DPP3 or $DPP3^{Y318F}$ similarly activated NRF2-dependent transcription when over-expressed (Fig. 2.3.I).

2.D.4 The ETGE motif is required for DPP3 binding to KEAP1.

Like WTX, PALB2, NRF2 and most of the ETGE-containing proteins evaluated, endogenous DPP3 associated with the KELCH domain of KEAP1 (Fig. 2.4.A). To validate that the ETGE motif is required for this binding, constructs encoding SBPHA-DPP3 or SBPHA-DPP3$^{\Delta ETGE}$ were transiently transfected into cells stably expressing FLAG-tagged full length KEAP1 or the KELCH domain. Wild-type DPP3 bound full length KEAP1 and the KELCH domain; however, DPP3$^{\Delta ETGE}$ was unable to bind KEAP1 or the KEAP1 KELCH domain (Fig. 2.4.B). Similarly, whereas endogenous KEAP1 failed to immunopurify with DPP3$^{\Delta ETGE}$, it did co-purify with DPP3-WT and the catalytic mutant DPP3^{Y318F} (Fig. 2.4.C). These data were confirmed by APMS of DPP3$^{\Delta ETGE}$ and DPP3^{Y318F}. Deletion of the ETGE motif within DPP3 may render the protein unstable and/or misfolded, which could account for lack of binding to KEAP1. To address this possibility, we tested the ability of a DPP3 alanine mutant to bind KEAP1. Like DPP3$^{\Delta ETGE}$, alanine point mutations within the domain (ETGE→AAGE) abolished KEAP1 binding (Fig. 2.4.D).

Crystallographic modeling revealed that NRF2 binds KEAP1 near the central pore of the KELCH β-propeller (Fig. 2.4.E) [10, 13, 32]. Using the crystal structure of DPP3, we asked if the ETGE motif within DPP3 and NRF2 adopt similar tertiary conformations. The ETGE motif in DPP3 lies on an unstructured loop on the surface of the protein (Fig. 2.4.F) [33]. It is therefore in a sterically favorable position to bind to KEAP1, as opposed to being buried within the globular domains. Similar to the NRF2 peptide, three tyrosine residues and one phenylalanine residue defines the binding surface that accommodates the specific conformation of the ETGE peptide of DPP3 (Fig. 2.4.G). Strikingly, the ETGE motif of

DPP3 and NRF2 adopt identical conformations when superimposed, suggesting that the ETGE motif of both proteins may interact with KEAP1 in a similar manner (Fig. 2.4.H) (root mean square deviation ≈ 0.05 Å between Cα atoms).

2.D.5 DPP3 competes with endogenous NRF2 for binding to KEAP1.

We tested whether DPP3 association with KEAP1 displaces NRF2. As homodimeric KEAP1 binds a single NRF2 molecule via two amino acid motifs (Fig. 2.1.A), competition experiments required the isolation of monomeric KEAP1. We created two double stable cell lines: the first expressed both SBPHA-KEAP1 and FLAG-KEAP1, and the second expressed SBPHA-KEAP1 and the FLAG-tagged BTB domain of KEAP1. Sequential affinity purification with streptavidin and FLAG resins purified KEAP1 homodimer or a KEAP1-BTB "pseudo-monomer", allowing us to test whether DPP3 competes with NRF2 for KEAP1 binding (Fig. 2.5.A). Compared to a truncated form of WTX that does not interact with KEAP1 [25], DPP3 over-expression resulted in reduced NRF2 binding to the pseudo-monomer KEAP1 but not to the KEAP1 homodimer (Fig. 2.5.B; compare lanes 3 and 4 to lanes 7 and 8). In contrast, when $DPP3^{\Delta ETGE}$ was introduced into each double-stable cell line, NRF2 binding to both the KEAP1-KEAP1 homodimer and KEAP1-BTB heterodimer was maintained (Fig. 2.5.C; compare lane 5 to 6). These findings suggest that DPP3 competes with NRF2 for binding to KEAP1 in an ETGE-dependent manner.

The 'hinge-and-latch' model predicts that loss of binding of the NRF2 DLG motif to KEAP1 results in a reduction of NRF2 ubiquitination and subsequent degradation. Given that DPP3 competes for binding to KEAP1 via the ETGE motif (Fig. 2.5.A-C), we tested if DPP3

over-expression reduced NRF2 ubiquitination in an ETGE-dependent manner. Affinity purification of exogenous NRF2 followed by Western blot analyses revealed relative levels of NRF2 ubiquitination. As the amount of wild-type DPP3 increased, ubiquitination of NRF2 decreased, as compared to control (Fig. 2.5.D; compare lanes 2 and 6). Consistent with its inability to bind KEAP1, $DPP3^{\Delta ETGE}$ did not reduce NRF2 ubiquitination (Fig. 2.5.E). These data suggest that over-expression of DPP3 alters the architecture of NRF2 bound to dimeric KEAP1, and ultimately decreases NRF2 ubiquitination.

2.D.6 DPP3 activates NRF2 signaling in an ETGE-dependent fashion.

To establish functional significance of DPP3 as a regulator of NRF2 activity, we determined whether DPP3 gain-of-function and loss-of-function impacted NRF2 transcriptional activity. Over-expression of DPP3, but not $DPP3^{\Delta ETGE}$ or $DPP3^{ala\Delta}$ significantly induced NRF2-dependent expression of an antioxidant responsive firefly luciferase reporter (Fig. 2.6.A). For loss-of-function, we designed and tested the silencing efficacy of three non-overlapping siRNAs targeting DPP3 (Fig. 2.6.B). siRNA-mediated silencing of DPP3 suppressed NRF2-mediated transcription of the ARE reporter, similar to that of NRF2 silencing (Fig. 2.6.C). To validate this phenotype using endogenous NRF2 readouts, qPCR was employed to monitor the expression of two well-established NRF2 target genes: heme oxgenase-1 (*HMOX1*) and glutamate-cysteine ligase modifier (*GCSm*). In agreement with the reporter data, DPP3 siRNAs reduced *HMOX1* and *GCSm* transcript levels similar to that of *NRF2* silencing (Fig. 2.6.D). Finally, the model predicts that DPP3 gain-of-function or loss-of-function would not affect NRF2 activity after treatment with a pathway agonist, as NRF2 would already be in a sterically unfavorable conformation for KEAP1-

mediated ubiquitination and degradation (Fig. 2.1.A). Consistent with this hypothesis, neither DPP3 siRNAs (Fig. 2.6.F) nor over-expression of DPP3 (Fig. 2.6.G) were able to suppress NRF2-mediated transcription after treatment with the small molecule, tert-butylhydroquinone (tBHQ).

2.D.7 DPP3 expression and DNA copy number positively correlates with NRF2 activity in squamous cell lung cancer.

To establish physiological significance for DPP3 in controlling KEAP1-NRF2 signaling, we evaluated *DPP3* mRNA abundance and gene alterations in squamous cell lung carcinoma, using data from the TCGA consortium (15). First, we found that *DPP3* mRNA expression is increased in lung SQCC as compared to matched normal tissue (Fig. 2.7.A). Of the four established lung SQCC subtypes, DPP3 expression is highest in primitive-type tumors (Fig. 2.7.A and S.2.2) [34]. Second, DPP3 genomic copy number and mRNA expression positively correlated, suggesting that DPP3 gene amplification may drive DPP3 over-expression in lung SQCC (Fig. 2.7.B). Third, when segregated by genotype, *DPP3* mRNA levels were higher in NRF2 wild-type lung SQCC as compared to tumors with mutated NRF2, which is consistent the proposed DPP3-competition model (Fig. 2.7.C). Surprisingly, *DPP3* mRNA abundance was found to be increased in KEAP1 mutant tumors as compared to KEAP1 wild-type tumors (Fig. 2.7.D). This co-occurrence might be explained if the mutations in KEAP1 are hypomorphic, resulting in a partially compromised ability to suppress NRF2. If so, the presence of DPP3 may further drive NRF2 activity. To test this hypothesis, we cloned and expressed five distinct KEAP1 mutations from the TCGA lung SQCC dataset and evaluated their impact on NRF2 function. In both HEK293T cells

and KEAP1-/- mouse embryo fibroblasts, all five KEAP1 mutants displayed reduced but not absent activity in suppressing NRF2, thus supporting the notion that these somatic mutations are hypomorphic (Fig. 2.7.E and Fig. S2.3). Impressively, over-expression of DPP3 further activated NRF2 in the presence of all five KEAP1 hypomorphs (Fig. 2.7.F). Importantly, the KEAP1 somatic mutants analyzed maintained association with both DPP3 and NRF2 (Fig. S2.3). These data support a model wherein somatic mutation of KEAP1 partially impairs its ability to suppress NRF2, and the presence of 'ETGE'-containing proteins like DPP3 may further drive pathway activity in KEAP1 mutant tumors.

Finally, we tested whether DPP3 expression associated with NRF2 transcriptional activity across the lung SQCC cohort, as defined by the expression of a gene set signature consisting of 15 NRF2 target genes [3]. DPP3 expression and the NRF2 signature score strongly associated (Fig. 2.7.H). Together, these data suggest that through competitive binding to KEAP1, DPP3 genomic amplification and over-expression may promote NRF2 activity in squamous cell carcinoma of the lung.

2.E. DISCUSSION

Aberrant KEAP1/NRF2 signaling has emerged as a critical regulatory pathway in a multitude of disease pathologies, most notably cancer. Although substantial progress has been made to define how reactive cysteines within KEAP1 govern its ability to ubiquitinate NRF2, the role of proteins peripheral to the KEAP1/NRF2/CUL3 core complex has only just begun to be explored. Recent studies have revealed four proteins that bind KEAP1 or NRF2 and ultimately inhibit NRF2 ubiquitination. Of these, WTX and PALB2 employ an ETGE

motif to directly bind KEAP1, thus displacing and stabilizing NRF2. Given these discoveries and the likelihood that KEAP1-associated proteins contribute to NRF2 perturbation in human disease, particularly when genomic alterations within KEAP1 and NRF2 are lacking, we sought to establish the ETGE motif as a defining characteristic in KEAP1 associated proteins that functionally control NRF2 stability.

We found that the ETGE motif defines the most frequently observed four amino acid sequence within the KEAP1 protein interaction network (Table S2.2; Fisher's exact test, 1-tail, p=5.8e-13). Of 13 ETGE containing proteins identified, we tested 7 and found that all required the ETGE motif to bind KEAP1 (Fig. 2.2.E). Aside from this ETGE-dependent KEAP1 binding however, we noted very few similarities. For example, with the exception of WTX, DPP3, and PALB2, none of the proteins have been previously reported to contribute or respond to oxidative stress. Additionally, functional annotations for identified proteins are surprisingly diversified: DNA replication and licensing (MCM3, MCMBP), cytoskeletal dynamics (SLK, WDR1), transcription (TSC22D4), and apoptosis (SLK) [35-45]. Within the context of our data, these observations suggest that each ETGE protein may function to control KEAP1 activity or be controlled by KEAP1 in a context-dependent fashion.

Because it robustly activates NRF2-mediated transcription (Fig. 2.6.A), binds KEAP1 with near exclusivity (Fig. 2.3.A and B) and has established catalytic activity, we chose to focus our mechanistic studies on DPP3. Although DPP3 possesses exopeptidase activity *in vitro* [33, 46], we were unable to reveal a role for its catalytic activity in either contributing to the KEAP1 interaction (Fig. 2.3.D and 2.4.C) or regulating NRF2-mediated transcription

(Fig. 2.3.I). That said, our studies did not examine the temporal effects of DPP3 expression on NRF2 activity, but rather assessed pathway activity at steady-state. Focused studies are needed to determine whether DPP3 catalytic activity functions to control KEAP1 and NRF2 dynamics, as well as pathway activity *in vivo.* Given the pressing need of identifying new drug targets within the KEAP1-NRF2 pathway, the possibility of targeting DPP3 catalytic function to control KEAP1-NRF2 remains an important opportunity.

The ETGE motif of DPP3 resides in a flexible loop on the surface of the protein and adopts a similar conformation to the NRF2 ETGE peptide when bound to KEAP1 (Fig. 2.4.F and G). Loss of this motif, through deletion or point mutation, abrogates the KEAP1-DPP3 protein interaction (Fig. 2.4), perturbs the interaction between NRF2 and KEAP1 (Fig. 2.5), as well as alters NRF2 ubiquitination (Fig. 2.5). We interpret these data to support a model wherein DPP3 competes the low-affinity DLG motif of NRF2 off of the KEAP1 KELCH domain, resulting in a complex of KEAP1, DPP3, and NRF2. Additional experiments are needed to determine protein stoichiometry within complexes containing KEAP1, NRF2, and DPP3. These experiments will reveal whether competitors like DPP3 specifically compete off the DLG of NRF2, as opposed to the ETGE motif; based on the relative affinities of the DLG and ETGE motifs, competition with the DLG motif of NRF2 is most probable [10-11].

In cancer, over-expression of an ETGE-containing protein may promote NRF2 activity in the absence of inactivating KEAP1 mutations or activating NRF2 mutations. Indeed, high NRF2 activity in tumors lacking KEAP1 or NRF2 mutation has been reported in ovarian cancer, sarcoma and squamous cell lung cancer [5, 17, 47]. After defining the ETGE-

containing proteins within the KEAP1 protein interaction network (Fig. 2.1), we surveyed their expression and DNA copy number across tumor samples taken from the TCGA SQCC lung cohort. DPP3 demonstrated copy number gains and mRNA over-expression, and importantly both of which positively correlated with NRF2 activity (Fig. 2.7). Whether genomic amplification of DPP3 constitutes a 'driver' event in cancer remains an important question for future research. Interestingly, two studies have demonstrated DPP3 over-expression in ovarian cancer. Given our data in lung SQCC, DPP3 expression may similarly be driven by genomic amplification in ovarian carcinoma, possibly functioning as a NRF2 agonist [48-49].

Our cancer genomic analyses and functional annotation of cancer-derived mutations in KEAP1 suggest that some KEAP1 somatic mutations are hypomorphic, resulting in partial NRF2 activation. This contrasts NRF2 mutation, which we believe yields a maximally activated pathway, one insensitive to KEAP1 modifiers such as DPP3. This hypothesis is supported by the following: 1) DPP3 genomic amplification and mRNA over-expression was largely restricted to NRF2 wild type tumors, 2) DPP3 over-expression positively associated with KEAP1 mutant tumors, 3) DPP3 over-expression in the presence of mutant KEAP1 further activated NRF2 signaling, and 4) as a tumor suppressor frequently targeted in cancer, KEAP1 is somewhat unique in that it is rarely deleted through homozygotic loss. Therefore, from a therapeutic and prognostic perspective, KEAP1 mutant tumors are not equivalent to NRF2 mutants. Future studies are needed to challenge this model. For example, does forced DPP3 expression drive NRF2 activity in mouse models of lung cancer, and would synergy be seen between DPP3 expression and KEAP1 mutation *in vivo*? Given that multiple different

cancer types have recently been found to exhibit constitutive NRF2 activation—some of which in the absence of NRF2 or KEAP1 mutations—our data collectively support a model where the expression of ETGE-containing proteins drive NRF2-mediated signaling via a competitive binding mechanism.

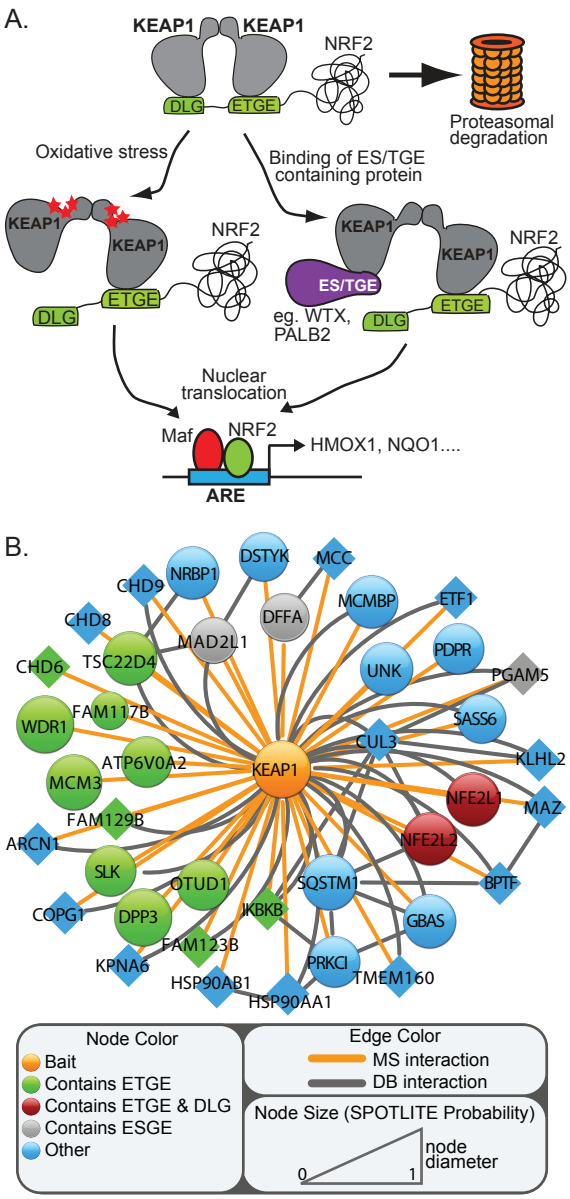

Figure 2.1. The KEAP1 protein interaction network is enriched for ETGE-containing proteins. (A) Cartoon schematic of NRF2 ubiquitination by KEAP1. KEAP1 inactivation is shown through cysteine modification and the competitive association of ETGE-containing proteins. (B) Schematic representation of the KEAP1 protein interaction network as defined by affinity purification and mass spectrometry. Nodes and edges were sized and colored according to probabilistic scoring approach, sequence and source of data. Circular nodes were sized according to SPOTLITE probability (10% FDR). Triangular nodes represent borderline SPOTLITE scored interactions that were observed across multiple APMS runs.

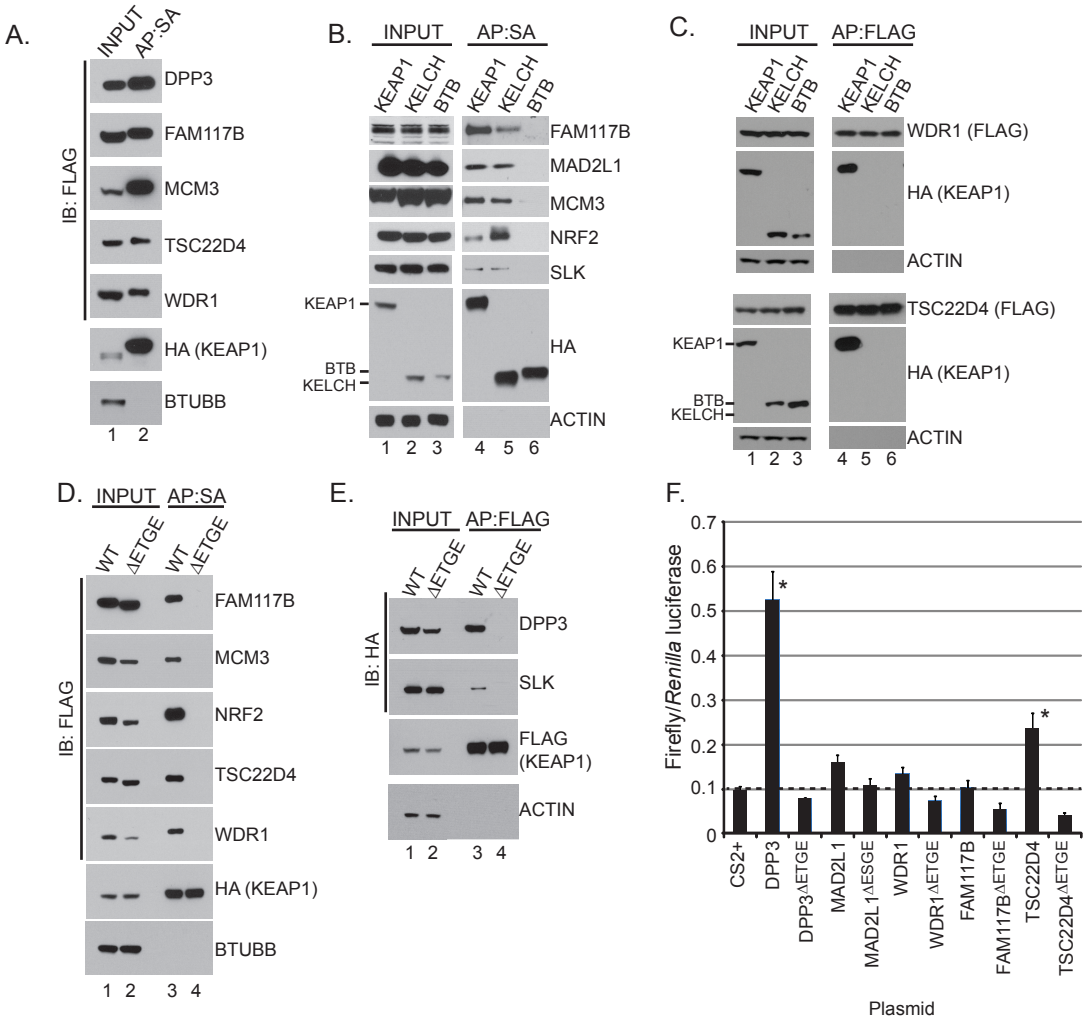

Figure 2.2. The ETGE motif is required for KEAP1 association. (A) Streptavidin affinity purified KEAP1 protein complexes from HEK293T cells were analyzed by Western blot for the indicated FLAG-tagged proteins (SBP, streptavidin binding peptide; HA, hemagglutinin). (B) Streptavidin affinity purified protein complexes from HEK293T cells stably expressing the indicated SBPHA-tagged proteins were analysed by Western blot for the indicated proteins. (C) HEK293T cells stably expressing FLAG-tagged WDR1 or TSC22D4 were transfected with SBPHA-tagged KEAP1, KEAP1 BTB, or KEAP1 KELCH before FLAG affinity purification and Western blot (D) FLAG-tagged wild-type or ET/SGE-deletion constructs were expressed in HEK293T cells stably expressing SBPHA-KEAP1 before streptavidin affinity purification and Western blot. (E) HEK293T cells stably expressing FLAG-tagged KEAP1 were transfected with the indicated expression constructs; associated proteins were revealed by FLAG immunoprecipitation and Western blot. (F) HEK293T cells were transfected with the indicated ET/SGE-containing protein, constitutively expressed Renilla luciferase and the NRF2-driven Firefly luciferase (NQO1-ARE). Error bars represent standard deviation from the mean from 3

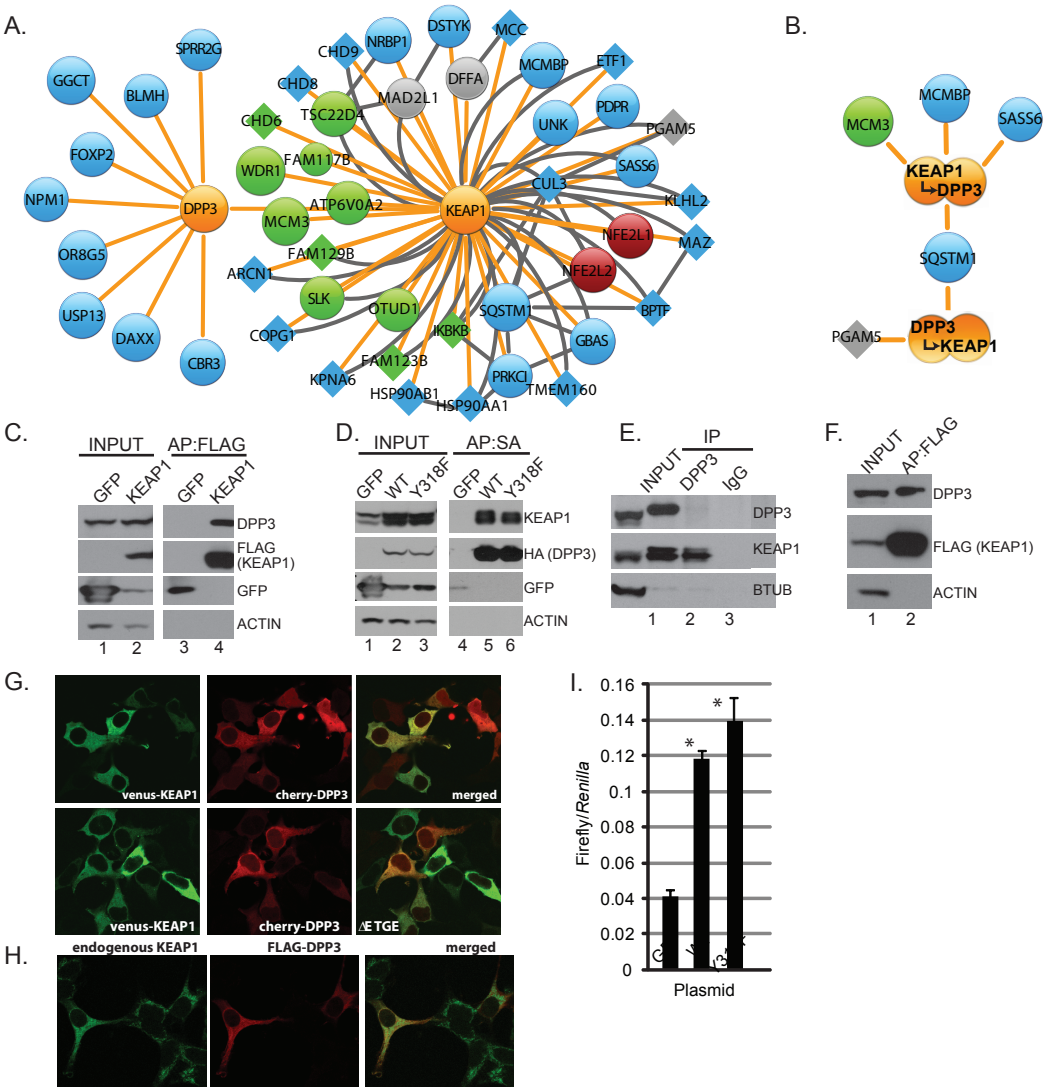

Figure 2.3. DPP3 is a KEAP1 interacting protein. (A) Schematic protein interaction network for DPP3 and KEAP1. Node and edge coloring and sizing are consistent with Figure 1. (B) HEK293T cells expressing KEAP1 and DPP3 were lysed and subjected to two sequential rounds of affinity purification before mass spectrometry. Data shown represent biological duplicate experiments, wherein the order of affinity purifications was reversed. (C) Protein complexes from HEK293T cells expressing FLAG-KEAP1 were affinity purified and analyzed by W. blot. (D) Protein complexes from HEK293T cells expressing SBPHA-DPP3 or SBPHA-DPP3-Y318F were streptavidin affinity purified and analyzed by W. blot. (E) Endogenous DPP3 from HEK293T cells was immunopurified and analyzed by W. blot for the indicated proteins. (F) Protein complexes were FLAG affinity purified from the lung adenocarcinoma cell line H2228 expressing FLAG-KEAP1 and analyzed by W. blot. (G) HEK293 cells were transfected with venus-KEAP1 and the indicated mCherry-fused DPP3 expression construct. (H) HEK293T cells transfected with FLAG-DPP3 and stained for FLAG and endogenous KEAP1. Scale =20 um. (I) HEK293T cells were transfected with NQO1-ARE-luciferase, constitutively active *Renilla* luciferase and the indicated expression plasmid before luciferase quantification (* $P<0.05$ across three biological replicate experiments).

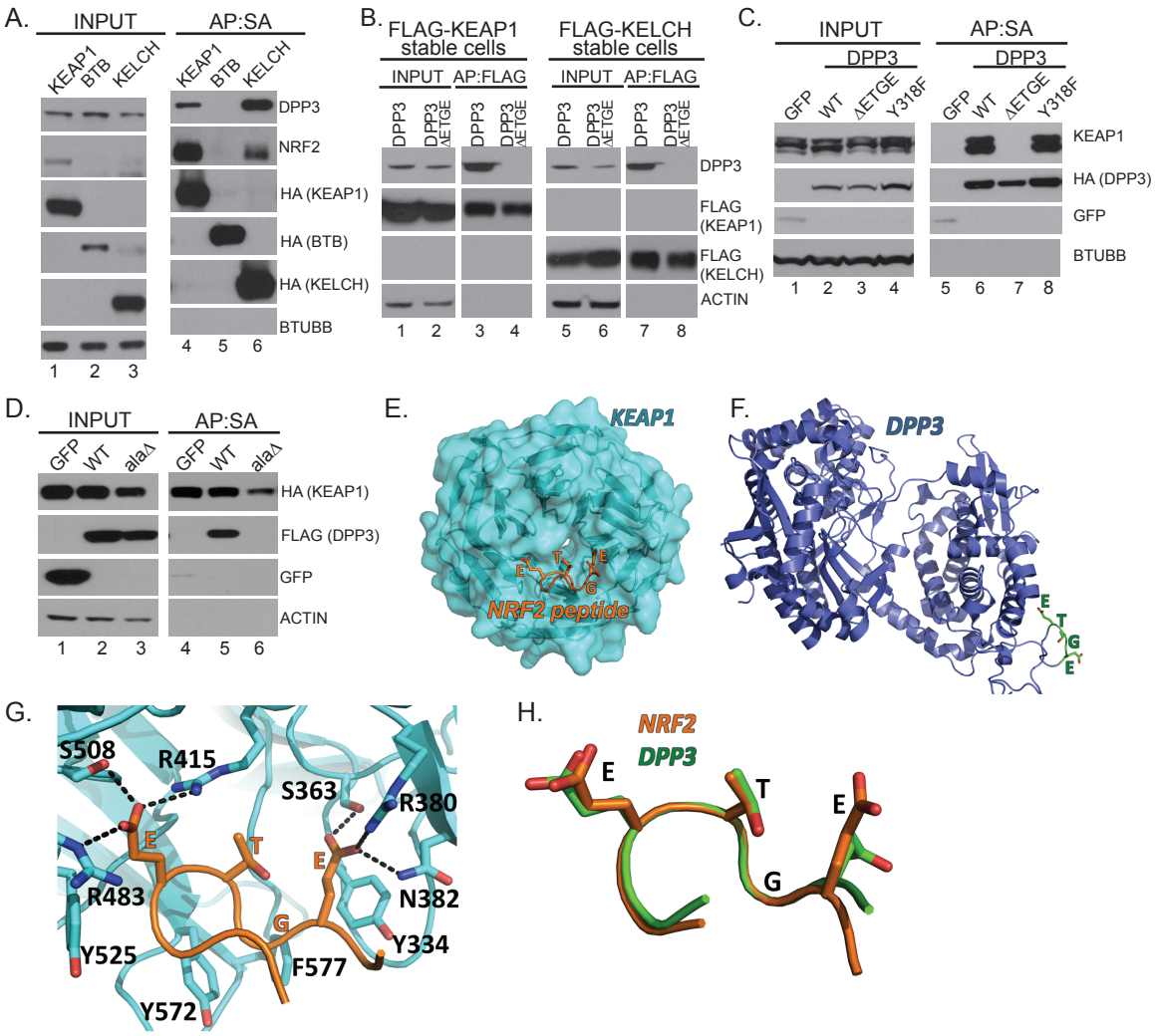

Figure 2.4. DPP3 interacts with the KELCH domain of KEAP1 via its ETGE motif. (A) Protein complexes from HEK293T cells stably expressing SBPHA-KEAP1, SBPHA-BTB, and SPBHA-KELCH were Streptavidin affinity purified and analyzed by Western blot. (B) Cells stably expressing FLAG-KEAP1 or the FLAG-KELCH domains of KEAP1 were transfected with the indicated SBPHA-DPP3 construct before affinity purification and Western blot. (C) Protein complexes from HEK293T cells stably expressing the indicated fusion protein were Streptavidin affinity purified and analyzed by Western blot. (D) HEK293T cells were transiently co-transfected with FLAG-GFP, FLAG-DPP3-WT, or FLAG-DPP3-AAGE (alaΔ) before FLAG-affinity purification and Western blot. (E) The KELCH domain of Keap1 (PDB ID 1X2R) adopts a six-bladed β-propeller structure (cyan). The ETGE motif of NRF2 (orange) binds near the central pore of the β-propeller. (F) The structure of human DPP3 (PDB ID 3FVY, blue) reveals an ETGE motif (residues 480-483, green) in an unstructured surface loop. (G) KEAP1 binding to the ETGE peptide (orange sticks) is stabilized by both hydrogen bonds (to serine and asparagine residues, cyan sticks) and electrostatic interactions (to arginine residues, cyan sticks) with KEAP1. (H) Superposition of the NRF2 ETGE motif bound to KEAP1 with the ETGE motif of DPP3 reveals similar conformations.

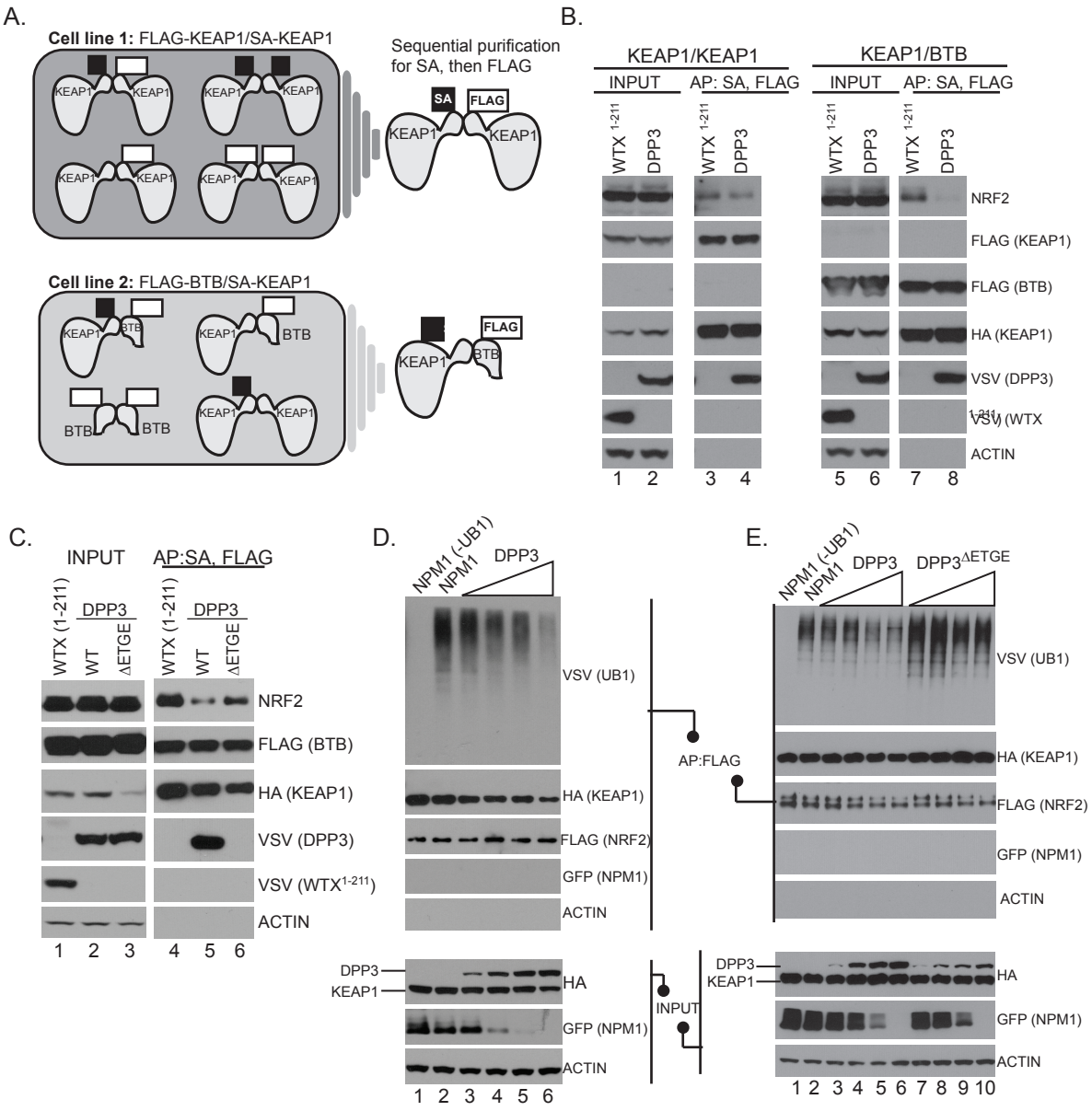

Figure 2.5. DPP3 competes with NRF2 for KEAP1 binding. (A) Schematic representation of the sequential affinity purification approach employed to purify a KEAP1-KEAP1 homodimer or KEAP1-BTB domain pseudo-monomer. (B) HEK293T cells stably expressing FLAG-KEAP1 and SBPHA-KEAP1 or FLAG-BTB and SBPHA-KEAP1 were transfected with VSV-DPP3-WT or VSV-WTX1-211 truncation mutant before sequential streptavidin and FLAG affinity purification and Western blot. (C) HEK293T cells stably expressing FLAG-BTB and SBPHA-KEAP1 were co-transfected with VSV-WTX(1-211), VSV-DPP3 or VSV-DPP3ΔETGE. Protein complexes were affinity purified with Streptavidin then FLAG and analyzed by Western blot. (D and E) HEK293T cells stably expressing SBPHA-KEAP1 were co-transfected with VSV-UB1, FLAG-NRF2, and either SBPHA-DPP3-WT, SBPHA-DPP3ΔETGE or negative control Venus-NPM1. NRF2 was FLAG affinity purified and analyzed by Western blot.

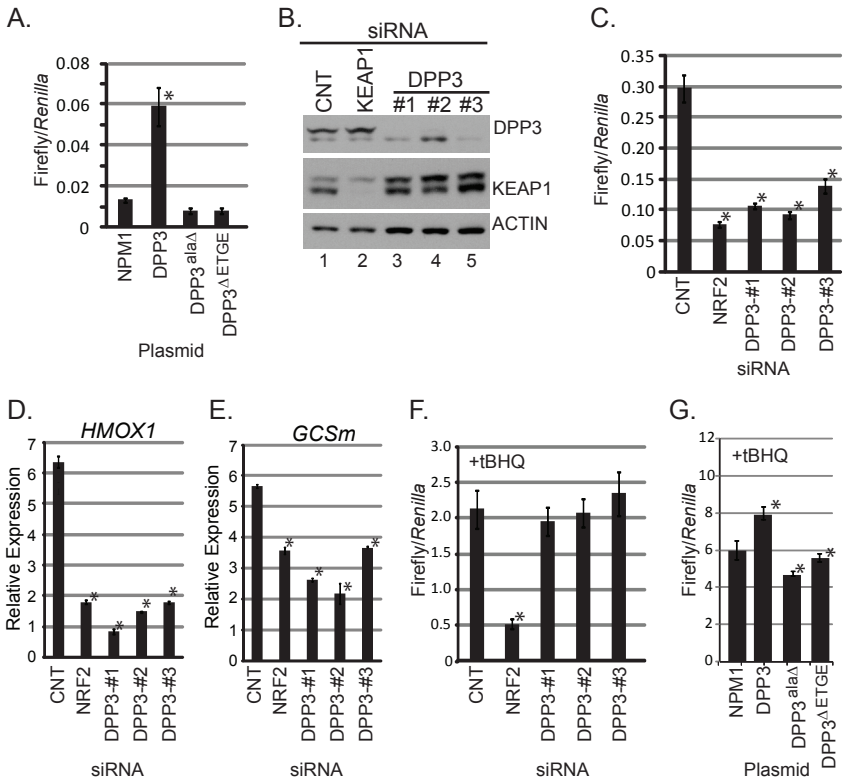

Figure 2.6. DPP3 is an activator of NRF2-mediated transcription. (A) HEK293T cells were transfected with the indicated plasmid along with constitutively expressed Renilla luciferase and the NQO-1 promoter driving Firefly luciferase (NQO1-ARE). Cells were lysed and luciferase values were normalized to Renilla. Error bars represent standard deviation from the mean over 3 biological replicates, * $p<0.05$; Students T-test as compared to NPM1. (B) HEK293T cells were transfected with 10nM of the indicated siRNA. Protein lysate was analyzed by Western blot for the indicated endogenous protein. (C) HEK293T cells stably expressing the ARE reporter and Renilla luciferase were transfected with the indicated siRNA. Error bars represent standard deviation from the mean from 3 biological replicates. * $p<0.05$; Students T-test as compared to CNT. (D and E) HEK293T cells were transfected with siRNAs against the indicated mRNAs before mRNA isolation and qPCR for the indicated endogenous target genes. Relative expression was calculated based on expression of endogenous target gene transcript normalized to GAPDH. Error bars represent standard deviation from the mean from 3 biological replicates. * $p<0.05$; Students T-test as compared to CNT. (F and G) HEK293T cells were transfected with the indicated siRNAs or plasmids. Cells were treated with 50uM tBHQ 18 h priors to lysis. Error bars represent standard deviation from the mean from 3 biological replicates. * $p<0.05$; Students T-test.

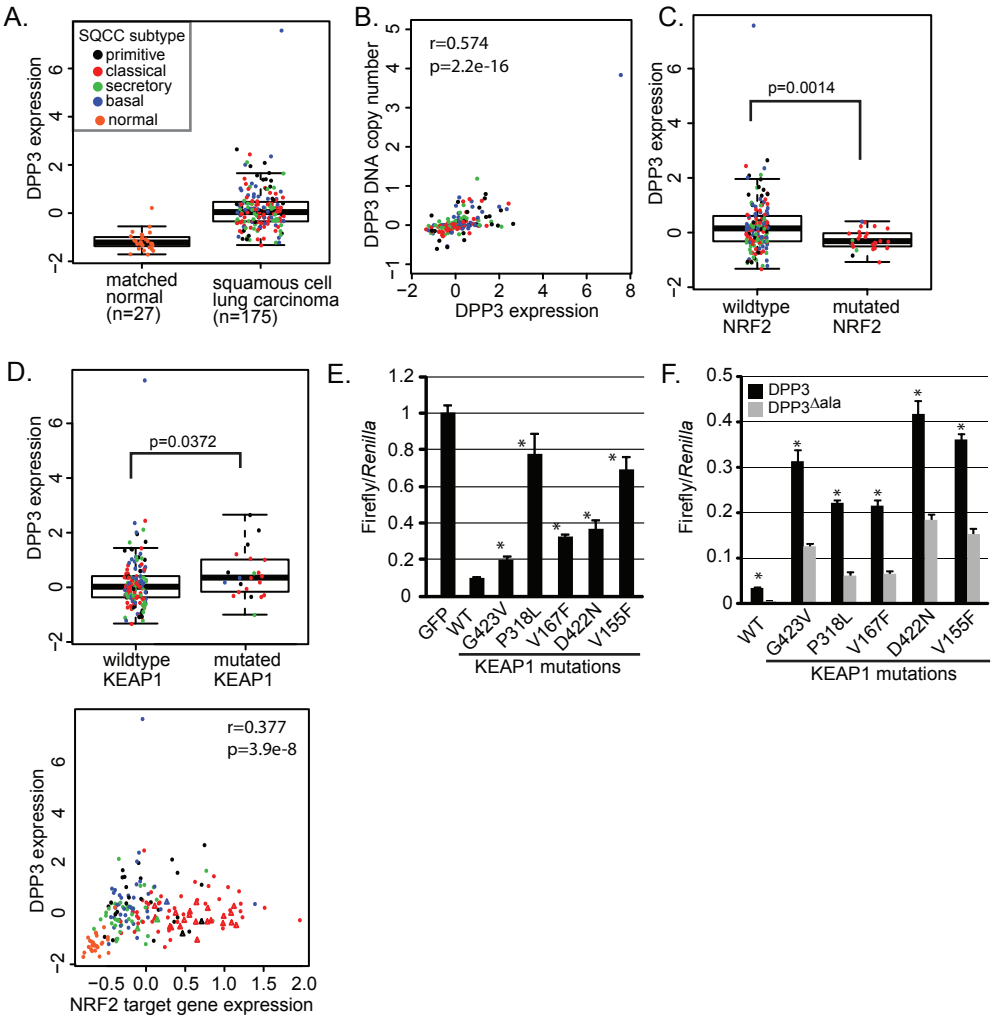

Figure 2.7. DPP3 expression positively associates with NRF2 activity in squamous cell lung cancer. (A) DPP3 mRNA abundance in 175 squamous cell lung carcinomas and 27 matched normal tissues (p=4.601e-14; Kruskal-Wallis test). Normal or lung SQCC subtype is indicated by color. With respect to tumor subtype, DPP3 is over-expression is enriched within the primitive subtype (p=0.03334; Kruskal-Wallis test; see also Figure S2). (B) DPP3 mRNA expression positively correlates with DPP3 genomic copy number (Spearman rank correlation). (C) Correlation of DPP3 mRNA expression with NRF2 mutational status (p=0.00141; Kruskal-Wallis test). (D) Correlation of DPP3 mRNA expression with KEAP1 mutational status (p=0.03718; Kruskal-Wallis test). (E and F) HEK293T cells were transiently transfected with NQO1-ARE-luciferase reporter, constitutively active Renilla reporter and the indicated expression plasmids (* P<0.05 across at least three biological triplicate experiments) (G) DPP3 mRNA expression positively associates with NRF2 target gene expression (Spearman rank correlation test). The NRF2 gene signature consists of 15 genes (3). Triangles represent tumors with NRF2 mutations. (See also Figure S2 and S3).

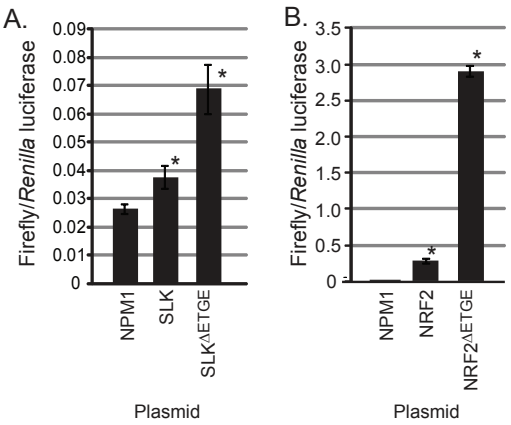

Figure S2.1. SLK promotes NRF2-mediated transcription. HEK293T cells were transiently transfected with the NQO1-ARE-luciferase reporter, constitutively active *Renilla* luciferase, and the indicated expression plasmids. Data plotted represent triplicate biological replicte experiments. ($p<0.05$).

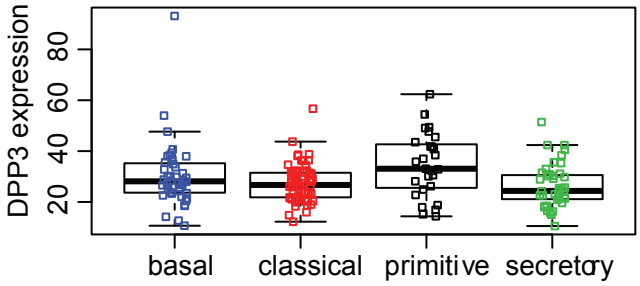

Figure S2.2. DPP3 expression is highest within the primitive subtype of lung SQCC. mRNA expression data and tumor subtyping was taken from the TCGA lung SQCC dataset. (p=0.03334; Kruskal-Wallis test).

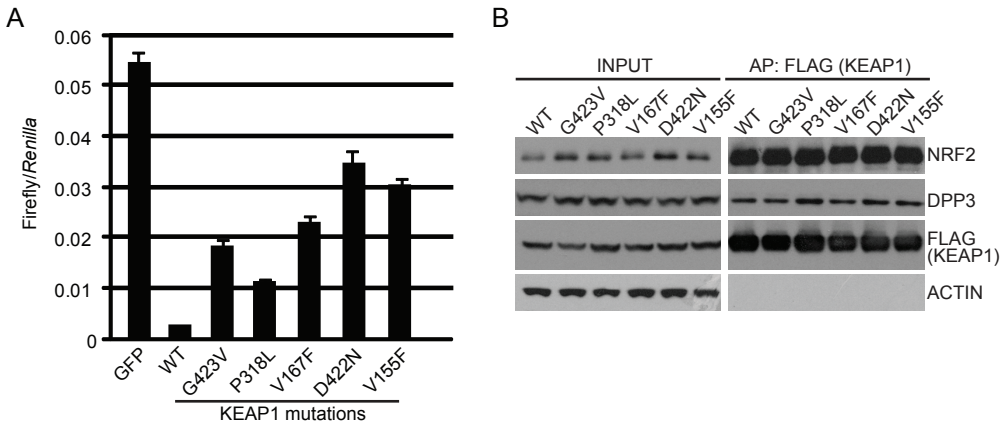

Figure S2.3. DPP3 promotes NRF2 activity in the presence of hypomorphic KEAP1 mutation. (A) KEAP1 -/- MEFs were transfected with the following constructs before luciferase quantitation: NQO1-ARE-luciferase reporter, constitutively active Renilla luciferase, and the indicated expression plasmid. (B) HEK293T cells were transiently transfected with the indicated FLAG-KEAP1 constructs before lysis and FLAG affinity purification. Western blot analysis was performed to visualize the association of endogenous NRF2 and DPP3. Cells were trated with 10uM MG132 4 hours prior to lysis.

Table S2.1 Sequence enrichment among KEAP1 interactors

Sequence	P-value proteome[a]	Rank	Sequence containing proteins[b]
ETGE	5.80e-13*	1	ATP6V0A2, DPP3, MCM3, NFE2L1, NFE2L2, OTUD1, SLK, TSC22D4, WDR1
IREL	1.31e-5	2	MCMBP, SASS6, SLK, TSC22D4, UNK
ESGE	4.03e-3	220	DFFA, MAD2L1, PGAM5

[a] Fisher's exact test using human proteome as background (see methods).
[b] Interactions passing a 10% FDR within SPOTLITE were used for testing.
* Significant using Bonferroni adjusted alpha level of 3.77e-6 (0.05 / 13265).

REFERENCES

1. Kensler, T.W., N. Wakabayashi, and S. Biswal, *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 89-116.

2. Sykiotis, G.P. and D. Bohmann, *Stress-activated cap'n'collar transcription factors in aging and human disease.* Sci Signal, 2010. **3**(112): p. re3.

3. Singh, A., et al., *RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy.* Cancer Res, 2008. **68**(19): p. 7975-84.

4. Ohta, T., et al., *Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth.* Cancer Res, 2008. **68**(5): p. 1303-9.

5. Singh, A., et al., *Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer.* PLoS Med, 2006. **3**(10): p. e420.

6. Takahashi, T., et al., *Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer.* J Surg Oncol, 2010. **101**(6): p. 500-6.

7. Cullinan, S.B., et al., *The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase.* Mol Cell Biol, 2004. **24**(19): p. 8477-86.

8. Furukawa, M. and Y. Xiong, *BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase.* Mol Cell Biol, 2005. **25**(1): p. 162-71.

9. Kobayashi, A., et al., *Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2.* Mol Cell Biol, 2004. **24**(16): p. 7130-9.

10. Ogura, T., et al., *Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening, double glycine repeat, and C-terminal domains.* Proc Natl Acad Sci U S A, 2010. **107**(7): p. 2842-7.

11. McMahon, M., et al., *Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex.* J Biol Chem, 2006. **281**(34): p. 24756-68.

12. Tong, K.I., et al., *Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism.* Biol Chem, 2006. **387**(10-11): p. 1311-20.

13. Tong, K.I., et al., *Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model.* Mol Cell Biol, 2006. **26**(8): p. 2887-900.

14. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends Biochem Sci, 2009. **34**(4): p. 176-88.

15. Hammerman, P.S., et al., *Comprehensive genomic characterization of squamous cell lung cancers.* Nature, 2012.

16. Imielinski, M., et al., *Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing.* Cell, 2012. **150**(6): p. 1107-20.

17. Konstantinopoulos, P.A., et al., *Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer.* Cancer Res, 2011. **71**(15): p. 5081-9.

18. Lister, A., et al., *Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy.* Mol Cancer, 2011. **10**: p. 37.

19. Li, Q.K., et al., *KEAP1 gene mutations and NRF2 activation are common in pulmonary papillary adenocarcinoma.* J Hum Genet, 2011. **56**(3): p. 230-4.

20. DeNicola, G.M., et al., *Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis.* Nature, 2011. **475**(7354): p. 106-9.

21. Hanada, N., et al., *Methylation of the KEAP1 gene promoter region in human colorectal cancer.* BMC Cancer, 2012. **12**: p. 66.

22. Frohlich, D.A., et al., *The role of Nrf2 in increased reactive oxygen species and DNA damage in prostate tumorigenesis.* Oncogene, 2008. **27**(31): p. 4353-62.

23. Muscarella, L.A., et al., *Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome.* Epigenetics, 2011. **6**(3): p. 317-25.

24. Je, E.M., et al., *Mutational and expressional analyses of NRF2 and KEAP1 in sarcomas.* Tumori, 2012. **98**(4): p. 510-5.

25. Camp, N.D., et al., *Wilms tumor gene on the X chromosome (WTX) inhibits the degradation of NRF2 through competitive binding to KEAP1.* J Biol Chem, 2012.

26. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response.* Mol Cell, 2009. **34**(6): p. 663-73.

27. Komatsu, M., et al., *The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1.* Nat Cell Biol, 2010. **12**(3): p. 213-23.

28. Ma, J., et al., *PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function.* Mol Cell Biol, 2012. **32**(8): p. 1506-17.

29. Liu, Y., et al., *A genomic screen for activators of the antioxidant response element.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5205-10.

30. Sardiu, M.E., et al., *Probabilistic assembly of human protein interaction networks from label-free quantitative proteomics.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1454-9.

31. Salopek-Sondi, B., et al., *Functional tyrosine residue in the active center of human dipeptidyl peptidase III.* Biol Chem, 2008. **389**(2): p. 163-7.

32. Lo, S.C., et al., *Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling.* EMBO J, 2006. **25**(15): p. 3605-17.

33. Baral, P.K., et al., *The first structure of dipeptidyl-peptidase III provides insight into the catalytic mechanism and mode of substrate binding.* J Biol Chem, 2008. **283**(32): p. 22316-24.

34. Wilkerson, M.D., et al., *Lung squamous cell carcinoma mRNA expression subtypes are reproducible, clinically important, and correspond to normal cell types.* Clin Cancer Res, 2010. **16**(19): p. 4864-75.

35. Canterini, S., et al., *Subcellular TSC22D4 localization in cerebellum granule neurons of the mouse depends on development and differentiation.* Cerebellum, 2012. **11**(1): p. 28-40.

36. Gibson, S.I., R.T. Surosky, and B.K. Tye, *The phenotype of the minichromosome maintenance mutant mcm3 is characteristic of mutants defective in DNA replication.* Mol Cell Biol, 1990. **10**(11): p. 5707-20.

37. Madine, M.A., et al., *MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells.* Nature, 1995. **375**(6530): p. 421-4.

38. Nguyen, T., et al., *Interactions of the human MCM-BP protein with MCM complex components and Dbf4.* PLoS One, 2012. **7**(4): p. e35931.

39. Takahashi, N., et al., *The MCM-binding protein ETG1 aids sister chromatid cohesion required for postreplicative homologous recombination repair.* PLoS Genet, 2010. **6**(1): p. e1000817.

40. Sakwe, A.M., et al., *Identification and characterization of a novel component of the human minichromosome maintenance complex.* Mol Cell Biol, 2007. **27**(8): p. 3044-55.

41. Cybulsky, A.V., et al., *The Ste20-like kinase SLK promotes p53 transactivation and apoptosis.* Am J Physiol Renal Physiol, 2009. **297**(4): p. F971-80.

42. Pombo, C.M., et al., *Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway.* EMBO J, 1996. **15**(17): p. 4537-46.

43. Cvrckova, F., et al., *Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast.* Genes Dev, 1995. **9**(15): p. 1817-30.

44. Kueh, H.Y., et al., *Actin disassembly by cofilin, coronin, and Aip1 occurs in bursts and is inhibited by barbed-end cappers.* J Cell Biol, 2008. **182**(2): p. 341-53.

45. Fujibuchi, T., et al., *AIP1/WDR1 supports mitotic cell rounding.* Biochem Biophys Res Commun, 2005. **327**(1): p. 268-75.

46. Barsun, M., et al., *Human dipeptidyl peptidase III acts as a post-proline-cleaving enzyme on endomorphins.* Biol Chem, 2007. **388**(3): p. 343-8.

47. Homma, S., et al., *Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer.* Clin Cancer Res, 2009. **15**(10): p. 3423-32.

48. Simaga, S., et al., *Dipeptidyl peptidase III in malignant and non-malignant gynaecological tissue.* Eur J Cancer, 1998. **34**(3): p. 399-405.

49. Simaga, S., et al., *Tumor cytosol dipeptidyl peptidase III activity is increased with histological aggressiveness of ovarian primary carcinomas.* Gynecol Oncol, 2003. **91**(1): p. 194-200.

III. CHAPTER THREE: CANCER-DERIVED MUTATIONS IN THE KEAP1 UBIQUITIN LIGASE IMPAIR NRF2 DEGRADATION BUT NOT UBIQUITINATION

3.A. OVERVIEW

Recent cancer genomics and functional analyses define the NRF2 transcription factor as an oncogene. Mutations in NRF2 localize to one of two binding interfaces, both of which mediate docking with KEAP1, an E3 ubiquitin ligase that promotes its proteasome-dependent degradation. Somatic mutations within KEAP1 are also common in human cancer, and expectedly co-mutation of KEAP1 and NRF2 is not observed. Consistent with its role as a tumor suppressor, mutations within KEAP1 are distributed throughout the full-length of the protein; whether, how and to what extent specific mutations affect KEAP1 activity is largely not known. Here we functionally and biochemically characterized 18 KEAP1 mutations taken from The Cancer Genome Atlas lung squamous cell carcinoma tumor set. Three mutations behaved as wild-type KEAP1, and are likely passenger events. The R554Q, W544C, N469fs, P318fs, and G333C mutant proteins did not bind NRF2 or suppress NRF2 activity. The remaining mutations exhibited hypomorphic suppression of NRF2, binding both NRF2 and CUL3. Proteomic study revealed that the R320Q, R470C, G423V, D422N, G186R, S243C, and V155F mutations resulted in an increased association between KEAP1 and NRF2. Intriguingly, these ‘superbinder’ mutants do not suppress NRF2 transcriptional activity and show reduced KEAP1-mediated degradation of NRF2. Cell-based and *in vitro* biochemical

analyses demonstrate that despite its inability to suppress NRF2 activity, the R320Q 'superbinder' mutant maintains its ability to ubiquitinate NRF2. These data connect KEAP1 genotype with NRF2 activity in human tumors, and provide new insight into KEAP1 mechanics.

3.B INTRODUCTION

In contrast to the mutational clustering seen in oncogenes, where a few residues are frequently affected, mutations in tumor suppressor proteins lack focal enrichment. This creates uncertainty as to the impact of specific mutations on protein function; mutations may be phenotypically silent 'passenger' events, they may result in a spectrum of hypomorphs, or produce a functionally dead protein. Catalogued associations between specific cancer genotypes and protein function will instruct many principles of cancer biology and oncology, including patient stratification for targeted therapy.

The Cancer Genome Atlas (TCGA) recently reported the characterization of 178 squamous cell lung carcinomas (SQCC), revealing at least 10 recurrently mutated genes. Among these were activating mutations in the *NFE2L2* (*NRF2*) oncogene and presumed loss-of-function mutations within the *KEAP1* tumor suppressor gene, at 15% and 12% of tumors, respectively [1]. KEAP1 functions as a substrate recognition module within the CUL3-based E3 ubiquitin ligase, which targets the NRF2 transcription factor for proteasomal degradation [2]. Regardless of tissue origin, nearly all somatic mutations within NRF2 fall to either the ETGE or the DLG motif, two regulatory short amino acid sequences within NRF2 that contact KEAP1 [3]. As such, these mutations liberate NRF2

from KEAP1-mediated ubiquitination. Comparatively, a survey of cancer genomic data revealed 213 somatic mutations dispersed across the full length of the KEAP1 protein (Fig. S3.1), a pattern consistent with the mutational spread often seen in tumor suppressor genes. Like many discoveries from genomic sequencing efforts, the functional consequences of these KEAP1 mutations are largely not known.

The lung SQCC analysis revealed that as expected, *KEAP1* mutations and *NRF2* mutations do not co-occur in the same tumor, and that tumors with *KEAP1* or *NRF2* mutations express relatively high levels of NRF2-target mRNAs [1, 4]. NRF2 target genes include a host of stress response genes, such as heme oxygenase 1 (*HMOX1*), NADPH dehydrogenase quinone 1 (*NQO1*), and genes involved in gluthathione synthesis [5]. The expression of these genes strengthens the cellular defense system to neutralize reactive oxygen species (ROS), clear xenobiotic agents, and reprogram protein degradation machinery to restore homeostasis. Recent studies also establish a role for NRF2 in modulating anabolic pathways to suit the metabolic demands of cancer cell growth, effectively yielding an increase in cancer cell proliferation [6]. Although comprehensive data are not complete, several studies have reported that NRF2 activity correlates with poor prognosis and chemotherapeutic resistance [7-9].

The now established importance of KEAP1-NRF2 in promoting cancer cell growth and survival underscores the need to elucidate how cancer evolution leads to pathway activation. Several mechanisms are easily recognized from cancer genomic studies: activating mutations in NRF2 free it from KEAP1 association [10], copy number amplifications of the NRF2 genomic locus increases protein expression, and KEAP1

promoter hypermethylation decreases its mRNA and protein expression [11-12]. What remains uncertain is which somatic mutations within KEAP1 affect its function, to what degree do they impact function, and mechanistically how its function is compromised. Recent efforts from several groups have identified correlations between cancer genotype and phenotype, and these findings may have a significant impact on clinical interventions [13-17]. With these concepts in mind, we functionally tested and biochemically characterized KEAP1 mutations found within lung SQCC. Our data connects cancer-derived KEAP1 genotypes with NRF2 phenotype. Unexpectedly, we found that many KEAP1 mutant proteins bind and ubiquitinate NRF2, but do not promote its proteasomal degradation or suppress its transcriptional activity.

3.C. MATERIALS AND METHODS

Tissue culture, transfections, and siRNAs.

HEK293T, A549, and H2228 cells were obtained from the American Tissue and Culture Collection, which authenticates cells line using short tandem repeat analysis. Cell lines were not passaged for more than 6 months after resuscitation. The KEAP1$^{-/-}$ MEFs were kindly provided by Thomas Kensler and Nobunao Wakabayshi. HEK293T cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS and 1% GlutaMAX (Life Technologies) in a 37°C humidified incubator with 5% CO_2. KEAP1$^{-/-}$ mouse embryo fibroblasts (MEF) were cultured in IMDM supplemented with 10% FBS. A549 and H2228 cells were grown in RPMI supplemented with 10% FBS. Expression constructs were transfected in HEK293T cells with Lipofectamine 2000 (Life Technologies). A549 cells and KEAP1$^{-/-}$ MEFs were transfected with Fugene HD

(Roche). Transfection of siRNA was done with Lipofectamine RNAiMAX (Life Technologies). siRNA sequences for CUL3 are as follows: (A) 5'-GGU CUC CUG AAU ACC UCU CAU UAU U, (B) 5'-GAA UGU GGA UGU CAG UUC ACG UCA A, (C) 5'-GGA UCG CAA AGU AUA CAC AUA UGU A.

Antibodies and buffers employed for Western blot analysis:

anti-FLAG M2 monoclonal (Sigma), anti-HA monoclonal (Roche), anti-βactin polyclonal (Sigma, A2066), anti-βtubulin monoclonal (Sigma, T7816), anti-KEAP1 polyclonal (ProteinTech, Chicago IL), anti-GFP (abcam, ab290), anti-NRF2 H300 polyclonal (Santa Cruz, Santa Cruz CA), anti-SLK (Bethyl, A300-499A), anti-DPP3 (abcam, ab97437), anti-MCM3 (Bethyl, A300-123A), anti-WTX [18], anti-IKKβ (Cell Signaling, 2678), anti-p62/SQSTM (Santa Cruz, sc25575), HMOX1 (abcam, ab13248), anti-CUL3 (Cell Signaling, 2759), anti-MEK1/2 (Cell Signaling, 8727), anti-histone 3 (Cell Signaling, 4499), anti-GST (Cell Signaling, 2622), and anti-VSV polyclonal (Bethyl, A190-131A). 0.1% NP-40 lysis buffer: 10% glycerol, 50mM HEPES, 150 mM NaCl, 2mM EDTA, 0.1% NP-40; RIPA buffer: 0.1% NP-40, 0.1% SDS, 10% glycerol, 25mM Tris HCl, 0.25% sodium deoxycholate, 150mM NaCl, 2mM EDTA.

Affinity purification, cell fractionation, and Western blotting.

For FLAG affinity purification, cells were lysed in 0.1% NP-40 lysis buffer. Cell lysates were cleared by centrifugation and incubated with FLAG resin (Sigma) before washing with lysis buffer and eluting with NuPAGE loading buffer (Life Technologies). For immunoprecipitation of endogenous NRF2, cells were lysed in 0.1% NP-40 lysis

buffer. Cell lysates were cleared by centrifugation, and pre-cleared for 1 hour with Protein A/G resin (Pierce). Lysates were then incubated with NRF2 H-300 antibody (Santa Cruz) overnight at 4 degree Celsius, and then incubated for 1 hour with Protein A/G resin before eluting with NuPAGE loading buffer. For siRNA, HEK293T cells were transiently transfected and lysed in RIPA buffer 60 hours post transfection. All antibodies and buffers used for Western analysis are listed in Supplementary Methods. Cell fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific).

Plasmids, expression vectors, and site-directed mutagenesis.

Expression constructs for the KEAP1 mutants were generated by PCR-based mutagenesis and sequence verified before use (GENEWIZ). The P318fs mutation was generated by PCR-based mutagenesis (Stratagene Quick Change) and sequence verified prior to use. The reporter gene fusion construct for human hNQO1-ARE-luciferase was a kind gift from Jeffrey Johnson.

ARE luciferase quantification.

Cells were transfected with expression constructs, FLAG-KEAP1, FLAG-NRF2, hNQO1-ARE luciferase, and a control plasmid containing *Renilla* luciferase driven by a constitutive cytomegalovirus (CMV) promoter. Approximately 24 hours post-transfection, NRF2-mediated transcription was measured as the ratio of Firefly to *Renilla* luciferase activity (Promega Dual-Luciferase Reporter Assay System).

NRF2 ubiquitination experiments.

Ubiquitination of NRF2 under denaturing conditions was performed in HEK293T cells stably expressing FLAG-KEAP1 wild type or R320Q, VSV-UB1, FLAG-NRF2, and Venus-NPM1. Cells were first lysed in denaturing buffer (25mM Tris, 150mM NaCl, 1% SDS, 1mM EDTA), then diluted with 0.1% NP-40 buffer, followed by immunoprecipitation of NRF2. For in vitro ubiquitination studies, GST-tagged wild-type Keap1 and the R320 mutant were over-expressed in Hi5 insect cells and purified using a glutathione affinity column. After removal of the GST tag, the proteins were further purified by ion exchange chromatography. For the *in vitro* ubiquitination assay, wild-type KEAP1 or the R320 mutant was mixed with recombinant human E1, UbcH5, CUL3-RBX1, ubiquitin and GST-tagged NRF2 NEH2 domain (GST-NRF2-Neh2) in buffer containing 40 mM Tris-HCl pH 8.0, 5 mM MgCl2, 2 mM DTT and 4 mM ATP. Ubiquitination was carried out at 37 °C and the products were analyzed by Western blot with anti-GST antibody.

Immunostaining.

HEK293T cells were cotransfected with the indicated plasmids and plated on 10 μg/mL fibronectin-coated coverslips. Cells were fixed in 4% paraformaldehyde in cytoskeletal buffer for 15 minutes, and coverslips were mounted to slides using the Prolong Gold antifade reagent (Molecular Probes). Images were acquired using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope equipped with ×63/1.42 Oil PlanApo objective lenses.

Affinity purification and mass spectrometry.

For streptavidin and FLAG affinity purification, cells were lysed in 0.1% NP-40 lysis. Cell lysates were incubated with streptavidin or FLAG resin and washed 5 times with lysis buffer. The precipitated proteins were trypsinized directly on beads using the FASP Protein Digestion Kit (Protein Discovery).

Protein identification, filtering, and bioinformatics.

All raw data were converted to mzXML format before a search of the resultant spectra using SorcererTM-SEQUEST® (build 4.0.4, Sage N Research) and the Transproteomic Pipeline (TPP v4.3.1). Data were searched against the human UniProtKB/Swiss-Prot sequence database (Release 2011_08) supplemented with common contaminants, i.e. porcine (Swiss-Prot P00761) and bovine (P00760) trypsin, and further concatenated with its reversed copy as a decoy (40,494 total sequences). Search parameters used were a precursor mass between 400 and 4500 amu, up to 2 missed cleavages, precursor-ion tolerance of 3 amu, accurate mass binning within PeptideProphet, semi-tryptic digestion, a static carbamidomethyl cysteine modification, variable methionine oxidation, and variable phosphorylation of serines, threonines, and tyrosines. False discovery rates (FDR) were determined by ProteinProphet and minimum protein probability cutoffs resulting in a 1% FDR were selected individually for each experiment. PeptideProphet/ProteinProphet results for each APMS experiment were stored in a local Prohits database. To determine an interacting protein's abundance relative to WT, prey spectral counts were bait normalized by dividing by the bait spectral count, followed by calculating the number of standard deviations from WT (similar to a

Z-score), where the standard deviation was computed for each prey individually. Unfiltered data and spectral count normalizations are provided as Supplementary Table S1.

3.D. RESULTS

3DB.1 Connecting cancer-derived KEAP1 mutations with NRF2 activity.

A search of the literature and public domain revealed 213 somatic mutations in KEAP1, observed across 17 cancer types and multiple cell lines (Table S3.1). Mapping these mutations onto the KEAP1 primary amino acid sequence revealed a relatively uniform distribution of affected residues (Fig. 3.1.A). The distribution of mutations specifically found in squamous cell lung carcinoma further reiterated the lack of a 'mutation cluster region' (Fig. 3.1.A, blue ovals). Of the 18 mutations found in lung SQCC, only two mutations resulted in a truncated protein product (N469fs and P318fs). The remaining 16 missense mutations included the addition of three new cysteine residues (G333C, W544C, and S243C), which might alter KEAP1 reactivity to electrophilic agents. One mutation, V155F occurred in two separate tumors, and interestingly, none of the mutations in KEAP1 were in residues that directly interface with NRF2 [19]. Given the importance of KEAP1-NRF2 signaling in cancer and our inability to predict the functional consequences of KEAP1 mutation, we cloned and comparatively evaluated each of the 18 lung SQCC mutations.

To test whether cancer-derived mutations in KEAP1 affect NRF2-driven transcription, we used an engineered reporter system, wherein the luciferase gene is

expressed in a NRF2-dependent manner. Ectopic expression of wild-type KEAP1 suppressed NRF2-dependent luciferase expression in HEK293T cells (Fig. 3.1.B). By comparison, the KEAP1 mutants displayed variable suppression of NRF2-driven transcription. Specifically, L231V, S224Y, and R71L suppressed NRF2 as well as wild-type KEAP1; these genotypes represent possible passenger mutations within KEAP1. By contrast, N469fs, P318fs, and G333C exhibited a null phenotype. Most surprisingly, of the 18 mutants examined, 12 retained partial ability to suppress NRF2-driven transcription. To further validate these data, we tested the panel in the KEAP1 mutant lung adenocarcinoma cell line A549 and in KEAP1 knockout mouse embryo fibroblasts (MEFs). In all three cell lines tested, we observed a largely consistent pattern of KEAP1-mediated NRF2 suppression (Fig. 3.1.C and D). These data further suggest that the genotype-phenotype relationships observed in HEK293T cells were not influenced by endogenously expressed wild-type KEAP1 protein.

At its core, this work sought to isolate and functionally annotate specific KEAP1 genotypes so that clinical correlations and predictions might be drawn from genome sequence data alone. As such, we tested whether the relative activities of each KEAP1 mutant correlated with the expression of 15 NRF2 target genes within the lung SQCC TCGA cohort [1, 4]. Comparing luciferase activity (Fig. 3.1.B-D) to the NRF2 transcriptional gene signature, we found that mutants that suppress like wild-type KEAP1 associate with decreased NRF2 activity, whereas mutants unable to suppress NRF2 correlate with increased NRF2 target gene expression ($p=0.049$; two-sided Wilcoxon

Rank Sum Test) (Fig. S3.1.A). Any attempt to further segregate mutations based on luciferase activity did not show a statistically significant correlation in the patient data.

3.D.2 Biochemical characterization of the KEAP1 mutants.

Next, we sought molecular insight into how specific mutations differentially impacted KEAP1 function. First, we determined whether the mutants expressed at levels similar to wild-type KEAP1, as non-synonymous mutations often impair protein folding to decrease protein stability. Transient expression from plasmid DNA indicates that the majority of KEAP1 mutants expressed at levels similar to wild-type protein (Fig. 3.2.A and S3.1.B). Further study is required to determine if the reduced expression of mutants R554Q, W544C, N469fs, P318fs, G480W, and G333C is due to altered protein or mRNA stability. To extend these data, the subcellular localization of KEAP1 and each KEAP1 mutant was evaluated in HEK293T cells; all mutants exhibited a localization pattern indistinguishable from wild-type KEAP1 (Fig. S3.1.C).

KEAP1 functions as a critical sensor of oxidative stress, wherein multiple cysteine residues act as biosensors for ROS and xenobiotic molecules [10, 20-21]. In cells, KEAP1 is thought to exist as a homodimer, creating a 2:1 stoichiometry with the NRF2 substrate. Following cysteine modification, either by reactive oxygen species or electrophilic agents like *tert*-butylhydroquinone (tBHQ), a conformational change within the KEAP1 homodimer creates an SDS-resistant form which is readily visualized under denaturing electrophoresis [22-23]. When treated with the pathway agonist, all 18 mutants formed an SDS-resistant dimer, suggesting that the mutations do not impair

dimerization (Fig. 3.2.A). To more rigorously test this, wild-type KEAP1 tagged with streptavidin binding peptide and the hemagglutinin epitope (SBPHA) was transfected into HEK293T cells stably expressing each FLAG-tagged KEAP1 mutant. FLAG affinity purification of the mutant protein, followed by Western blot for the HA-tagged wild-type protein was performed to evaluate KEAP1 dimerization (Fig. 3.2.B). Each KEAP1 mutant protein retained the ability to homodimerize with wild-type KEAP1.

The most likely molecular explanation for how KEAP1 mutations compromise its ability to suppress NRF2 is that the mutations impact either the KEAP1-NRF2 association or the KEAP1-CUL3 association. We evaluated whether the KEAP1 mutants maintain their ability to interact with endogenous CUL3. Affinity purification and Western blot analysis revealed that all of the KEAP1 mutants interact with CUL3 (Fig. 3.2.C and S3.2.A). Further analysis is needed to determine if the subtle differences in CUL3 binding reflect differential affinities or expression variability (Fig 3.2.C, compare lanes 15, 16, 20). Next we determined if the KEAP1-NRF2 association was maintained among the mutants. Western blot analysis of affinity purified KEAP1 and mutant KEAP1 protein complexes showed that the R554Q, W544C, N469fs, P318fs, and G333C mutants failed to bind NRF2 (Fig. 3.3.A and S3.2.B and S3.2.C). Surprisingly, however, the remaining 13 KEAP1 mutants retained NRF2 binding. Together, these data suggest that with the exception of R554Q, W544C, N469fs, P318fs, and G333C, SQCC-derived KEAP1 mutants maintain their ability to bind both NRF2 and CUL3.

Mass spectrometry-based proteomic analysis of KEAP1 revealed 42 high confidence associated proteins [4]. To gather a global perspective of how the mutations affect KEAP1 protein interactions, we performed two experiments. First, we tested the association of 7 high confidence interacting proteins by affinity purification and Western blot analysis. The data show a distinct pattern among the KEAP1 mutants; those that do not bind NRF2 fail to bind several of the known interactors, including SLK, AMER1 (WTX), MCM3, DPP3, and IKBKB (IKKβ) (Fig. 3.3.A and S3.2.C). Interestingly, all of these proteins contain an ETGE motif [4]. Two mutations, G480W and S224Y, show decreased binding to SLK, MCM3, and DPP3 as compared to NRF2 (Fig. 3.3.A, lanes 8, 15). Second, we employed affinity purification and shotgun mass spectrometry to define and compare the protein interaction network for wild-type KEAP1 and the following mutants: R554Q, R320Q, R470C, G480W, G423V, D422N, G186R, S243C, and V155F (Fig. 3.3.B and S3.2). The unbiased proteomic screens confirm the Western blot results and further expand the pattern of altered protein interactions.

3.D.3 A class of KEAP1 mutants with increased NRF2 binding.

We were particularly intrigued with a subset of mutants that consistently bound more NRF2 than wild-type KEAP1. Although we collectively refer to these mutants as the 'superbinders', relative protein affinity is not meant to be inferred. The superbinder mutants include R320Q, R470C, G423V, D422N, G186R, S243C and V155F (Fig. 3.3.A and S3.2.C, lanes 3, 5, 6, 9, 14, 16, 20). Label-free mass spectrometry comparing wild-type KEAP1 and two of these mutants (R320Q and R470C) further confirmed an increased abundance of NRF2 with these mutant protein complexes as compared to wild-

type KEAP1. For comparative purposes, we also performed quantitative proteomic analysis on two non-superbinder mutant proteins: R554Q, which cannot bind NRF2 and G480W, which binds NRF2 similarly to wild type (Fig. 3.3.B). With the exception of R554Q, in which no NRF2 was detected, both R554Q and G480W exhibited a protein interaction network similar to wild-type KEAP1 (Fig. 3.3.A).

Despite an increased level of associated NRF2, the superbinder mutants were unable to suppress NRF2-mediated transcription of an artificial reporter gene (Fig. 3.1.B-D). To confirm this using endogenous metrics of NRF2 activity, HEK293T cells, H2228 cells or A549 cells were transiently transfected with wild-type KEAP1 or the superbinder mutants before Western blot analysis of NRF2 and the NRF2 target gene HMOX1. Transient expression of each superbinder strongly increased the levels of NRF2 and HMOX1 in the H2228 and A549 cell lines (Fig. 3.4.A and B). Subcellular fractionation of the HEK293T cells further revealed that KEAP1 superbinder expression increased the levels of NRF2 within the nuclear compartment (Fig. 3.4.C and S3.3).

3.D.4 KEAP1 'superbinder' mutants facilitate NRF2 ubiquitination but not degradation.

Our functional and biochemical examination revealed 7 KEAP1 mutations that show significantly impaired ability to suppress NRF2, but yet unexpectedly bind more NRF2 than wild-type KEAP1. To gain further insight, we evaluated NRF2 protein turnover and ubiquitination following KEAP1 superbinder expression. Using a cycloheximide pulse-chase approach, NRF2 protein half-life was evaluated in HEK293T cells stably expressing: 1) wild-type KEAP1, 2) the R320Q superbinder, 3) R470C

superbinder, 3) R554Q which does not bind NRF2, or 5) G480W which behaves like wild type. The expression of R320Q or R470C dramatically stabilized the NRF2 protein as compared to wild type or G480W (Fig. 3.5.A). The increased NRF2 stability occurred as a result of binding R320Q or R470C, as unbound NRF2 in the flow-through eluate showed elevated levels but dynamic turnover (Fig. 3.5.B, compare flow-through to KEAP1 affinity purification). Together, these data suggest that the superbinder mutations within KEAP1 result in the stabilization of KEAP1-associated NRF2 and elevated levels of free NRF2, although the free NRF2 is still subject to dynamic turnover.

Given the increased NRF2 association and protein stability, we hypothesized that R320Q and other superbinder mutants impair NRF2 ubiquitination. To test this, we performed two complementary experiments to evaluate NRF2 ubiquitination by wild-type KEAP1 or the R320Q superbinder. First, Western blot analysis of immunoprecipitated NRF2, after denaturation, showed robust ubiquitination by both wild-type KEAP1 and R320Q (Fig. 3.5.C). Second, we performed *in vitro* ubiquitination reactions using purified proteins (Fig. 3.5.D). Remarkably, both experimental approaches demonstrate that wild-type KEAP1 and R320Q ubiquitinate NRF2.

3.E. DISCUSSION

With some latitude, we can classify the 18 KEAP1 mutations into three classes. First, the L231V, S224Y, and R71L mutations did not impact the KEAP1-NRF2 association or the suppression of NRF2 activity. These mutations likely represent passenger events within KEAP1, at least with respect to NRF2. Second, and not

surprisingly, the frame shift mutations N469fs and P318fs, as well as G333C, R554Q and W544C did not bind NRF2 and did not suppress NRF2-mediated transcription. These genotypes represent null or near-null alleles. Third, the remaining ten mutations fell within a hypomorphic phenotypic range, with suppression occurring between 30-60% of the wild-type KEAP1. Biochemically, the hypomorphic mutants displayed either reduced NRF2 binding or surprisingly, increased binding (the superbinders).

Mutations in tumor suppressor genes often results in complete loss of protein expression or the expression of a truncated protein product [24]. It is therefore intriguing to consider why KEAP1 is rarely lost through genomic deletion, despite being located between the SMARCA4 and STK11 tumor suppressor genes on 19p (cBioPortal). A number of loosely connected observations raise the possibility that KEAP1 may exert cancer-relevant functions that extend beyond regulation of oxidative stress and NRF2. First, we found that many KEAP1 mutations result in a hypomorphic phenotype, rather than a genetic null. Second, in general, these hypomorphic mutations do not affect the global KEAP1 protein interaction network, suggesting that some KEAP1 protein interactions are retained in the absence of NRF2 suppression (Fig. 3.3.B). Indeed, KEAP1 associated proteins regulate a number of disparate cellular processes, including cell cycle, migration, and apoptosis [4, 25-33]. Third, while the presence and importance of NRF2-independent KEAP1 functions remain unknown, we and others have established that several KEAP1 interacting proteins drive NRF2 activation via a competitive binding mechanism [4, 18, 34-36]. Previously, we found that hypomorphic KEAP1 mutants can be further inactivated by the ETGE-containing competitive binding protein, DPP3.

Coupled with the observed over-expression of DPP3 in lung squamous cell carcinoma, these observations suggest that from the perspective of cancer cell fitness, the presence of a hypomorphic KEAP1 mutation may be more valuable than a null mutant.

The most surprising and perhaps exciting discovery we observed was the identification of the 'superbinders'—those that do not suppress NRF2-mediated transcription, exhibit enhanced binding to NRF2, and facilitate NRF2 ubiquitination. Three points of discussion are appropriate. First, by what mechanism could the 'superbinder' mutations affect NRF2 stability? Several possibilities exist, including an increased affinity between KEAP1 and NRF2 as a means to suppress substrate turn-over. Analogously, the expression of a superbinder variant SH2 domain antagonizes epidermal growth factor signaling via competitive inhibition [37]. That said, although studies are ongoing, the lack of a focal enrichment within the tertiary structure casts some doubt on this possibility (Fig. 3.5.E). CRL E3 ubiquitin ligases cycle through an active and inactive state, and this neddylation-dependent transitioning is required for substrate turnover. A second possibility is that the superbinder mutations simply slow the rate of CUL3 neddylation. Finally, proteasome-mediated substrate degradation requires several steps, including recognition, unfolding, translocation, and deubiquitination prior to proteolysis [38]. The striking observation that the enhanced NRF2 binding class of KEAP1 mutants ubiquitinate NRF2 suggests that the mutations functionally hinder one of the steps prior to proteolysis, but after ubiquitination. Here, immediate questions include whether the superbinder mutations affect the ubiquitin chain linkage on NRF2 or whether they perturb the interaction of KEAP1 with the proteasome. All three of these putative

mechanisms to describe the superbinder phenotype would inactivate KEAP1 and stabilize NRF2 in a manner consistent with the widely accepted “saturation model” [21]. Importantly, as the KEAP1 mutants described in this study exhibit hypomorphic phenotypes, the superbinders could represent a novel mechanism cancer cells employ to enhance cellular fitness without compromising all cellular functions of multifunctional proteins.

Second, it is now widely accepted that elevated levels of NRF2 are associated with enhanced cell viability in several tumor types [7, 39-41]. Although we show that ‘superbinder’ mutations result in NRF2 transcriptional activation, further studies are required to determine whether this KEAP1 mutant class is capable of enhancing cancer cell fitness *in vivo,* and whether that depends upon prolonged activation of NRF2. Additionally, given emerging evidence identifying other putative KEAP1 substrates in cancer-relevant pathways, such as IKKβ within NF-κB signaling [5, 42], investigating how—if it all—superbinder mutations impact these proteins could also have clinical significance. Looking at the full set of KEAP1 mutant tumors and the expression of 15 NRF2 target genes, a marginal but statistically significant difference was observed between phenotypically ‘silent’ KEAP1 mutations and mutations which suppress KEAP1-driven NRF2 degradation (Fig. S3.1.A). Our attempts to more precisely correlate KEAP1 genotype with the cell-based phenotypic scoring failed to reach statistical significance. This is not surprising given the multitude of signaling and metabolic inputs that control KEAP1.

Third, from a structural perspective, we noted weak correlation between the tertiary position of a mutation and whether the mutation produced a KEAP1 superbinder (Fig. 3.5.E). Although speculative, the superbinder mutations appear to be localized at positions that might orient the relative position of IVR and KELCH domains; experiments testing this model are ongoing. Intriguingly, of the 181 missense mutations reported in KEAP1, 6 directly target the R320 superbinder residue, making it the most commonly affected amino acid in KEAP1 (Fig. 3.1.A). Beyond the superbinder mutations, mapping all SQCC 19 mutations onto the KEAP1 structure failed to reveal a discernible pattern. Likewise, side-chain biochemistry for the mutations varies widely, including those within the superbinder class. Cysteine reactivity depends upon the local chemical microenvironment, which is largely dictated by the surrounding amino acids in a protein tertiary structure. Hence, for a cysteine-dependent biosensor like KEAP1, oncogenesis may partially suppress KEAP1 activity by selecting for mutations which add cysteines (S243C, G333C, R470C) or which reduce the relative pKa of existing cysteines, making them more sensitive to electrophilic attack [43]. Clearly, spatial constraints preclude the random addition of cysteines as a means to increase the reactivity of KEAP1 to oxidative stress. New cancer-derived cysteines of functional importance would occupy specific localizations within the folded protein. By extension of this idea, cancer-derived mutations that create ‘hyperactive’ cysteines within KEAP1 would be expected to produce a hypomorphic phenotype, as we have observed. Further study is needed to support these ideas, perhaps through the functional and biochemical characterization of the other 213 cancer-derived mutations in KEAP1. The resulting data

may better enable predictions of genotype-phenotype relationships; based on the data presented here, it is not possible to derive functional conclusions from mutation location or residue substitution.

In summary, we describe the functional and biochemical characteristics of 18 mutations in the E3 ligase adaptor protein KEAP1, which were found in patient-derived lung squamous cell carcinomas. We show that while most of these mutations maintain similar protein interactions to wild-type KEAP1, all but three exhibit hypomorphic or null activity with respect to suppression of NRF2-mediated transcription. Intriguingly, a subset of these mutations exhibit enhanced binding to NRF2 despite an inability to suppress NRF2 activity. Functional analysis of one of these mutants, R320Q, revealed that these mutants are still able to ubiquitinate NRF2, but appear to be unable to facilitate its degradation. Further studies are required to elucidate the mechanism of this class of KEAP1 mutations, including how they interact with the proteasome, as well as whether these mutants enhance viability of cancer cells via prolonged activation of NRF2.

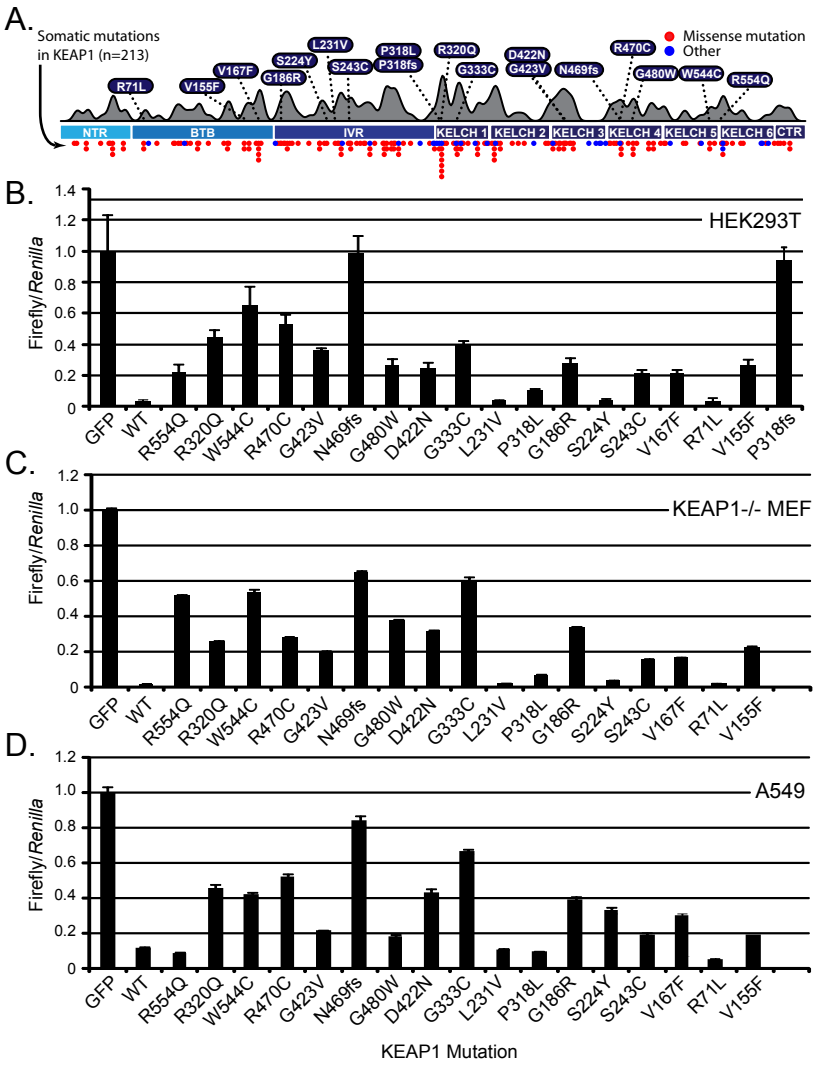

Figure 3.1. Mutations in KEAP1 positively correlate with increased NRF2 activity. (A) Probability density function of KEAP1 mutations were approximated using kernel density estimation. Mutations from SQCC are annotated above; mutations from the literature and public domain are represented by red hash marks. B-D, HEK293T cells (B), KEAP1 knockout mouse embryo fibroblasts (C), and A549 cells (D) were transiently transfected with the indicated plasmids along with constitutively expressed Renilla, and the NQO1 promoter driving Firefly luciferase. Cells were lysed and luciferase values were normalized to Renilla. Error bars represent SD from the mean over three biological replicates.

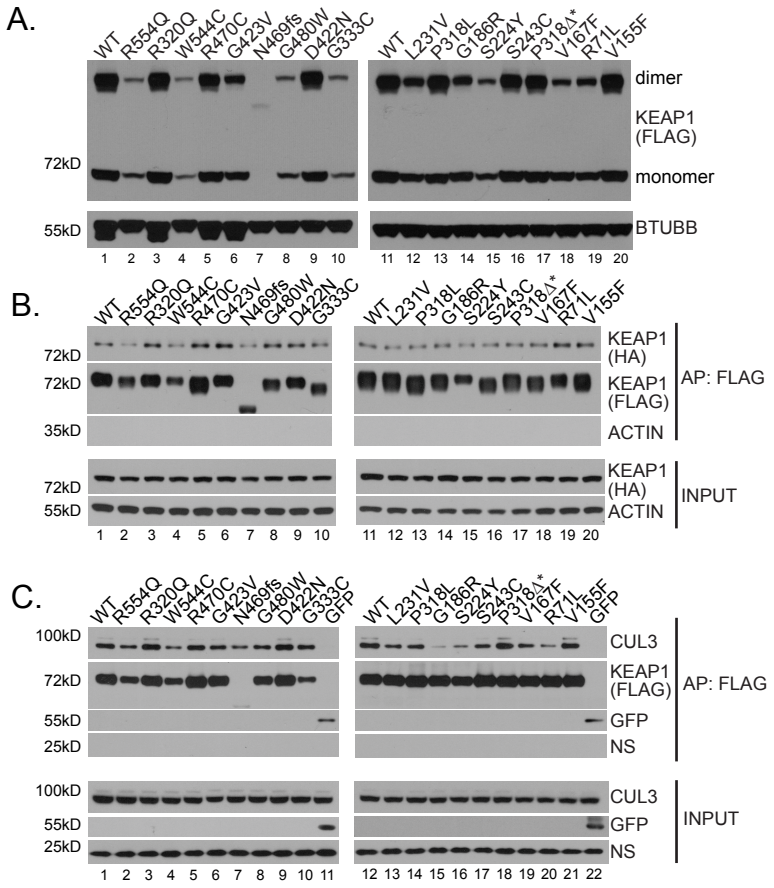

Figure 3.2. SQCC KEAP1 mutants retain the ability to dimerize and interact with the CUL3 E3 ubiquitin ligase. (A) HEK293T cells were transiently transfected with the indicated KEAP1 mutant plasmids, and treated with 50uM tert-butylhydroquinone (tBHQ) for one hour. Cells were lysed in RIPA buffer and expression of FLAG tagged mutants was analyzed by Western blot for the indicated proteins. (B) HEK239T cells stably expressing the indicated FLAG-tagged KEAP1 mutants were transiently transfected with SBPHA-KEAP1. Cells were lysed in 0.1% NP-40 buffer and affinity purification of the FLAG-tagged protein complexes were analyzed by Western blot for the indicated proteins (SBP, streptavidin binding peptide; HA, Hemagglutinin). (C) FLAG-tagged protein complexes were affinity purified from HEK293T cells stably expressing the indicated KEAP1 mutants and analyzed by Western blot for the indicated proteins.

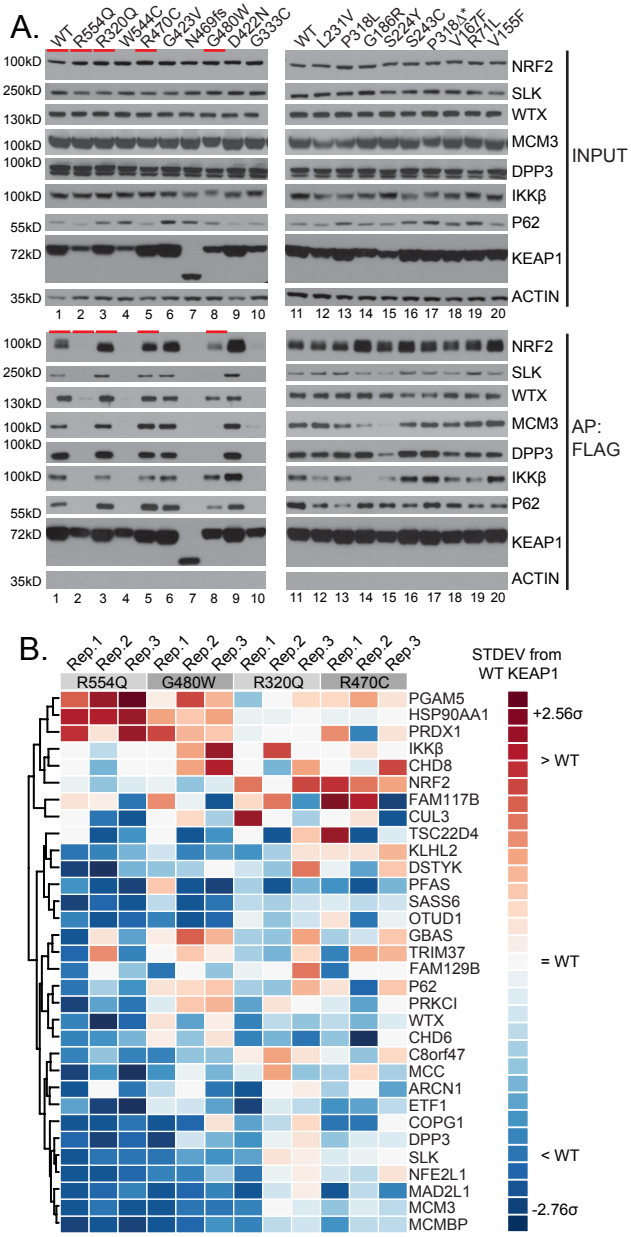

Figure 3.3. KEAP1 mutant proteins exhibit differential binding to interacting proteins. (A) HEK293T cells stably expressing the indicated KEAP1 mutants were treated with 10 uM MG132 for one hour, followed by FLAG affinity purification. Protein complexes of the FLAG-tagged mutants were analyzed by Western blot for the indicated proteins. Red lines indicate KEAP1 mutants that were analyzed by mass spectrometry as indicated in (B). B, APMS experiments were performed via affinity purification of streptavidin-tagged KEAP1 mutants from stable HEK293T cells followed by MS analysis of the bound proteins. Colors represent normalized spectral counts – semi-quantitative values that reflect protein abundance – from the APMS experiments. Proteins displayed are previously identified high-confidence KEAP1 interactors.

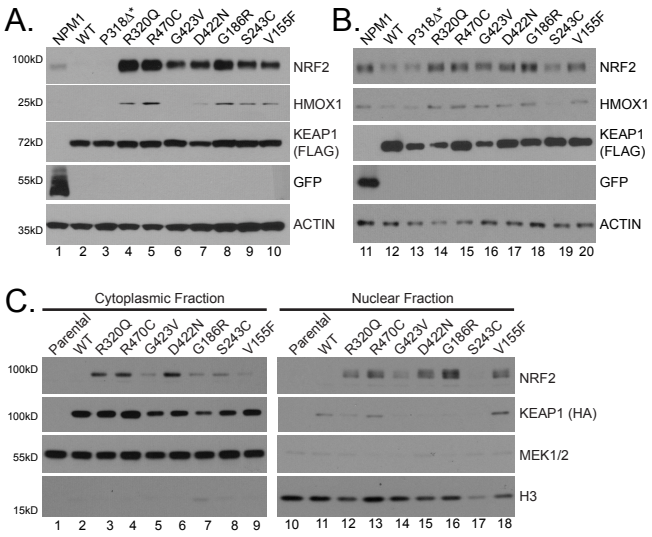

Figure 3.4. Expression of KEAP1 superbinder mutants enhances nuclear localization of NRF2. (A) H2228 cells were co-transfected with the indicated FLAG-tagged KEAP1 mutant plasmid and NRF2 plasmid. Cells were lysed in RIPA buffer and analyzed by Western blot for the indicated proteins. (B) A549 cells were transiently transfected with the indicated KEAP1 mutants, and protein lysates were analyzed as described in (A). (C) HEK293T cells were transiently transfected with the indicated FLAG-tagged KEAP1 mutants. Cells were fractionated into nuclear and cytoplasmic fractions and lysates were analyzed by Western blot for the indicated proteins.

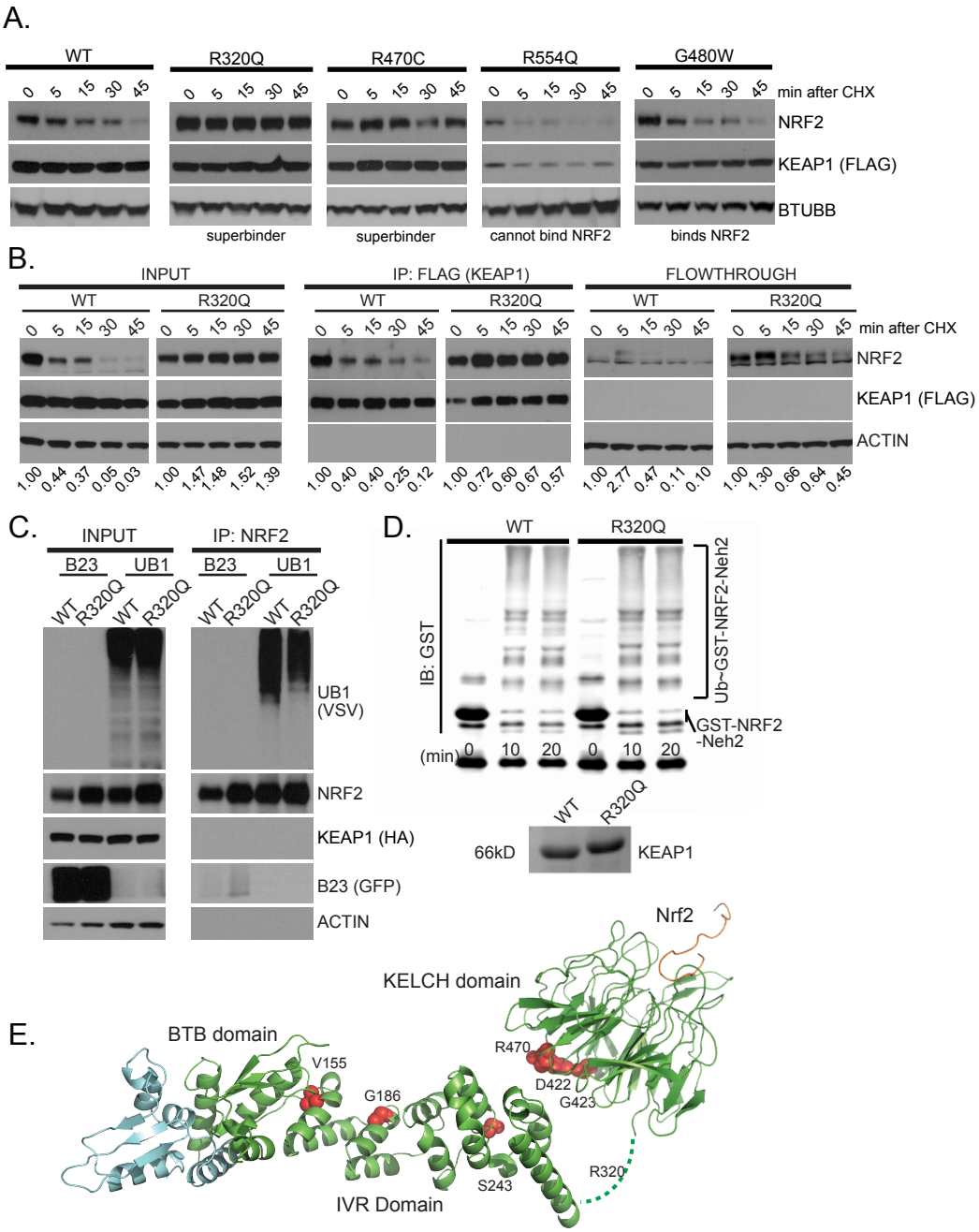
A.
WT
R320Q
R470C
R554Q
G480W
0 5 15 30 45
min after CHX
NRF2
KEAP1 (FLAG)
BTUBB
superbinder
superbinder
cannot bind NRF2
binds NRF2
B.
INPUT
IP: FLAG (KEAP1)
FLOWTHROUGH
WT
R320Q
min after CHX
NRF2
KEAP1 (FLAG)
ACTIN
1.00 0.44 0.37 0.05 0.03
1.00 1.47 1.48 1.52 1.39
1.00 0.40 0.40 0.25 0.12
1.00 0.72 0.60 0.67 0.57
1.00 2.77 0.47 0.11 0.10
1.00 1.30 0.66 0.64 0.45
C.
INPUT
IP: NRF2
B23
UB1
WT
R320Q
UB1 (VSV)
NRF2
KEAP1 (HA)
B23 (GFP)
ACTIN
D.
WT
R320Q
IB: GST
Ub~GST-NRF2-Neh2
GST-NRF2 -Neh2
(min) 0 10 20 0 10 20
66kD
KEAP1
E.
Nrf2
KELCH domain
BTB domain
V155
G186
R470
D422
G423
S243
R320
IVR Domain

Figure 3.5. KEAP1 superbinder mutants cannot degrade NRF2 but maintain the ability to ubiquitinate NRF2. (A) HEK293T cells stably expressing the indicated FLAG-tagged KEAP1 mutants were transiently transfected with NRF2. Cells were treated with 50 ug/mL cycloheximide (CHX) for the indicated time, and cell lysates were analyzed by Western blot for the specified proteins. (B) HEK293T cells stably expressing the indicated FLAG-tagged KEAP1 mutants were treated with CHX as described in (A). FLAG affinity purification was performed to isolate protein complexes containing the indicated KEAP1 mutants. Whole cell lysate (INPUT), affinity purified complexes (IP:FLAG), and eluate (FLOWTHROUGH) were analyzed by Western blot for the indicated proteins. Values represent NRF2 quantitation relative to FLAG-tagged KEAP1 expression. (C) HEK293T cells stably expressing either FLAG-tagged wild-type KEAP1 or the R320Q mutant were transfected as described in (A). Cells were lysed under denaturing conditions, and then diluted to physiological pH in 0.1% NP-40 lysis buffer. Immunopurification of NRF2 was performed, and protein complexes were analyzed by Western blot. (D) Purified KEAP1 or the R320 mutant was mixed with recombinant human E1, UbcH5, CUL3-RBX1, ubiquitin and GST-tagged NRF2 NEH2 domain. Ubiquitinated NRF2 was detected by Western blot analysis. (E) The BTB and IVR domains of Keap1 (green) were modeled by the I-TASSER server. The BTB domain of the second copy of KEAP1 within the dimer is shown in cyan. Superbinder residues are shown in red spheres. R320 is located in a predicted short linker connecting the BTB-IVR domain and KELCH domain.

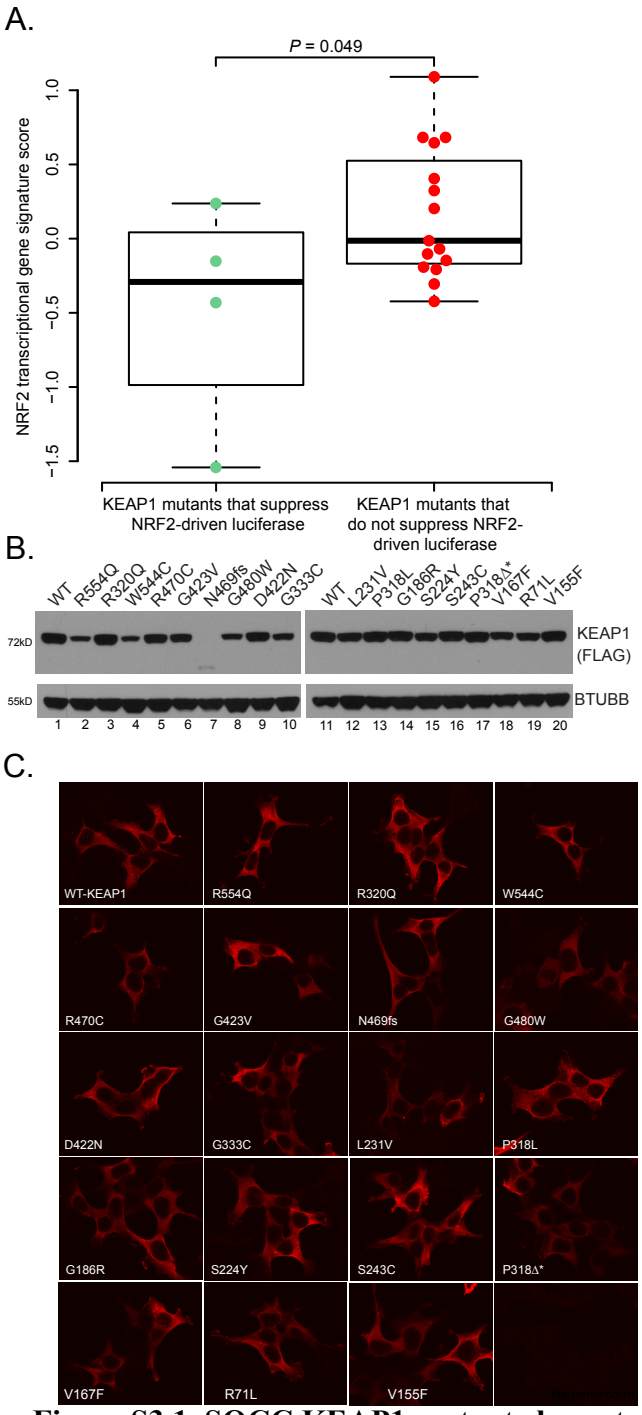

Figure S3.1. SQCC KEAP1 mutant characterization.
(A) Activity of KEAP1 mutants, as based on luciferase activity (B-D), positively correlates with NRF2 target gene expression (P=0.049, Kruskal-Wallis test). (B) HEK293T cells were transiently transfected with the indicated FLAG-tagged KEAP1 mutants and cell lysates were analyzed by Western blot.
(C) HEK293T cells were transiently transfected with the indicated FLAG-KEAP1 mutants and stained for FLAG. Scale, 20 um.

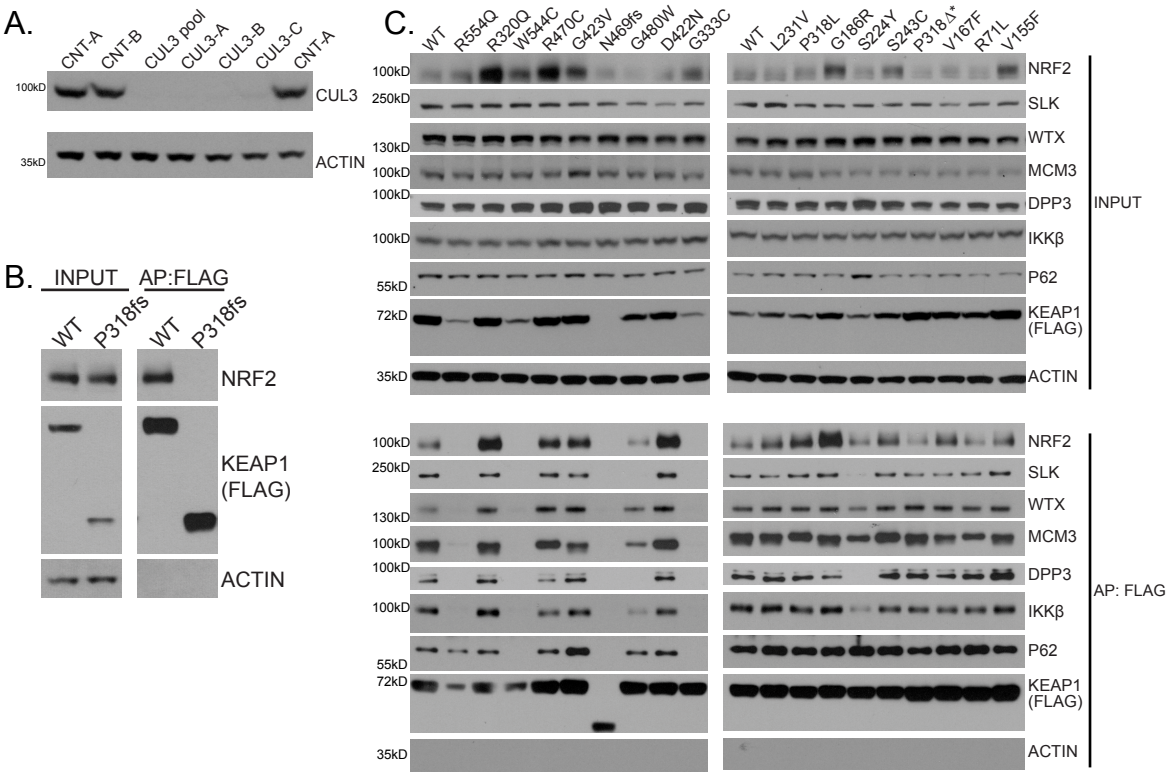

Figure S3.2. KEAP1 mutants differentially bind to interacting proteins.
(A) Validation of CUL3 siRNAs used to identify endogenous CUL3 band in Western blot. HEK293T cells were transfected with 10nM of the indicated siRNAs to CUL3. Cell lysates were analyzed by Western blot. (B) Mutant P318fs* (as seen in Figures 2, 3A, 4A and B, S1, and S2C) was originally synthesized incorrectly. The correct mutant, P318fs, was synthesized and HEK293T cells were transfected followed by affinity purification for the tagged mutant protein. Cell lysates and affinity purified protein complexes were analyzed by Western blot to assess expression of the P318fs mutant, as well as NRF2 association. (C) HEK293T cells stably expressing the indicated FLAG-tagged KEAP1 mutants were affinity purified and lysates were analyzed by Western blot.

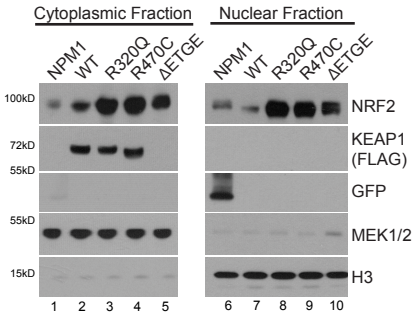

Figure S3.3. KEAP1 superbinder mutants enhance nuclear localization of ectopic NRF2. HEK293T cells were transiently co-transfected with the indicated FLAG-tagged KEAP1 mutants and NRF2. Cells were fractionated into a cytoplasmic and nuclear fraction, and cell lysates were analyzed by Western blot.

REFERENCES

1. *Comprehensive genomic characterization of squamous cell lung cancers.* Nature, 2012. **489**(7417): p. 519-25.

2. Furukawa, M. and Y. Xiong, *BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase.* Mol Cell Biol, 2005. **25**(1): p. 162-71.

3. Tong, K.I., et al., *Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model.* Mol Cell Biol, 2006. **26**(8): p. 2887-900.

4. Hast, B.E., et al., *Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination.* Cancer Res, 2013. **73**(7): p. 2199-210.
5. Wakabayashi, N., et al., *When NRF2 talks, who's listening?* Antioxid Redox Signal, 2010. **13**(11): p. 1649-63.

6. Mitsuishi, Y., et al., *Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming.* Cancer Cell, 2012. **22**(1): p. 66-79.

7. Jiang, T., et al., *High levels of Nrf2 determine chemoresistance in type II endometrial cancer.* Cancer Res, 2010. **70**(13): p. 5486-96.

8. Mahaffey, C.M., et al., *Multidrug-resistant protein-3 gene regulation by the transcription factor Nrf2 in human bronchial epithelial and non-small-cell lung carcinoma.* Free Radic Biol Med, 2009. **46**(12): p. 1650-7.

9. Rushworth, S.A., et al., *The high Nrf2 expression in human acute myeloid leukemia is driven by NF-kappaB and underlies its chemo-resistance.* Blood, 2012. **120**(26): p. 5188-98.

10. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends Biochem Sci, 2009. **34**(4): p. 176-88.

11. Muscarella, L.A., et al., *Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer.* Epigenetics, 2011. **6**(6): p. 710-9.

12. Hanada, N., et al., *Methylation of the KEAP1 gene promoter region in human colorectal cancer.* BMC Cancer, 2012. **12**: p. 66.

13. Jaiswal, B.S., et al., *Oncogenic ERBB3 Mutations in Human Cancers.* Cancer Cell, 2013. **23**(5): p. 603-17.

14. Safaee, M., et al., *Overexpression of CD97 Confers an Invasive Phenotype in Glioblastoma Cells and Is Associated with Decreased Survival of Glioblastoma Patients.* PLoS One, 2013. **8**(4): p. e62765.

15. Hakimi, A.A., et al., *Adverse Outcomes in Clear Cell Renal Cell Carcinoma with Mutations of 3p21 Epigenetic Regulators BAP1 and SETD2: a Report by MSKCC and the KIRC TCGA Research Network.* Clin Cancer Res, 2013.

16. Meng, X., et al., *TRPM7 mediates breast cancer cell migration and invasion through the MAPK pathway.* Cancer Lett, 2013. **333**(1): p. 96-102.

17. Wu, Y.M., et al., *Identification of Targetable FGFR Gene Fusions in Diverse Cancers.* Cancer Discov, 2013.

18. Camp, N.D., et al., *Wilms tumor gene on the X chromosome (WTX) inhibits the degradation of NRF2 through competitive binding to KEAP1.* J Biol Chem, 2012.

19. Lo, S.C., et al., *Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling.* EMBO J, 2006. **25**(15): p. 3605-17.

20. Cullinan, S.B., et al., *The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase.* Mol Cell Biol, 2004. **24**(19): p. 8477-86.

21. Zhang, D.D., et al., *Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex.* Mol Cell Biol, 2004. **24**(24): p. 10941-53.

22. Rachakonda, G., et al., *Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3.* Chem Res Toxicol, 2008. **21**(3): p. 705-10.

23. Fourquet, S., et al., *Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation.* J Biol Chem, 2010. **285**(11): p. 8463-71.

24. Levine, A.J., *The tumor suppressor genes.* Annu Rev Biochem, 1993. **62**: p. 623-51.

25. Fujibuchi, T., et al., *AIP1/WDR1 supports mitotic cell rounding.* Biochem Biophys Res Commun, 2005. **327**(1): p. 268-75.

26. Kato, A., et al., *Critical roles of actin-interacting protein 1 in cytokinesis and chemotactic migration of mammalian cells.* Biochem J, 2008. **414**(2): p. 261-70.

27. Takei, Y. and G. Tsujimoto, *Identification of a novel MCM3-associated protein that facilitates MCM3 nuclear localization.* J Biol Chem, 1998. **273**(35): p. 22177-80.

28. Kubota, Y., et al., *Identification of the yeast MCM3-related protein as a component of Xenopus DNA replication licensing factor.* Cell, 1995. **81**(4): p. 601-9.

29. Young, M.R. and B.K. Tye, *Mcm2 and Mcm3 are constitutive nuclear proteins that exhibit distinct isoforms and bind chromatin during specific cell cycle stages of Saccharomyces cerevisiae.* Mol Biol Cell, 1997. **8**(8): p. 1587-601.

30. Wagner, S., et al., *Association of the Ste20-like kinase (SLK) with the microtubule. Role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading.* J Biol Chem, 2002. **277**(40): p. 37685-92.

31. Wagner, S., et al., *FAK/src-family dependent activation of the Ste20-like kinase SLK is required for microtubule-dependent focal adhesion turnover and cell migration.* PLoS One, 2008. **3**(4): p. e1868.

32. Sabourin, L.A. and M.A. Rudnicki, *Induction of apoptosis by SLK, a Ste20-related kinase.* Oncogene, 1999. **18**(52): p. 7566-75.

33. Hao, W., et al., *Induction of apoptosis by the Ste20-like kinase SLK, a germinal center kinase that activates apoptosis signal-regulating kinase and p38.* J Biol Chem, 2006. **281**(6): p. 3075-84.

34. Ma, J., et al., *PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function.* Mol Cell Biol, 2012. **32**(8): p. 1506-17.

35. Komatsu, M., et al., *The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1.* Nat Cell Biol, 2010. **12**(3): p. 213-23.

36. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response.* Mol Cell, 2009. **34**(6): p. 663-73.

37. Kaneko, T., et al., *Superbinder SH2 domains act as antagonists of cell signaling.* Sci Signal, 2012. **5**(243): p. ra68.

38. Finley, D., *Recognition and processing of ubiquitin-protein conjugates by the proteasome.* Annu Rev Biochem, 2009. **78**: p. 477-513.

39. Ohta, T., et al., *Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth.* Cancer Res, 2008. **68**(5): p. 1303-9.

40. Konstantinopoulos, P.A., et al., *Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer.* Cancer Res, 2011. **71**(15): p. 5081-9.

41. Cong, Z.X., et al., *ERK and PI3K signaling cascades induce Nrf2 activation and regulate cell viability partly through Nrf2 in human glioblastoma cells.* Oncol Rep, 2013.

42. Kim, J.E., et al., *Suppression of NF-kappaB signaling by KEAP1 regulation of IKKbeta activity through autophagic degradation and inhibition of phosphorylation.* Cell Signal, 2010. **22**(11): p. 1645-54.

43. McMahon, M., et al., *Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18838-43.

CHAPTER IV: DISCUSSION

4.A. Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination

In this study we demonstrate that proteins within the KEAP1 interaction network containing the ETGE amino acid sequence comprise a group of interactors that compete with NRF2 for binding to the KELCH domain of KEAP1. Some of the proteins identified, such as DPP3, are able to indirectly activate NRF2 signaling via a competitive binding mechanism, which suggests that the low affinity DLG motif of NRF2 is displaced from KEAP1 by the ETGE motif of the competitive interactor. Data suggestive of a competitive binding mechanism for activation of NRF2 outside of oxidative stress has been supported for several years [1-4]. Our study conclusively demonstrates that the ETGE motif is overrepresented in the KEAP1 PIN, and that competitive binding is likely a ubiquitous mechanism for NRF2 activation.

4.A.1 Exploring the potential role of DPP3 expression in cancer

With respect to DPP3, this paper further supports the notion that overexpression of *DPP3* may have a defined role in cancer cell survival and progression. *DPP3* is overexpressed in ovarian and endometrial cancer, and this expression correlates with poor prognosis for ovarian cancer [5, 6]. A role for aberrant KEAP1-NRF2 signaling in ovarian cancer progression, malignancy, and chemotherapeutic resistance has also emerged [7], and

although not directly linked in the literature, it is possible that DPP3 overexpression may contribute to enhanced NRF2 activation in this pathology. Prior to our study, expression of DPP3 had not been investigated in lung cancer. While further studies are required to confirm that DPP3 is overexpressed, particularly at the protein level, we were surprised to find a strong correlation between *DPP3* mRNA expression and the *NRF2* gene signature in the squamous cell lung carcinoma TCGA dataset (Fig. 2.7) [3, 8]. Co-expression of DPP3 with KEAP1 mutations from the TCGA sequencing consortium was also found to further activate NRF2-mediated transcriptional activity (Fig. 2.7.F). This data further supports both the competitive binding mechanism for NRF2 activation, as well as speculation that KEAP1 mutations are largely hypomorphic with respect to suppression of NRF2. While the importance of overexpression of ETGE-containing proteins, like DPP3, in *KEAP1* mutant backgrounds will be discussed in a later section, several other unanswered questions remain with respect to the function and regulation of both DPP3 and other ETGE-containing proteins.

4.A.1.a Validation of the Competitive Binding Model

The competitive binding model is built on the foundation that the ETGE motif of NRF2 is required for KEAP1 interaction, whereas the DLG motif is largely required only for ubiquitination and degradation [9-11]. When the DLG motif is “latched” onto one of the KELCH domains of the KEAP1 homodimer, the seven lysine residues within the N-terminal alpha helix of NRF2 are in a sterically favorable conformation for ubiquitination. “Unlatching” the DLG following an oxidative stress-induced conformational change in KEAP1 results in a sterically unfavorable condition for NRF2 ubiquitination. In the

competitive binding model, an ETGE-containing interacting protein would act similarly to cysteine modification by ROS to dissociate the DLG of NRF2 from KEAP1. While this model most ideally agrees with current data describing the KEAP1-NRF2 interaction—namely by the relative affinities of DLG and ETGE, the observed maintained KEAP1-NRF2 interaction under both oxidative stress [12-14], and expression of ETGE-containing proteins [1-4]—we cannot rule out the possibility that ETGE-containing proteins could completely liberate NRF2 from KEAP1. The following experiments may be performed to validate the competitive binding model. Note that DPP3 will be used as an example ETGE-containing protein in the following experiments, however any confirmed KEAP1 ETGE interacting protein could be used as well.

In vitro binding to establish formation of a KEAP1-NRF2-DPP3 complex

In vivo experiments suggest that DPP3 can bind to the KEAP1-NRF2 complex to form a trimer (Fig. 2.5.C); however, *in vitro* binding assays will validate the existence of this putative protein complex. Equal molar ratios of affinity-tagged KEAP1, NRF2, and DPP3 should be incubated in buffered Tris using the following combinations: KEAP1 and NRF2 only, KEAP1 and DPP3 only, KEAP1, NRF2, and DPP3, and NRF2 and DPP3 only. Purification of KEAP1 using the appropriate affinity bead resin, followed by Western blot analysis can be used to determine which protein complexes exist after *in vitro* binding.

The expected results of this experiment would include identification of an interaction between KEAP1 and NRF2, as well as KEAP1 and DPP3. Additionally, this experiment will further establish the presence of a KEAP1-NRF2-DPP3 trimer, in agreement with the data

presented in Figure 2.5.C. A follow-up to this experiment would include modulating levels of the DPP3, while keeping KEAP1 amounts consistent. For example, while holding KEAP1 and NRF2 amounts constants, increasing amounts of DPP3 should be added, followed by affinity purification and analysis of protein complexes by Western blot. A non-specific protein such as GST, as well as DPP3-ΔETGE or the alanine mutant of DPP3 should be used as negative controls. One would expect to not see displacement of NRF2 until concentrations of the DPP3 are prohibitively high, while formation of a KEAP1-NRF2-DPP3 complex is observed in Western blot. It should be noted, however, that while *in vitro* binding experiments are more than sufficient for identifying a KEAP1-NRF2-DPP3 trimer, it does not account for the relative endogenous amounts of each protein. Consequently, titrating levels of DPP3 may entirely displace NRF2 from KEAP1, even if this is an extremely unlikely event *in vivo*. Collection of the flowthrough for each fraction after affinity purification may be used to determine if and/or when the DPP3 fully displaces NRF2, as NRF2 protein would be detectable in Western blot of the eluate.

Surface Plasmon Resonance to identify KEAP1-NRF2-DPP3 complex, and determine the affinity of NRF2 and DPP3 for binding to KEAP1

Surface Plasmon Resonance (SPR) is a technique that uses changes in reflected light to determine dynamics of biomolecular interactions in a liquid. Changes in the refractive index of the liquid at the surface of the crystal, which are detected because at least one protein component is immobilized on the surface, alter how light is reflected. Consequently, these changes in reflected light can be extrapolated to determine how protein-protein interactions are changing within the solution [15].

To identify dynamic changes in the KEAP1-NRF2 complex, purified KEAP1 should be immobilized on the surface of the SPR crystal. A solution containing purified NRF2 will be flowed over the surface, followed by a solution containing purified DPP3. Purified DPP3-ΔETGE or alanine mutant may be used as negative controls. In a typical SPR assay in which the binding affinity of a protein or ligand is determined, after flowing solution containing the ligand over the SPR surface, a buffer solution is used to cause dissociation of the ligand. Flowing a solution with purified DPP3 over the SPR surface after NRF2 should not cause dissociation of NRF2 at low concentrations of DPP3, but will cause subtle changes in the refractive index of the liquid if DPP3 binds to KEAP1 with NRF2. If increasing molar solutions of DPP3 are used, however, one may expect to see an additional change in the refractive index, which may result from DPP3 inducing full displacement of NRF2. Although once again exceedingly high concentrations of DPP3 may fully displace NRF2 from KEAP1, which may be irrelevant at endogenous concentrations, utilization of SPR will further validate the formation of a KEAP1-NRF2-DPP3 complex.

SPR can also be employed to determine the binding affinities of DPP3 and NRF2 for KEAP1. Similar to the experiments previously outline, KEAP1 would be immobilized on the SPR crystal surface, but rather than sequentially flowing solutions containing both NRF2 and DPP3 over the surface, a single solution containing only NRF2 or DPP3 would be used. To dissociate NRF2 or DPP3 from KEAP1 and find the Kd for each protein, a peptide designed by the Kuhlman lab (KEAP1 monobody), which binds to KEAP1 with greater affinity than the ETGE peptide of NRF2 [16] will be used. This experimental design can be further

augmented to establish whether DPP3 or NRF2 would dissociate first from KEAP1. Briefly, after flowing a solution of NRF2 and DPP3 over immobilized KEAP1 to establish a KEAP1-NRF2-DPP3 trimer, a solution of increasing concentrations of KEAP1 monobody will flowed over the SPR surface. We would expect the lower affinity protein to dissociate first. If DPP3 dissociates prior to NRF2, we can conclude that endogenously, it is likely that DPP3 only competes with the DLG motif of NRF2, thus supporting the competitive binding model. In the event NRF2 dissociates first, we will still be unable to refute complete displacement of NRF2 from KEAP1. This observation would not prove the competitive binding model incorrect; it would simply add an additional component in which complete dissociation of NRF2 is possible, and likely dependent on the relative affinities of NRF2 and the ETGE-containing protein of interest.

4.A.1.b Weighing the odds for a role of DPP3 peptidase activity in KEAP1-NRF2 signaling

Interest in *DPP3* as a regulator of KEAP1-NRF2 activity was two-fold. First, *DPP3* was already published as a pathway activator and our gain-of-function assays agreed with this observation. Second, it has a confirmed catalytic activity as a peptidase [17-19], and is therefore a potential therapeutic target if the peptidase activity is required for activation of NRF2. Particularly intriguing was that the peptidase activity of DPP3 was specific to the N-terminus of a substrate. The DLG motif of NRF2 comprises residues 29-31, and is largely considered to be more critical for regulation of NRF2 degradation and stabilization than that ETGE motif [9, 11]. In the event DPP3 is a processive peptidase, it could cleave residues from the N-terminus of NRF2 through the DLG motif. Should this occur, even if DPP3 dissociated from KEAP1, the N-terminus of NRF2 could not re-bind to KEAP1, thus

perpetuating NRF2 activation via the saturation model. While this model would not likely affect initial activation of NRF2, as this occurs via competitive binding and displacement of the DLG, it could prolong activation of NRF2, particularly if DPP3 interaction with and dissociation from KEAP1 does not permanently alter tertiary complex structure like is thought to occur with cysteine modification [20, 21].

Several biochemical and structural properties of DPP3 support the hypothesis that its catalytic activity could possibly impact modulation of KEAP1-NRF2 pathway activity. First, the substrate binding cleft of DPP3 is very large, which indicates it could accommodate a diverse set of substrates [22]. Furthermore, it has been proposed that the large binding pocket of DPP3 may be indicative of substrate specificity being attained by interaction with other proteins [22, 23]. Interaction with KEAP1 via the ETGE motif could mediate the "specificity" required for NRF2 to be a DPP3 substrate. Second, another factor mediating N-terminal peptidase specificity is recognition of a four to five amino acid stretch in the substrate that occurs around a scissile bond in the active site of the peptidase [23, 24]. Unlike most peptidases in the same family, DPP3 does not have this requirement, further suggesting that DPP3 has the potential to act on a wide range of substrates. Finally, the DLG and ETGE regions of NRF2 are thought to be largely unstructured. Consequently, accommodating the region containing the DLG into the substrate binding cleft of DPP3 is likely more easily attained than if the region had a defined secondary structure, such as the alpha helix that separates the DLG and ETGE.

The experiments presented in Chapter Two suggest that the catalytic activity of DPP3 is not required for activation of NRF2 (Fig. 2.3.I and 2.4.C). One potential criticism of this data, however, is that all of the experiments were performed at steady state. To more rigorously test if the catalytic activity of DPP3 catalytic activity affects NRF2 stability, a cell extract-based degradation assay could be employed. Incubating S^{35}-labeled NRF2 with KEAP1 and either wild-type DPP3 or the Y318F catalytic mutant would allow for DPP3 to cleave the N-terminus of NRF2 once bound to KEAP1 [4]. Following exposure to DPP3, the labeled NRF2 would be added to cell free extracts for determination of half-life. If DPP3 cleaves NRF2, a prolonged NRF2 half-life would be expected in samples treated with wild-type DPP3 compared to the Y318F mutant. A caveat of this assay is that a prolonged NRF2 half-life could also be indicative of a DPP3-mediated cleavage event in KEAP1 that renders KEAP1 inactive. To help resolve this issue, incubation of recombinant NRF2 with wild-type or Y318F mutant DPP3 prior to addition to the cell-free extract can be done. Additionally, mass spectrometry-based techniques, such as N-terminal specific labeling with a reagent like *N*-hydroxysuccinimide [25], of NRF2 pre- and post-incubation with DPP3 would reveal N-terminal cleavage events specific to NRF2.

In addition to our experimental data suggesting the catalytic activity of DPP3 does not enhance NRF2 activity, additional biochemical characteristics of DPP3 suggest the probability of NRF2 being a substrate is very low. First, currently the only known endogenous substrates of DPP3 are enkephalins, a family of pentapeptides that modulate nociception [26-28]; cleavage of enkephalins by DPP3 results in their subsequent degradation [26]. DPP3 cleaves strictly at the N-terminus and only two amino acid residues

at a time [22, 23]. Coupled with the proposed substrates consisting only of pentapeptides, it is unlikely that DPP3 acts processively. In addition, despite having a comparatively large substrate binding cleft, the accepted length of peptides that can fit in this pocket is limited to 3-10 residues, and the C-terminus of a putative substrate will encounter much greater steric hindrance due to the shape of the cleft [23, 29]. The alpha helix of NRF2 begins at residue 39; further structural studies would be required to validate this secondary structure would not fit within the binding cleft of DPP3, although the scenario is unlikely.

4.A.1.c Investigating whether DPP3 enhances cell viability

Overexpression of NRF2 is associated with enhanced cell viability in response to chemotherapeutic insult [30-32], and similarly, siRNA-mediated knockdown of *NRF2* enhances sensitivity to chemotherapeutic insult [32]. As DPP3 activates NRF2-mediated transcription, it stands to reason that overexpression of DPP3 should also enhance cell viability.

To establish if DPP3 can mediate cell viability in a NRF2-dependent manner, DPP3 wild-type and DPP3-ΔETGE should be overexpressed, followed by treatment with several chemotherapeutics, such as doxorubicin, cisplatin, and etoposide. Cell viability may be assessed with multiple assays, including MTT and caspase 3/7, as well as Western blot analysis for apoptotic markers (cleaved PARP, caspase-3). In addition to cell viability, assays that measure cytosolic redox should be used to evaluate whether DPP3 overexpression also reduces global intracellular ROS levels. These assays would be set up similarly to viability assays, but use of oxidation-sensitive dyes, such as DCF (dichlorofluorescein), are used to

assess redox status. Although the model of DPP3-mediated NRF2 activation acknowledges a phenotype associated with DPP3 overexpression, endogenous DPP3 may promote some level of basal NRF2 activation. A role for endogenous levels of DPP3 supporting a basal level of NRF2 activation is supported by Figure 2.6, which demonstrates siRNA-mediated knockdown of *DPP3* reduces NRF2-mediated transcription. For completeness, siRNA-mediated knockdown of *DPP3* should also be assessed in the previously described assays for its potential to decrease cell viability and intracellular redox potential.

4.A.1.d Exploring the role of DPP3 in tumorigenesis: would a mouse model be worthwhile?

An emerging model of cancer progression states that mutations in "driver genes" that support cellular proliferation and growth compound to ultimately result in a malignant tumor. Importantly, a "gateway" mutation must first occur, after which subsequent mutations further promote tumorigenesis [33]. Collectively, this model suggests that malignant progression is not the result of one mutation, but rather the combination of several mutagenic events that increase global cancer cell fitness. In addition to mutation, other genetic events, like copy number amplification, translocation, and promoter modifications, also contribute to tumorigenesis. These genes may be referred to as "epi-driver genes", rather than "mut-driver genes". Typically, epi-driver genes are not frequently mutated, and mut-driver genes are not associated with epigenetic events [33]. Intriguingly, *NRF2* appears to fall into a classification as both an epi-driver gene and a mut-driver gene. Several cancers have been found to have mutations in the DLG or ETGE motif [34-36], suggesting *NRF2* has a role as a mut-driver gene. Other cancers define *NRF2* as an epi-driver gene, as *KEAP1* inactivation or *NRF2* copy number amplification result in increased expression of *NRF2* [37-42]. Still, other cancers

such as lung cancer, exhibit both activating mutations in *NRF2*, in addition to *NRF2* overexpression [8, 39-41, 43-46].

While the exact role that NRF2 plays in tumorigenesis has yet to be fully elucidated, several studies demonstrate that NRF2 expression promotes cell survival and tumor growth, particularly with concurrent KRAS mutations [47, 48]. The growing acceptance of the competitive binding model as a biologically relevant mechanism for activating NRF2 provides an interesting opportunity to investigate how *NRF2* as a driver gene is affected by other genetic events to contribute to tumor progression. *DPP3* expression correlates with both poor prognosis in ovarian cancer [5] and *NRF2* gene signature in squamous cell lung cancer [3]; however, based on the data presented in Chapter Two, it is unlikely that DPP3 overexpression alone will be able to promote tumorigenesis. Introducing *DPP3* into a mouse xenograft model that couples *NRF2* overexpression with a gateway mutation, such as an activating KRAS mutation, could provide valuable insight into how ETGE-containing proteins affect cancer cell survival *in vivo*.

Incorporating *DPP3* overexpression into a cancer model with NRF2 would aid in elucidating whether correlations with DPP3 and poor prognosis, such as that in ovarian cancer, are indicative of an underlying mechanism of promoting cancer cell fitness. Performing these experiments in *Nrf2* knockout animals would also confirm that any correlation between *DPP3* expression and prognosis is indeed NRF2-dependent. Furthermore, linking *DPP3* overexpression with *NRF2* activation in a tumor model would

certainly establish a greater clinical relevance for the competitive binding model, and provide precedence for potentially investigating other ETGE-containing proteins in a similar manner.

4.A.2 The ETGE-dependent interactors: Novel substrates, KEAP1-NRF2 pathway modulation, and NRF2-independent functions of KEAP1

The experiments presented in Chapter Two introduce several other ETGE-containing proteins, some of which may have a functional role in activation of NRF2 signaling, or in modulating putative NRF2-independent functions of KEAP1. An interesting observation of the KEAP1 PIN, particularly with respect those proteins that contain an ETGE motif, is that these proteins function in a highly diverse set of cellular functions. It should be noted that whereas some of these functions could be linked to signal transduction pathways and processes known to be associated with KEAP1 and NRF2, still others impact biological pathways that currently have no correlation to KEAP1-NRF2 signaling. Whether the endogenous function of these interacting proteins impacts, or is impacted by, interaction with KEAP1 requires further study. Validation of proteins such as TSC22D4 and SLK as NRF2 activators may not only expand the growing list of competition-based pathway agonists, but also may illuminate currently unappreciated pathways in which NRF2 activation has a role. Proteins like WDR1, FAM117B, and MCM3, which appear to not significantly affect NRF2 activation, may be suggestive of NRF2-independent functions of KEAP1.

4.A.2.a Are ETGE-containing interactors novel KEAP1 substrates?

There are currently five known substrates for KEAP1: NRF2, IKKβ, BCL-2, BCL-XL, and PGAM5. With the exception of BCL-2, which interacts with KEAP1 via three

residues that do not share homology to an ETGE motif [49], substrates bind to KEAP1 via an ETGE motif, or in the case of BCL-2, indirectly through PGAM5 [50]. Consequently, it is reasonable to speculate that some of the ETGE-containing proteins within the KEAP1 PIN are novel substrates of KEAP1. Determining which, if any, of these ETGE proteins are novel substrates of KEAP1 will provide valuable clues into which cellular pathways are affected by KEAP1-NRF2 signaling, or by NRF2-independent functions of KEAP1. Several experiments can be conducted to facilitate validation of novel KEAP1 substrates:

Investigating enhanced stability of ETGE-containing proteins under proteasome inhibition or siRNA-mediated knockdown of KEAP1

Treating cells with a proteasome inhibitor such as bortezomib or MG132, or transfecting cells with siRNAs to *KEAP1* causes stabilization of NRF2. This same approach may be used to determine if any of the ETGE-containing proteins are stabilized in a manner similar to NRF2. A complement to this experiment would be detecting levels of ETGE-containing protein expression in *Keap1* knockout mouse embryo fibroblasts (MEF). Expression of NRF2 is elevated in *Keap1*$^{-/-}$ MEFs, and the expectation is that other ETGE proteins would also exhibit elevated expression in this cell line if they were KEAP1 substrates. Finally, it is well established that treatment with proteasome inhibitors increases the amount of NRF2 that is bound to KEAP1 following KEAP1 immunoprecipitation. Any ETGE-containing proteins that are novel KEAP1 substrates should have the same pattern of increased interaction with KEAP1 following inhibition of the proteasome. Although NRF2 has a short half-life, which allows for relatively rapid detection of stabilization after proteasome inhibition, it should not be assumed that other putative KEAP1 substrates exhibit

the same rapid turnover. Consequently, several time points should be selected to evaluate the half-life of putative substrates, as well as changes in intracellular levels following proteasome inhibition to account for slower turnover.

Determining half-life and KEAP1-mediated ubiquitination of ETGE-containing interactors

The experiments described above address only steady state expression of ETGE proteins; however, to fully vet the possibility that these proteins are novel KEAP1 substrates, investigating half-life and ubiquitination status is required. Determining whether the half-life of ETGE proteins is affected by pathway modulation can be accomplished with a pulse-chase experiment in which *de novo* protein synthesis is inhibited with cycloheximide. For example, siRNA-mediated knockdown of *KEAP1* should increase the half-life of an ETGE protein that is a substrate of KEAP1. However, the half-life of a ΔETGE mutant would not be expected to change following knockdown of *KEAP1*.

Lastly, should any of the ETGE-containing proteins show promise as a KEAP1 substrate, analysis of KEAP1-dependent ubiquitination should be performed. Two approaches may be taken—an *in vitro* ubiquitination assay, and cell-based ubiquitination. For analysis of cell-based ubiquitination, the ETGE protein of interest, tagged ubiquitin, and KEAP1 or a negative control protein are transfected into *KEAP1*-deficient cells, such as *Keap1*$^{-/-}$ MEFs. Following a brief treatment with MG132, cells are lysed under denaturing conditions. After titrating the lysate back to physiological pH, ubiquitinated protein complexes of the ETGE-containing protein can be analyzed by either immunoprecipitation of the ETGE protein, or by affinity purification of the tagged ubiquitin. Substrates of KEAP1

should only show ubiquitin laddering in cells that were transfected with *KEAP1*, and not the negative control protein. A complementary assay to this would be to perform the same experiment in cells with endogenous KEAP1. Rather than comparing transfected *KEAP1* or negative control protein, siRNAs to *KEAP1* or a scramble control are compared. Conditions in which *KEAP1* siRNAs are used are expected to have reduced ubiquitination of ETGE proteins that are KEAP1 substrates.

An *in vitro* ubiquitination assay should also be performed to eliminate any experimental artifacts that may occur due to other endogenous proteins present in cell lysate. Briefly, recombinant CUL3, KEAP1, ubiquitin, the ETGE protein of interest, and an E1 and E2 are combined and incubated to allow ubiquitination to occur, followed by immunoprecipitation of the ETGE protein. Ubiquitin-dependent laddering of the ETGE protein should only occur when KEAP1 is included in the reaction.

4.A.2.b Novel regulators of KEAP1-NRF2 signaling: TSC22D4 and SLK

In addition to DPP3, TSC22D4 and SLK were the only ETGE-containing proteins that also activated NRF2-mediated transcription. The ability to activate NRF2 is where any commonalities stop, however, as these proteins have very disparate cellular functions from each other. Further study is required to both validate TSC22D4 and SLK as novel activators of KEAP1-NRF2 signaling, as well as determine whether KEAP1 interaction impacts cell function.

TSC22D4: a transcription factor with links to modulation of metabolic pathways

TSC22D4 is part of the larger *TSC22D* leucine zipper transcription factor family, and collectively these proteins act in several diverse cellular functions. For example, TSC22D1 exhibits tumor suppressor activity, and TSC22D3 antagonizes cellular proliferation in an AP1- and NFκB-dependent manner [51-53]. Functional roles for TSC22D4 are largely unknown; however, studies demonstrate that TSC22D4 plays a role in modulating apoptosis in neuronal cells [52], as well as lipid metabolism. Specifically, TSC22D4 is induced in mouse models of cancer cachexia and impair lipogenesis in liver [53]. Furthermore, *TSC22D4* was found to regulate several lipogenic genes in both mouse and human hepatocytes.

Follow-up studies investigating whether the metabolic changes induced by TSC22D4 can be linked to the NRF2-mediated metabolic reprogramming employed by cancer cells could provide insight into how TSC22D4 acts as a positive regulator of NRF2 signaling. As depicted in Figure 2.2.F, overexpression of *TSC22D4* activates NRF2. Consequently, siRNAs to *TSC22D4* should be used to determine if suppression of NRF2-mediated transcription occurs following knockdown of *TSC22D4*. Furthermore, gain-of-function and loss-of-function for *TSC22D4* followed by quantitative PCR for metabolic genes regulated by NRF2, including *G6PD, PGD, TKT*, *ME1*, and *TALDO1*, can be performed to determine if TSC22D4 contributes to NRF2-dependent metabolic changes.

SLK: an ETGE-independent activator of NRF2-mediated transcription

While only three of the ETGE-containing proteins—DPP3, TSC22D4, and SLK—activate NRF2 above control, all of the ETGE proteins have a subtle but reproducible reduction in NRF2 transcriptional activity when the ETGE motif is deleted (Fig. 2.2.F). The one exception is SLK (Ste20-like kinase), which not only still activates the NRF2 reporter when the ETGE motif is deleted, but also does so to a greater degree than wild-type SLK (Fig. S.2.1.A). Intriguingly, SLK-ΔETGE cannot bind to KEAP1, despite maintaining the ability to activate NRF2 (Fig. 2.2.E). The only other ETGE protein we investigated that exhibits a similar pattern of activation is NRF2 itself, which begs the question of whether SLK is the ETGE-containing protein most likely to be a novel KEAP1 substrate. In addition to potentially acting as a KEAP1 substrate, SLK has been implicated in several biological processes, which warrant investigation into any role SLK may have in modulating KEAP1-NRF2 signaling.

Ste20-like kinase (*SLK)* is a serine/threonine kinase that has physiological roles primarily linked to regulating cytoskeletal dynamics. Co-immunoprecipitation experiments show that SLK interacts with tubulin and polymerizing microtubules during normal adhesion and cell spreading [54]. Overexpression of SLK disrupts actin stress fibers, and decreases focal adhesion stability [54-56]. In addition to regulating the cytoskeleton, SLK has been found to induce apoptosis in some cell lines [57, 58], and its expression and activation is increased during kidney development [59], as well as acute ischemic injury in the kidney [59, 60]. Furthermore, SLK associates with the mitotic spindle during mitosis [61, 62], and siRNA-mediated knockdown or use of a kinase dead construct have been shown to inhibit

cell cycle progression, particularly through G2 [61]. Downstream signaling targets of SLK include upregulation of c-Jun N-terminal kinase (JNK), p53, and p38 MAPK cascades [56, 57, 63]. Collectively, beyond regulation of the cytoskeleton, studies of SLK demonstrate that it is capable of mediating cellular processes involved in cell survival during development, injury, and repair.

The emerging functional roles that SLK has in regulating conditions of cellular stress are particularly appealing considering that SLK also activates NRF2-mediated transcription. Moreover, several cytosolic kinases have been found to phosphorylate NRF2 and modulate NRF2 function, including the SLK downstream target p38 MAPK kinases. To investigate whether the kinase activity of SLK impacts NRF2 signaling, a kinase dead mutant of SLK can be used in luciferase-based assays for NRF2 function. Furthermore, *in vitro* phosphorylation experiments comparing wild-type and kinase dead SLK will establish whether NRF2 is a kinase substrate. Some debate exists as to whether p38 MAPK-mediated regulation of NRF2 is activating or suppressing [64-66]. Similar experiments as those previously described can be performed to determine if 1) SLK acts upstream of p38 MAPK kinases rather than directly on NRF2, and 2) whether activating effects of p38 MAPK signaling on NRF2 occur via SLK.

Despite an appealing link between SLK-mediated regulation of stress pathways and activation of NRF2, we cannot rule out the possibility that the KEAP1-SLK interaction is affecting cytoskeletal dynamics in some manner. Gain-of-function and loss-of-function studies for KEAP1 to determine if modulating KEAP1 expression can affect cytoskeletal

phenotypes associated with SLK function, including focal adhesion stability and turnover, actin network stability, and actin stress fiber stability should be performed for completeness. Furthermore, with respect to both TSC22D4 and SLK, it is possible that activation of NRF2 is entirely independent of the function of either gene, and, like DPP3, is due solely to the competitive binding model. More thorough study of whether SLK and TSC22D4 are found to be overexpressed in cancer, as we found with DPP3, will help parse the likelihood that the observed interactions between KEAP1 and these proteins is due to competitive binding only, or if they also serve to regulate other functions of KEAP1 and NRF2.

4.A.2.c NRF2-independent functions of KEAP1: does KEAP1 regulate cell cycle?

The KEAP1 protein interaction network consists of a variety of proteins with very disparate cellular functions; however, we found several proteins involved in cell cycle regulation. The cell cycle-related proteins, including minichromosome maintenance complex component 3, (*MCM3*), MAD2 mitotic arrest deficient-like 1 (*MAD2L1*), and minichromosome maintenance complex binding protein (*MCMBP*), comprise the only group of proteins from the KEAP1 PIN with obviously linked functions. Consequently, we are particularly intrigued by the possibility that KEAP1 has a defined role in cell cycle regulation and progression. MCM3 is one of several MCM proteins in the pre-replication complex, which is critical for initiation of genomic replication [67-69]. While MCMBP is not an ETGE-containing protein, it is a validated component of the KEAP1 PIN, and also participates in the replication complex with MCM3 [70, 71]. Finally, MAD2L1 is a component of the mitotic spindle assembly checkpoint, which inhibits anaphase until chromosomes are properly aligned on the metaphase plate [72-74]. Collectively, if one or all

of these proteins are either novel substrates of KEAP1, or use interaction with KEAP1 for their functional roles, it suggests that KEAP1 may have a previously unappreciated role in cell cycle progression.

Experiments to determine whether MCM3 and MAD2L1, the two ETGE-containing proteins, are putative KEAP1 substrates as described above, may be complicated by the fact that those experiments are performed in a heterogeneous cell population. Synchronizing the cell population with a treatment such as nocodazole, prior to performing experiments correct for this possible impediment, because it will provide a homogeneous population of cells for each phase in the cell cycle. Identifying whether MCM3 and MAD2L1 are substrates in synchronized cell populations will require immunoprecipitation of KEAP1 during various stages of the cell cycle. The results of these co-immunoprecipitation assays will not only potentially illuminate if MCM3 and MAD2L1 are KEAP1 substrates, but the findings will also indicate whether the KEAP1-MCM3 and KEAP1-MAD2L1 interaction is cell cycle dependent. Additionally, Western blot analysis of synchronized cell populations will reveal whether KEAP1 expression is regulated in a cell cycle-dependent manner.

In conjunction with identifying protein interaction dynamics during the cell cycle, modulation of KEAP1 protein levels by siRNA-mediated knockdown, or overexpression will illustrate whether KEAP1 expression is required for normal cell cycle progression. Thymidine incorporation assays, which are a direct measure of cell proliferation and DNA synthesis during mitosis can be employed. Briefly, cells stably expressing KEAP1, or transfected with siRNAs to KEAP1 will be arrested with nocodazole. Upon release following

nocodazole washout, synchronized cells will be analyzed for thymidine incorporation. If modulation of KEAP1 expression impacts cell cycle progression in some manner, the expected results would be increased or decreased thymidine incorporation in KEAP1 gain-of-function or loss-of-function samples compared to negative control.

4.B Cancer-derived Mutations in the KEAP1 Ubiquitin Ligase Impair NRF2 Degradation but not Ubiquitination

Chapter Three describes the characterization of 18 mutations in *KEAP1* that were found in patient-derived squamous cell lung carcinomas. Beyond determining that these mutants are largely unable to promote NRF2 degradation and suppress NRF2 transcriptional activity, several additional observations were made:

a) Mutations in *KEAP1* are primarily hypomorphic with respect to modulation of NRF2 activity.
b) With few notable exceptions, the protein interaction networks of KEAP1 mutants are comparable to wild-type KEAP1.
c) The relative expression of *KEAP1* mutant alleles compared to wild-type varied, although at this time it is not known whether these differences are due to mRNA or protein instability.
d) *KEAP1* mutants can be grouped into several general classes based on suppression of NRF2 activity and biochemical characteristics.

Not only may these observations impact how KEAP1-NRF2 signaling is viewed as a cancer-relevant pathway, but also our findings, as well as similar studies, may alter how mutations in cancer are approached from a global therapeutic perspective. Specifically, our data suggests

that any given group of mutations in a given gene are not equal with respect to the functional consequence each mutation has on cancer cell fitness. Consequently, the context in which a mutation occurs—including temporal regulation, relative allelic expression, and incidence of other mutations or expression differences of interacting proteins—is likely more important than the a single mutation alone.

4.B.1 Mutations in KEAP1 are largely hypomorphic

A particularly interesting, and somewhat surprising, observation regarding mutations in *KEAP1* was that 13 of the 18 mutations exhibited hypomorphic activity with respect to suppression of NRF2-mediated transcription. The two frameshift mutations (N469fs and P318fs), both of which result in a truncated protein product, behave as an inactive protein. It should be noted, however, that the most "null" phenotype of these mutants was observed in HEK293T cells, which have an intact endogenous KEAP1-NRF2 pathway. The other two cell lines tested, A549 and *Keap1*$^{-/-}$ MEFs, have a hypomorphic and absent endogenous pathway, respectively. Further study is required to determine if the two truncating mutations are dominant negative. Not only is there a precedent for mutations in *KEAP1* to act as dominant negative proteins [75], but the relatively low expression of the truncation mutations (Fig. 3.2.A and S3.2.B) paired with the nearly null transcriptional phenotype (Fig. 3.1.B-D) even in cells with intact KEAP1-NRF2 signaling, suggest these mutations may be dominant negative mutants as well. Only three of the mutants, L231V, S224Y, and R71L, as well as an incidental incorrectly made mutation P318L, suppress NRF2 as well as wild-type KEAP1. These mutations likely represent silent, or "passenger" mutations.

The remaining 13 mutations cannot suppress NRF2-mediated transcription as well as wild-type KEAP1, but also fall short of a null phenotype or the GFP negative control. We consider these mutations to be hypomorphic. As depicted in Figure 3.2.A, the relative stability of the KEAP1 mutants is variable as evidenced by some differences in protein expression. Therefore, we cannot rule out the possibility that the observed differences in transcriptional assays may be due to discrepancies in expression. By and large, however, most of the mutants express as well as wild-type KEAP1, so we are reasonably comfortable defining these mutations as hypomorphic.

As stated in Chapter Three, the relative abundance of hypomorphic mutations in *KEAP1* within the lung squamous cell carcinoma cohort begs the question of why there seems to be a preference for hypomorphic mutations over null mutations. Indeed, the abundance of hypomorphic mutations is unlikely to be related to some requirement for basal NRF2 suppression, as activating mutations in *NRF2* occurred in 19% of the same lung squamous cell cohort in which mutations in *KEAP1* were found [8]. Furthermore, it is well established that constitutive NRF2 activity promotes cancer cell survival and proliferation [7, 31, 32, 34, 36, 44, 46, 76-81], so it stands to reason that alternative forces may be driving the "decision" of a cancer cell to not fully activate NRF2 via complete inactivation of KEAP1. These somewhat counterintuitive observations regarding KEAP1 mutation status can be reconciled by considering some putative advantages these hypomorphic mutations have by maintaining interaction with other binding partners, as well as accounting for potential NRF2-independent functions of KEAP1.

As shown in Figure 2.7.E and F, co-expression of DPP3 can enhance NRF2-mediated transcriptional activity in a *KEAP1* mutant background. Not only does this data further support the notion that these *KEAP1* mutations are hypomorphic, but it also introduces the possibility that any deficiency in augmented NRF2 activity from *KEAP1* hypomorphic mutation can be compensated for by modulating expression of other competitive binding proteins. This hypothesis is further supported by the observation that *DPP3* is overexpressed in lung squamous cell carcinoma (Fig. 2.7.A-D). Intriguingly, recent evidence suggests *DPP3* is also overexpressed in head and neck squamous cell carcinoma (Fig. 4.1), which also shows significant mutation of *KEAP1* and *NRF2*. These observations imply that overexpression of ETGE competitive binding proteins may be a ubiquitous mechanism of augmenting NRF2 activity in KEAP1 hypomorphic tumors. The growing number of competitive binding proteins with defined roles in cancer survival and proliferation, as well as specific examples of known competitive binding proteins, like DPP3, being overexpressed in cancers with dysregulated KEAP1-NRF2 activity undoubtedly warrants further exploration of how competitive binding proteins may affect hypomorphic KEAP1 phenotypes.

While interaction with competitive binding proteins enables further activation of NRF2 signaling in hypomorphic KEAP1 backgrounds [3], it is likely this means of activation represents only a compensatory mechanism for hypomorphic phenotypes, rather than an early driving force behind selection. Alternative substrates of KEAP1 may account for the proposed selective advantage of hypomorphic mutations, as alternative substrates are the most likely source of NRF2-independent functions of KEAP1. In support of this hypothesis

is the observation that mutations in KEAP1 rarely occur in residues that interface with NRF2, which are presumably the same residues that would interface with alternative substrates that also bind via an ETGE motif. As shown in Table S3.1, only 4 of the 213 annotated mutations—R483H, R415G, S555-60Δ, and Y334H—would result in a change of a residue that interfaces with NRF2; it should be noted that only the S555-60Δ would likely not result in a mutant KEAP1 phenotype [10, 21, 34, 82]. While only speculative thus far, if KEAP1 has a role in cell cycle progression, it is feasible that KEAP1 E3 ligase activity is required for progression to the next phase of the cell cycle. In this case, maintaining some ability to functionally contribute to cell cycle events through hypomorphic activity may achieve an ideal balance for cancer cells—pro-survival effects of enhanced NRF2 signaling and supported NRF2-independent functions of KEAP1.

A caveat of the aforementioned hypothesis, however, is that all known KEAP1 substrates would only serve to benefit cancer cell survival if KEAP1 function were impaired. Constitutive activation of NFκB, for example, is largely considered a pro-survival and pro-proliferative pathway in the context of cancer [83-85]. KEAP1 acts to suppress NFκB signaling through degradation of IKKβ; thus, KEAP1 inactivation through mutations would be favored [86]. Furthermore, impaired KEAP1 function supports the anti-apoptotic effects of both BCL-2 and BCL-X_L by freeing them from degradation so that they can bind pro-apoptotic BAX [49, 50, 87, 88]. Along these same lines, if KEAP1 functions to degrade a protein that acts at a cell cycle checkpoint decreased KEAP1 activity would slow cell cycle progression—an undesirable phenotype for a cancer cell. A potential resolution to this problem would be to account for the importance of timing in acquiring mutations in KEAP1.

The concept that *KEAP1* and *NRF2* are driver genes is becoming more widely accepted. The current paradigm of driver genes and passenger genes focuses on the idea that only genes that further support cancer cell fitness can be considered driver genes. Another important consideration in this hypothesis, however, is when a mutation may occur. Mutations in *NRF2* are an excellent example of how temporal appearance of a mutation may also be critical to cancer cell fitness. As described in Chapter One, activation of NRF2 can be both anti- and pro-tumorigenic, depending on the context in which the activation occurs [31]. Expression of NRF2 can prevent transformation of a normal cell into a cancer cell by mitigating ROS that may otherwise result in cancer-driving DNA mutations. Conversely, when a cell has already become autonomous with respect to growth and proliferation, constitutive activation of NRF2 promotes tumorigenesis by reducing ROS and driving transcription of cell survival and drug efflux genes. Consequently, it is unlikely that NRF2 could act as a gateway mutation in tumorigenesis. Indeed, this is supported by the inability of constitutive NRF2 activation to result in tumor formation [89, 90].

With respect to KEAP1, however, one can imagine a different scenario. Like activating mutations in *NRF2*, inactivating mutations in KEAP1 that result in a null phenotype, are likely not advantageous as gateway or early mutations in tumorigenesis, because they could result in early chemopreventive NRF2 activity. However, activation of the other substrates via mutational inactivation of KEAP1, namely IKKβ, could certainly be desirable from the perspective of cancer cell fitness. Hypomorphic mutations in KEAP1 have the potential to act as early driver mutations by contributing to pathways such as NF-κB signaling, as well as the enhanced stability of anti-apoptotic proteins like BCL-2 and BCL-

X_L. Constitutive NRF2 activation in this context will be reduced, possibly to the point of not supporting any anti-tumorigenic effects. At later stages of tumor progression, augmented NRF2 activation through alternative means, such as competitive binding proteins, would then facilitate cancer cell fitness.

Further experiments are required to determine exactly how hypomorphic mutations in KEAP1 may be more advantageous to inactivating mutations. It is likely that the selective processes behind hypomorphic mutations in KEAP1 are influenced by several factors, including the relative contributions of each pathway that is mediated by KEAP1 has in cancer progression. The major question that remains is whether these hypomorphic KEAP1 mutations enhance cancer cell viability, and if so, if the mechanism is NRF2-dependent. Furthermore, as mentioned in Chapter Three, a group of these hypomorphic mutations that we have termed "superbinders" appears to have an interesting—and potentially novel—mechanism mediating the inability to fully suppress NRF2. Experimental interrogation of these mutants will hopefully provide the insight required to elucidate how NRF2 function is affected, and whether this mutant class represents a novel mechanism of KEAP1-NRF2 regulation employed in cancer.

4.B.2 KEAP1 mutants: effects on cell viability and tumorigenesis, and the contribution of relative expression

4.B.2.a Cell viability and tumorigenesis

The data presented in Chapter Three demonstrate that 15 of the 18 KEAP1 mutations cannot suppress NRF2-mediated transcription (Fig. 3.1.B-D). Furthermore, a more detailed

analysis of several of these mutants shows that HMOX1 is upregulated, and NRF2 localizes to the nucleus following KEAP1 mutant expression (Fig. 3.4 and S3.3). Constitutive activation of NRF2 has been shown to enhance cell viability following chemotherapeutic insult. As elevated levels of NRF2 are observed following expression of KEAP1 mutants, it stands to reason that KEAP1 mutant expression should also correlate with resistance to chemotherapeutic insult. While conducting studies for the data presented in Chapter Three, we attempted several permutations of cell viability assays. We were unable to detect any robust or statistically significant differences between the KEAP1 mutants and wild-type KEAP1 (Fig. 4.2.A and B). Furthermore, contrary to previously published reports, wild-type NRF2 was unable to cause an appreciable rescue from cell death in our hands (Fig. 4.2.C and D). The following sections outline not only the experimental conditions and assays in our attempts to evaluate cell viability, but also describe how we may improve the assays for future analysis of KEAP1 mutant function in cell survival.

Cell-based assays: PrestoBlue, Caspase-3/7 Glo, and Cell Titer Glo

We first chose to pursue high-throughput assays to evaluate cell viability for several reasons: 1) Variations of these assays were used to establish the phenotype that NRF2 expression is able to enhance cell survival following apoptotic insult [32], 2) several assays for a variety of cell viability measures (mitochondrial respiration, ATP production, caspase activation, loss of membrane integrity) are commercially available, which would provide the most complete panel of cell survival, and 3) to produce the most clinically-relevant data, we would test a several chemotherapeutics that are currently used in the clinic to treat lung cancer.

The assays chosen were as follows:

A. PrestoBlue (Invitrogen). This assay uses a resazurin-based dye, similar to MTT, in which reduction of resazurin in the mitochondria produces a fluorogenic molecule. In this assay, cell viability is directly correlated with mitochondrial respiration.
B. CellTiter Glo (Promega). A luminescent signal is produced proportionally to the amount of ATP present. Similar to the PrestoBlue assay, CellTiter Glo will provide a measure of the number of cells present, which is proportional to viable cells within a sample.
C. Caspase-3/7 Glo (Promega). This assay provides a direct measure of apoptosis. It contains a proluminescent molecule that includes the consensus sequence for caspase cleavage. The luminescent readout produced is directly produced to the amount of active caspases present within a sample.

The experimental setup included a negative control protein, such as GFP or nucleophosmin (NPM1), wild-type KEAP1, KEAP1 mutants, and usually wild-type NRF2 or NRF2-ΔETGE as positive controls. Regardless of the assay employed, or the apoptotic insult, we were unable to detect any considerable pro-survival effect when the KEAP1 mutants were expressed (Fig. 4.2).

The most likely explanation for why we are unable to observe any type of rescue following expression of NRF2 is related to the cell line. All high-throughout assays were performed in HEK293T cells, which we suspect already have a higher basal level of KEAP1-NRF2 pathway activity. Initially we employed these cells because they are highly

transfectable, which allows for analysis of a more homogeneous cell population, and are amenable to creation of stable cell lines without the need for transduction. Experiments that establish the pro-survival role of NRF2, however, use other cell lines, including A549 and several breast cancer lines [32]. Future experiments to evaluate the effect KEAP1 mutants have on cell viability should include alternative cells lines, and creation of stable cell lines by virus should be employed to achieve greater homogeneity among the cell population. Furthermore, in addition to including either NRF2 or NRF2-ΔETGE as theoretical maximums for viability, a NRF2 siRNA control should also be included, as this would set a theoretical minimum.

It should also be noted that in addition to high-throughput assays to assess cell viability, Western blotting for apoptotic markers were also attempted. These experiments were conducted in several different cell lines, including HEK293T, A549, H2228, and H1299; however, regardless of the cell line used, we were unable to detect any differences in viability (Fig. 4.2). All Western blot analyses were performed with transient transfections. Follow up studies should include a similar experimental design, except with cell lines stably expressing KEAP1 mutants.

4.B.2.b Clinically relevant models of cell viability

High-throughput assays have the advantage of screening several different experimental conditions relatively quickly, which is ideal for evaluating 18 separate mutations following treatment with multiple clinically relevant drugs. These assays are effective at measuring a cells ability to survive chemotherapeutic insult, but they do not

necessarily assess the transformative properties of the gene(s) being assessed, nor do they provide insight into the proliferative abilities of these cells following insult. Anchorage-independent growth assays and colony formation (clonogenic) assays can surrogate as measures of cell transformation and proliferative capacity, respectively.

Anchorage-independent growth assays make use of immortalized cells that are unable to divide unless plated on a surface. Neither KEAP1 nor NRF2 have been shown to have transformative properties, and restoration of NRF2 expression has been shown to inhibit colony formation in anchorage-independent growth assays [91]. Consequently, it is unlikely that expression of a KEAP1 mutant alone will affect colony formation in an anchorage-independent growth assay. However, pairing expression of a KEAP1 mutant with expression of a protein already known to promote colony formation, such as KRAS, may illuminate an additive or synergistic effect KEAP1 mutant expression and cell proliferation. As with the high-throughput cell based assays, a negative control protein, wild-type KEAP1, and wild-type NRF2 and NRF2-ΔETGE should be included. Experimental conditions that include NRF2 are expected to have the most robust effect of enhancing colony formation. KEAP1 mutants that behave in transcriptional assays like wild-type KEAP1 should also be evaluated, as enhanced colony formation with these mutants may indicate NRF2-independent functions of KEAP1 that support cancer cell proliferation.

Clonogenic assays can be used as a complementary technique to anchorage-independent growth experiments. Unlike anchorage-independent growth assays, however, it is possible that pairing KEAP1 mutations with KRAS expression may be unnecessary. In

clonogenic assays cells are plated directly onto a cell culture medium, possibly rendering addition of a gene that induces anchorage-independent growth superfluous. Additionally, clonogenic assays are easily paired with cellular insult. Briefly, cell populations expressing KEAP1, mutant KEAP1, NRF2/NRF2-ΔETGE, or negative control protein are treated with chemotherapeutic drug. The cells are then re-plated at very low confluency into a separate vessel without drug and allowed to grow into colonies. As NRF2 regulates expression of several drug efflux genes, the expectation is that cells expressing NRF2 or KEAP1 mutants will form colonies, whereas wild-type KEAP1 and the negative control will not. The same assay can be performed but rather than re-plating cells in drug-free media, maintaining them in sub-lethal doses.

The gold standard in assessing whether a gene or mutation impacts tumor progression is utilization of mouse xenograft models. Similar to the anchorage-independent growth assay, it is likely that KEAP1 mutants will have to be paired with expression of an oncogene, such as KRAS. Rather than assessing if KEAP1 mutants promote tumorigenesis alone, these experiments would determine if KEAP1 mutants have the ability to enhance oncogene-mediated tumor growth. Again, the expected results are that inclusion of NRF2, or KEAP1 mutants that result in activated NRF2 will result in larger tumors than KRAS alone. Additionally, other genes known to impact xenograft models that are specifically correlated with squamous cell lung carcinoma, such as platelet derived growth factor receptor (*PDGFR*) and fibroblast growth factor receptor (*FGFR*), may also be studied for an additive effect with KEAP1 mutants [92-94]. Finally, response to chemotherapeutic treatment in mouse models

expressing *KEAP1* mutants can be used to evaluate whether there are practical therapeutic implications for determining *KEAP1* mutational status in a clinical setting.

4.B.2.c The impact of allelic expression of KEAP1 mutants on cell viability

As discussed in the introduction, at least a 75% reduction in KEAP1 activity is required to see a phenotypic effect on NRF2 [95]. Studies conducted on the importance of graded KEAP1 expression were conducted in mice with floxed wild-type *Keap1* alleles. Floxed alleles resulted in reduced expression of Keap1, even when only one allele was floxed, resulting in "knockdown" animals. Knockdown animals were viable, but still exhibited lessened phenotypes of the knockout animals, including hypertrophy of the esophagus and upper stomach. An interesting observation of these animals, however, was that they demonstrate greater resistance to hepatotoxicity compared to wild-type animals [95]. Furthermore, administration of the glutathione precursor *N*-acetylcysteine prior to hepatotoxic stress had a protective effect, whereas an inhibitor of GCL sensitized both knockdown and wild-type animals to hepatotoxicity. These results are suggestive of a NRF2-mediated mechanism of protection in the knockdown animals.

Given the importance of KEAP1 expression with respect to NRF2 activity, it is intriguing to think about how the relative expression of *KEAP1* mutant and wild-type alleles affects tumorigenesis and cancer cell viability in heterozygous tumors. As shown in Table 1.1, lung squamous cell tumors exhibit a wide variation of allelic expression. From a therapeutic standpoint, whether a tumor achieves a 75% reduction in KEAP1 activity—either by homozygous KEAP1 mutation, or a combination of preferentially expressed mutant allele

to wild-type—may have a significant impact on chemotherapeutic response. Therefore, understanding how these KEAP1 mutants may affect NRF2 activity in knockdown animals, and cell lines derived from knockdown animals may, in fact, be the most clinically relevant analysis of KEAP1 mutations.

The experiments detailed in this section are still adequate for addressing the importance of relative allelic expression of KEAP1. Rather than performing assays in non-specific cell lines like HEK293T, however, a floxed *Keap1* allele background should be used. Specifically, *KEAP1* mutants should be stably introduced into MEFs derived from *Keap1*$^{+/+}$, *Keap1*$^{flox/+}$, *Keap1*$^{flox/-}$, and *Keap1*$^{-/-}$ animals. Viability assays, including high-throughput and anchorage-independent growth and clonogenic assays, should be performed to evaluate the impact of KEAP1 mutant expression in a knockdown background using the experimental designs previously described.

4.B.3 Mutant Classification: limitations, superbinder identification, and determining mechanism of the superbinder mutant class

4.3.a Limitations of KEAP1 mutant classification

Initial characterization of the TCGA *KEAP1* mutants revealed that the mutants could be binned into four classes based on luciferase activity and NRF2 binding. The classes were as follows:

Class I—unable to suppress NRF2-mediated transcriptional activity, bound NRF2 to an equal or lesser extent than wild-type KEAP1

Class II—unable to suppress NRF2-mediated transcriptional activity, unable to bind NRF2

Class III—unable to suppress NRF2-mediated transcriptional activity, bound more NRF2 than wild-type KEAP1

Class IV—behaved similarly to wild-type in both transcriptional assays and Western blot analysis

The ultimate goal of utilizing mutational data from resources such as the TCGA sequencing consortium is having the ability to predict functional outcomes based on a particular genotype. Establishing a predictive genotype-phenotype relationship could direct therapeutic interventions without first requiring rigorous characterization of mutant behavior in a laboratory setting. Classifying mutations based on objective, quantifiable criteria, like luciferase activity and substrate affinity, is appealing because it offers the first steps towards predicting how a mutation may affect global cellular fitness in the context of cancer. However, further interrogation of individuals within a particular mutant class revealed greater variability than we initially anticipated. Much of the observed variability is attributable to limitations of the experiments used to establish the different mutant classes.

The four mutant classes were derived from two criteria—NRF2 binding and luciferase activity. Luciferase activity was described in a binary fashion, wherein any mutant that exhibited luciferase activity above wild-type control was considered a non-suppressor. Binding to NRF2 was assessed with Western blot analysis, and described on a graded scale; however, this scale was largely subjective, as we did not evaluate binding with a quantitative means, such as LI-COR or measurement of pixel density. The main limitation of both of these classification methods was that neither accounts for differences in mutant expression. Mutants unable to bind NRF2, for example, consistently expressed at lower levels than other

mutants or wild-type KEAP1. Consequently, drawing conclusions about the function of these mutants is difficult without first normalizing to mutant expression. Furthermore, the luciferase assay used to evaluate KEAP1-mediated suppression of NRF2 is highly variable, and extremely dependent on expression of the KEAP1 mutant constructs. Future studies should include Western blot analysis of the cell extracts used in luciferase assays to ensure the mutants are, at the very least, expressed.

In addition to limitations regarding the expression of the KEAP1 mutants, we must also account for variability among cell lines. As shown in Figure 3.1.B-D, each cell line tested exhibited the same relative pattern of suppression among KEAP1 mutants; however, the absolute values varied greatly between cell lines. Some of this variability can be ascribed to transfection efficiency of the cell lines used. Another consideration is the presence of endogenous KEAP1, particularly when there is a precedent for mutations in KEAP1 to act as a dominant negative [75]. Furthermore, in cell lines such as A549, which has both a homozygous KEAP1 mutation and reduced KEAP1 expression due to promoter methylation [41], it is difficult to predict whether these factors may impact exogenously expressed KEAP1 mutant function. As previously stated, the relative pattern of suppression among KEAP1 mutants is approximately equal, including suppression observed in KEAP1 knockout MEFs (Fig. 3.1.C), where the possible effects of endogenous KEAP1 are not an issue.

Even in a hypothetical situation where KEAP1 mutants are expressed equally in a luciferase assay, relating mutant function in a luciferase assay to the relative suppressive abilities of that mutation in a tumor for use in classification may not be feasible. As depicted

in Figure Table 1.1 the relative allelic expression of the wild-type and mutant allele can vary widely. Unless the tumor is homozygous mutant, attempting to achieve a comparable wild-type KEAP1 to mutant KEAP1 in a luciferase assay is likely not practical or useful for classification purposes. Indeed, when we attempted to correlate NRF2 gene signature of tumors with the luciferase activity of the corresponding mutant, further stratifying luciferase activity (beyond "suppressor" or "non-suppressor") did not improve the p-value (Fig. S.3.1.A). This is not to say that luciferase assays are not useful in characterizing KEAP1 mutant biology. Rather, luciferase assays to assess KEAP1 mutant function should be used as a complementary experimental tool in mutant characterization, and perhaps if luciferase assays will be used in mutant classification, they can be kept at a binary "suppressor" or "non-suppressor" variable.

4.3.b Identification of the KEAP1 superbinder mutant class

The KEAP1 superbinder class was initially defined as the class of mutants that was unable to suppress NRF2-mediated transcription in a luciferase-based assay, but bound more NRF2 than wild-type KEAP1 when KEAP1 was affinity purified. Quantitative analysis reveals that with the exception of S243C, superbinders bind at least twice as much NRF2 as wild-type (Fig. 4.3). We chose one of the superbinder mutants—R320Q—for further characterization, and found that NRF2 has an extended half-life when R320Q is expressed. The observed extended half-life seen in Western blot is not exclusively due to more NRF2 being bound to KEAP1 (Fig. 3.5.B). The most surprising characteristic of the superbinders, however, is that they do not appear to impede NRF2 ubiquitination (Fig. 3.5.C and D). Given the unique nature of this particular group of KEAP1 mutants, we investigated several other

characteristics, including protein interaction network changes, NRF2 binding affinity, and interaction with the proteasome, to provide insight into the potential mechanism of these mutants.

Protein interaction network differences of the superbinder mutants

With the exception of NRF2, the superbinder mutants do not exhibit vastly different protein interaction networks compared to wild-type KEAP1. Quantitative mass spectrometry analysis of the superbinders R320Q and R470C reveals that most KEAP1 interacting proteins, particularly ETGE-containing interactors, are present, although the relative amounts vary slightly compared to wild-type KEAP1 (Fig. 3.3.B). Furthermore, in a panel of five known protein interactors, the superbinders exhibit a remarkably consistent binding pattern to wild-type (Fig. 3.3.A, lanes 3, 5, 6, 9, 14, 16, 20). Two notable exceptions are reduced binding of IKKβ and MCM3 to G186R (Fig. 3.3.A, lane 14). Further quantitative analysis, including quantifying affinity purification and Western blot for the superbinder mutants, should be performed to validate the perceived similarities in the PIN for these mutants. At this time, however, the data regarding superbinder protein-protein interactions suggest that the most critical characteristic of these mutants regarding function and global cellular impact can be attributed to NRF2 interaction and degradation.

Identifying differential binding affinities for the NRF2 superbinder mutants

One possibility to explain why the superbinders bind more NRF2 than wild-type is that the mutation causes a structural and/or biochemical change in KEAP1 that enhances the affinity for NRF2. A change such as this could explain not only the greater amount of NRF2

bound to KEAP1, but also if the affinity of KEAP1 for NRF2 impacts proteasomal delivery and degradation of NRF2, it could also explain why NRF2 turnover appears to be slower with superbinder KEAP1 (Fig. 3.5.A and B). To begin to address the relative affinities of KEAP1 superbinder mutants, affinity purification followed by stringent salt wash was performed. The expected result of this experiment is that NRF2 would dissociate from wild-type KEAP1 at lower molarity salt than a superbinder mutant, because it would be more tightly bound to superbinder KEAP1. As shown in Figure 4.4, no appreciable difference was detected in the amount of NRF2 bound to affinity purified KEAP1 after stringent salt washes. It should be noted, however, that even at the highest concentration of salt, a substantial amount of NRF2 was bound to wild-type KEAP1. Consequently, it is possible that the experimental conditions require a higher concentration of salt, such that a more dynamic range of NRF2 dissociation from wild-type KEAP1 is achieved.

4.3.c Determining the mechanism of KEAP1 superbinder mutants

To ensure a comprehensive analysis of mutations in KEAP1, the experiments outlined in Chapter Three for the R320Q superbinder mutant to further characterize function, including NRF2 localization, NRF2 half-life, and NRF2 ubiquitination should be performed for all 18 KEAP1 mutants. At this time, however, the KEAP1 superbinder mutant class appears to be unique among the other mutants found in the lung squamous cell carcinoma dataset. Currently 7 of the 18 mutations are considered to be superbinder mutants. Although this group could become more stratified following further characterization, given the current data these mutants comprise the largest and most consistent group of KEAP1 mutants in the

lung squamous cell cohort. Consequently, we are interested in determining if KEAP1 superbinder mutants represent a novel mechanism of NRF2 activation in cancer.

Increased affinity for NRF2 vs. inefficient interaction with the proteasome

An initial step in understanding the superbinder mechanism of action is differentiating between whether these mutants actually have an enhanced affinity for NRF2, or whether the KEAP1-NRF2 complex cannot be as easily degraded via the proteasome, perhaps due to conformational changes that impair interaction with the proteasome. The saturation model predicts that inactivation of KEAP1 permits newly translated NRF2 to evade degradation, thus activating NRF2 transcription. Therefore, the outcome of either an increased affinity for NRF2 or a slower rate of turnover due to inefficient proteasomal recognition would be the same with respect to intracellular NRF2 activation. The significance in distinguishing between increased affinity or impaired turnover lies in considering NRF2-independent functions of KEAP1, as well as elucidating how KEAP1—and E3 ligases as a protein class—interact with the proteasome to facilitate substrate degradation.

An enhanced affinity for NRF2 may limit how easily NRF2 is released to the proteasome to undergo proteolysis. In turn, a decreased rate of NRF2 proteolytic degradation has the potential to disrupt the rates of degradation of other KEAP1 substrates as described in the saturation model. Additionally, as all known KEAP1 substrates bind to the KELCH domain like NRF2, it is also possible that the affinity for alternative substrates may also be

increased. The overall effects of changes in substrate affinity will likely need to be studied on an individual basis.

To determine if KEAP1 superbinder mutants have differential affinities for NRF2, surface plasmon resonance (SPR) experiments may be used. Wild-type KEAP1 and a superbinder mutant such as R320Q will be immobilized on the surface. Recombinant NRF2 would then be flowed over the immobilized KEAP1 to determine binding affinity. The same KEAP1 monobody as described earlier can be used to facilitate NRF2 dissociation for determination of Kd. Previously we have utilized this KEAP1 binding peptide to detect differential binding affinities of NRF2 between wild-type KEAP1 and the R320Q mutant. Similar to the salt washes in Figure 4.4, no appreciable difference was detected between wild-type KEAP1 and R320Q (Fig. 4.5). However, while we were able to detect NRF2 dissociation from KEAP1, it was not complete (Fig. 4.5, lanes 20, 23-26). Furthermore, at the highest concentrations unbound peptide was detectable in the flowthrough (Fig. 4.5, lanes 20 and 26), suggesting that the concentration of KEAP1 monobody was approaching a saturating level. It is also somewhat puzzling as to why the majority of NRF2 remains bound to KEAP1 despite the suggestion that peptide concentration is saturating (Fig 4.5, lanes 20 and 26). Flaws in experimental design could explain this discrepancy. Immunoprecipitation samples were prepared from whole cell lysate, which yields an unknown amount of purified KEAP1; consequently it is difficult to determine what concentrations of peptide are needed to adequately titrate NRF2 from KEAP1. This experiment should be repeated using recombinant R320Q, wild-type KEAP1, and NRF2 to eliminate disparities in purification for

each mutant, as well as artifacts that may result from using protein purified from whole cell lysate.

Another alternative is that the KEAP1 superbinder mutants are unable to facilitate delivery of NRF2 to the proteasome for degradation. As stated in the introduction, several regulatory events precede the peptide bond cleavage step of proteasome-mediated proteolysis. One of these upstream events includes recognition of a proteasome substrate, which occurs through protein interactions with regulatory factors, or ubiquitin receptors, associated with the proteasome [96, 97]. Mutations that alter E3 ligase structure to sterically impede association, or mutate residues required for interaction with these factors would result in an equilibrium shift towards NRF2 bound to KEAP1.

We previously tested interaction of wild-type KEAP1 compared to superbinder R320Q with one of these factors, CDC48/p97, which is known to assist in segregating ubiquitinated substrates from unmodified partners [96, 98]. As depicted in Figure 4.6 (lanes 5-8) no obvious difference between wild-type KEAP1 and R320Q was observed. Despite not detecting any decreased binding of CDC48/p97 associated with R320Q, several other ubiquitin receptors have been shown to mediate substrate recognition by the proteasome [96, 97]. Quantitative mass spectrometry analysis comparing wild-type KEAP1 with a representative superbinder, such as R320Q, may provide clues as to which of these other regulatory protein interactions may be altered due to KEAP1 mutation. In addition to mass spectrometry analysis, a limited siRNA screen of ubiquitin receptors may be performed. Briefly, the luciferase reporter for NRF2 activity (hQR41) can be used to initially distinguish

putative hits, as wild-type KEAP1 should no longer be able to suppress NRF2-mediated activity if a required ubiquitin receptor was knocked down. Validation of hits would include transfecting cells with the siRNA to a specific ubiquitin receptor, followed by affinity purification of KEAP1. The prediction would be that wild-type KEAP1 would behave similarly to a superbinder mutant if that particular ubiquitin receptor was mediating NRF2 recognition in a KEAP1-dependent manner.

USP15: KEAP1 ubiquitination and KEAP1-CUL3 complex formation

Ubiquitination of KEAP1 is an established means of KEAP1-NRF2 pathway regulation [99]. The theory behind this regulation rests on the observation that following treatment with some pathway agonists, such as tBHQ, ubiquitination switches from NRF2 to KEAP1 [100]. A switch in ubiquitination destabilizes the KEAP1-NRF2-CUL3 complex, as ubiquitinated KEAP1 cannot associate with CUL3 as we as the deubiquitinated form [99, 100]. Importantly, ubiquitination of KEAP1 does not target it for degradation; rather, ubiquitinated KEAP1 cannot assemble into the KEAP1-CUL3 ligase complex as efficiently. Recently it has been proposed that the deubiquitinase USP15 is responsible for maintaining KEAP1 in the deubiquitinated form to facilitate NRF2 degradation. Indeed, overexpression of *USP15* promotes NRF2 degradation, and siRNA-mediated knockdown activates NRF2-mediated transcription [100]. Furthermore, USP15 expression stabilizes the KEAP1-CUL3 interaction, which drives ubiquitination and degradation of NRF2. USP15 has also been shown to be required for the processing of ubiquitinated substrates that are bound to CDC48/p97 [100, 101]. Given these observations, an altered interaction with USP15 may be a possible mechanism to describe the KEAP1 superbinder mutant phenotype.

Superbinder mutations may result in a structural change in KEAP1 that impedes interaction with USP15. In this event, KEAP1 ubiquitination may be enhanced under basal conditions, thus destabilizing the KEAP1-CUL3 E3 ubiquitin ligase complex and promoting NRF2 stabilization. To begin analyzing the role of USP15 in superbinder function, KEAP1 ubiquitination status should be assessed. Immunoprecipitation of either wild-type KEAP1 or R320Q under denaturing conditions, followed by Western blot analysis for ubiquitination will determine if there is a difference in ubiquitination under basal conditions. If USP15 cannot interact with R320Q, the anticipated result is increased ubiquitination of R320Q compared to wild-type. Complementary to this experiment, knockdown of *USP15* by siRNA transfection should eliminate any difference between wild-type KEAP1 and R320Q ubiquitination. In addition to analysis of KEAP1 ubiquitination status, co-immunoprecipitation should be performed to compare USP15 association with wild-type KEAP1 and the R320Q mutant.

One piece of data that argues against USP15 regulating KEAP1 deubiquitination is Figure 3.5.C, in which *in vivo* ubiquitination of NRF2 is approximately equal between wild-type and R320Q. In an *in vitro* ubiquitination assay, R320Q also performs as well as wild-type KEAP1 in ubiquitinating NRF2, but as this experiment was conducted with recombinant protein, we may assume that the ubiquitination status of the mutant and wild-type were equal. The experiment depicted in Figure 3.5.C was performed in HEK293T cells, which contain endogenous KEAP1. As KEAP1 acts as a homodimer, it is possible that the presences of wild-type KEAP1-R320Q heterodimers may be able to compensate for R320Q homodimers that may not function as efficiently in the KEAP1-CUL3 complex due to enhanced

ubiquitination. Future studies should include *in vivo* experiments in KEAP1$^{-/-}$ MEFs to eliminate any possible compensatory mechanism from endogenous wild-type KEAP1.

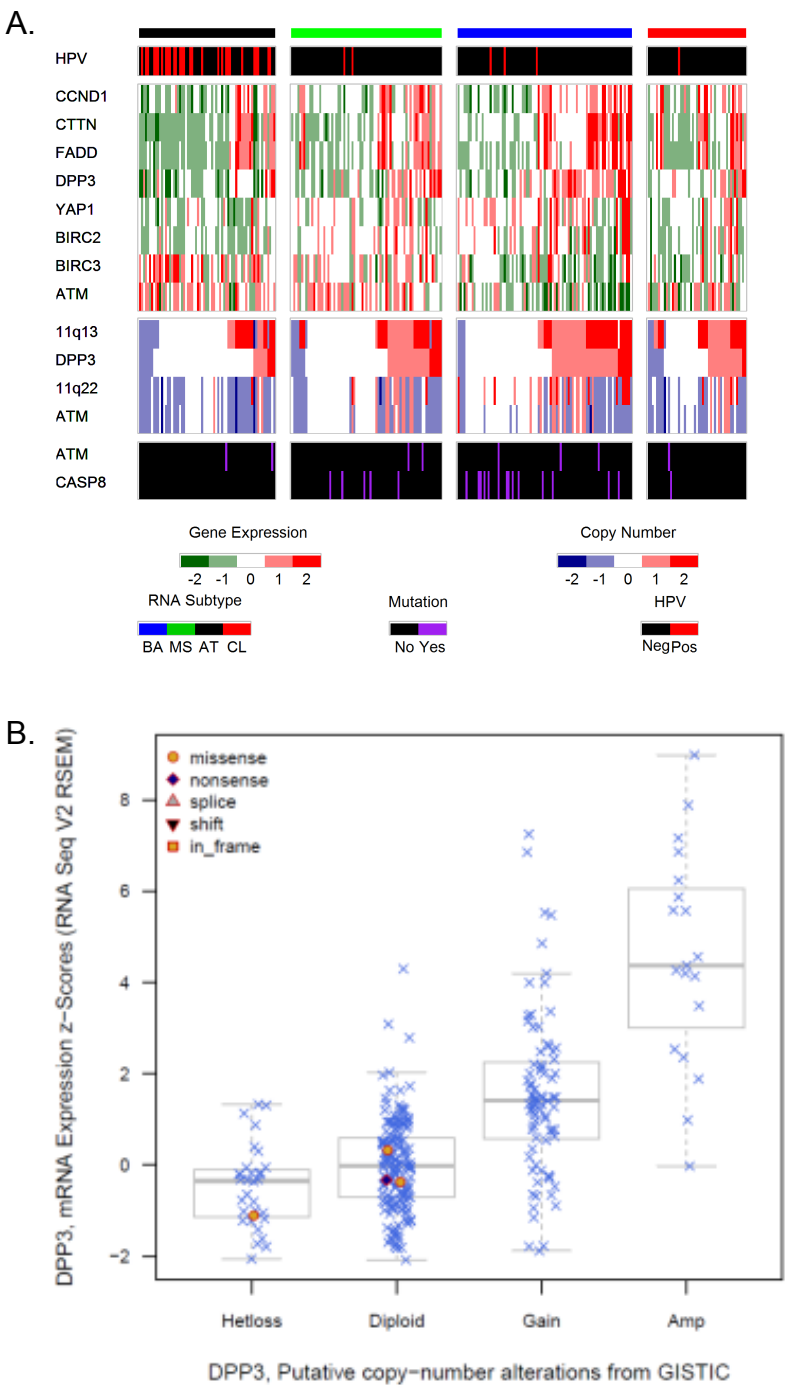

Figure 4.1. DPP3 is overexpressed in head and neck squamous cell carcinoma. (A) Gene expression and copy number analysis for head and neck squamous cell carcinoma subtypes. Tumor subtypes are displayed as columns; basal (BA), mesenchymal (MS), atypical (AT), classical (CL). (B) DPP3 expression (y axis) versus DPP3 copy number (x axis). Hetloss., heterozygous loss; Amp., amplification.

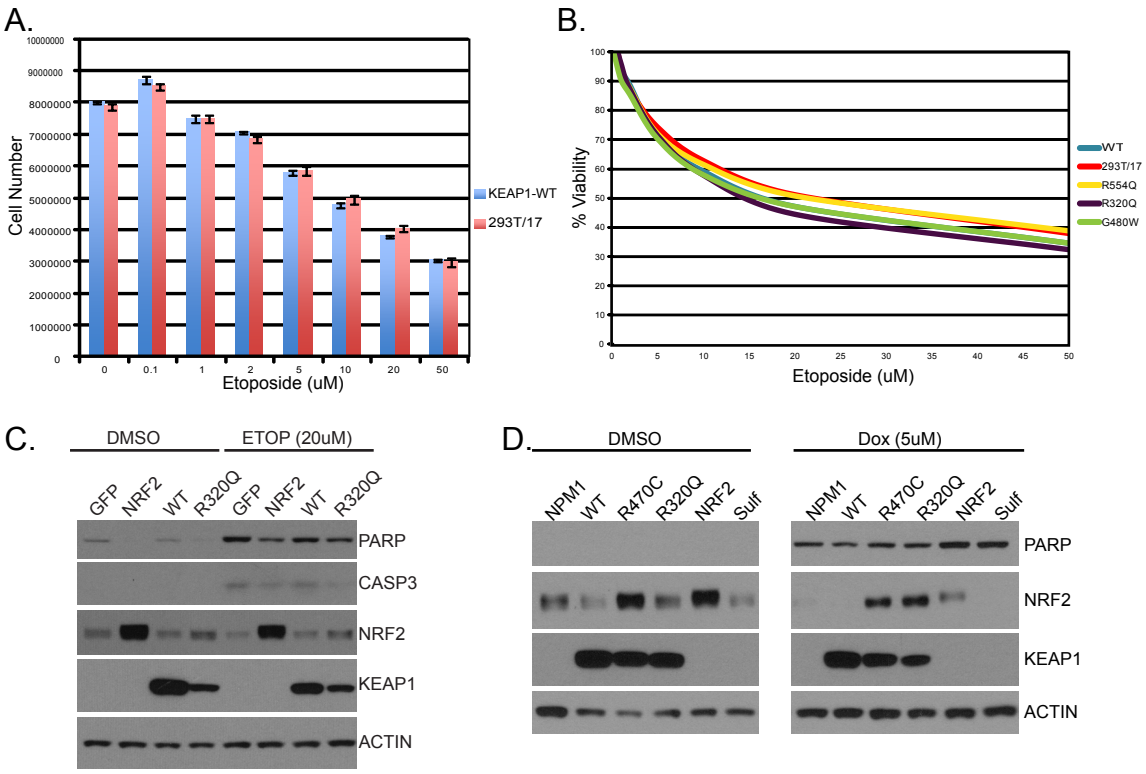

Figure 4.2. KEAP1 mutations do not significantly impact cell viability. (A) HEK293T cells stably expressing wild-type KEAP1 (KEAP1-WT) were treated with Etoposide for 48 hours. Cell viability was measured by mitochondrial repiration (PrestoBlue, Invitrogen) and compared to the parental line (293T/17). (B) HEK293T cells stably expressing a subset of KEAP1 mutations were treated with Etoposide for 48 hours. Cell viability was measured by total intracellular ATP (CellTiterGlo, Promega) and compared to the parental line (293T/17). Wild-type KEAP1, WT (C) A549 cells were transiently transfected with wild-type KEAP1 (WT), KEAP1-R320Q mutant (R320Q), NRF2, or negative control GFP for 24 hours. Cells were then treated with Etoposide for 12 hours, followed by Western blot for markers of apoptosis. PARP, cleaved PARP; CASP3, cleaved caspase-3. (D) A549 cells were transiently transfected with wild-type KEAP1 (WT), KEAP1-R470C mutant (R470C), KEAP1-R320Q mutant (R320Q), NRF2, or negative control nucleophosmin (NPM1) for 24 hours. A 12 hour pre-treatment with Sulforaphane (20uM) was also performed (Sulf). Cells were then treated with Etoposide for 48 hours, followed by Western blot for markers of apoptosis. PARP, cleaved PARP.

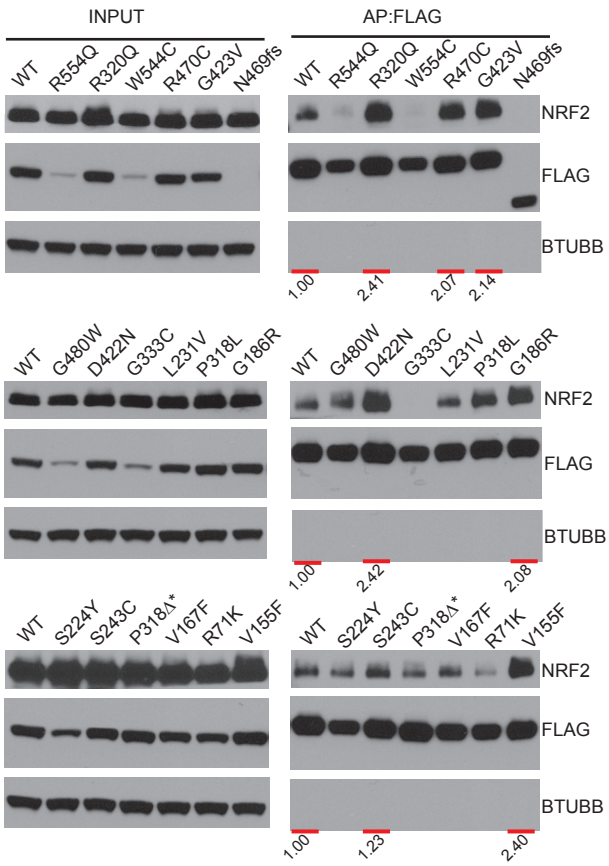

Figure 4.3. KEAP1 superbinder mutants bind more NRF2 than wild-type KEAP1. HEK293T cells were transiently transfected with wild-type KEAP1 (WT) or a mutant construct. Affinity purification of KEAP1 followed by Western blot for NRF2 was performed to assess NRF2 binding. Relative abundance of NRF2 binding for the superbinder mutants (annotated with red lines) was performed using ImageJ. NRF2 binding was normalized to the amount of KEAP1 (FLAG).

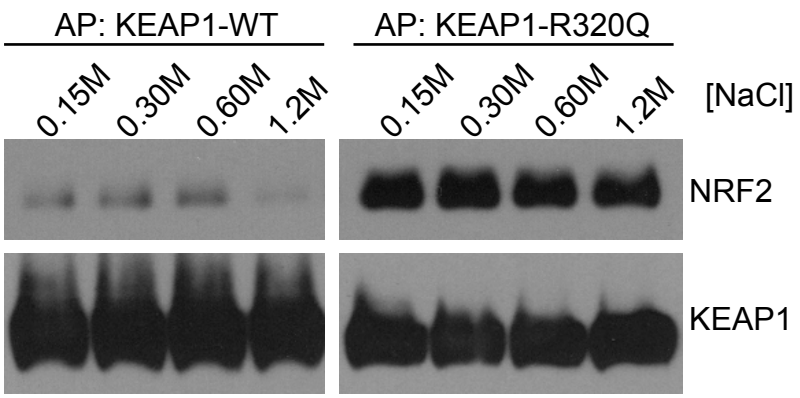

Figure 4.4. Wild-type KEAP1 and the R320Q superbinder bind NRF2 with similar affinities. HEK293T cells stably expressing wild-type KEAP1 (KEAP1-WT) or the R320Q mutant (KEAP1-R320Q) were lysed in 0.1% NP-40 lysis buffer (see Chapter 2 Materials and Methods for recipe), followed by affinity purification for KEAP1. The affinity resin was washed in 0.1% NP-40 lysis buffer with the indicated concentrations of NaCl and protein complexes were analyzed by Western blot to assess NRF2 binding.

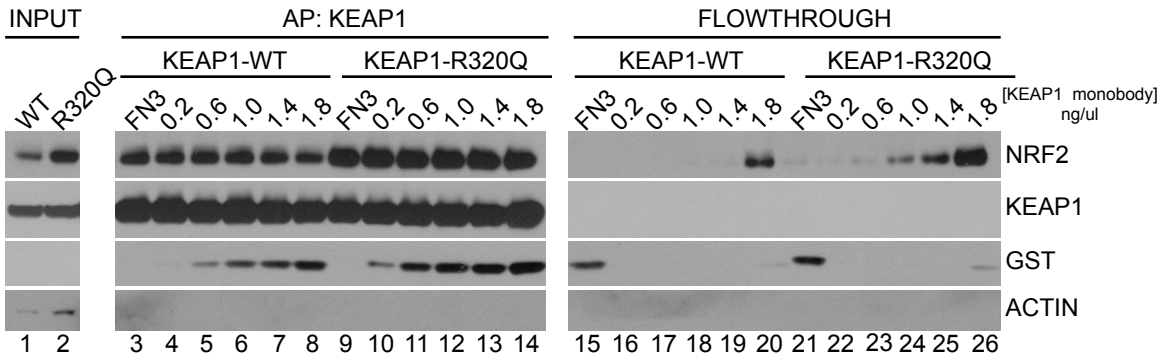

Figure 4.5. The KEAP1 monobody cannot compete NRF2 off of wild-type KEAP1 or the R320Q superbinder mutant. HEK293T cells stably expressing wild-type KEAP1 (KEAP1-WT) or the R320Q superbinder mutant (KEAP1-R320Q) were lysed in 0.1% NP-40 lysis buffer (see Chapter 2 Materials and Methods for recipes) and affinity purified for KEAP1. The affinity resin was resuspended in lysis buffer, and then incubated with negative control (FN3), or the indicated increasing concentrations of monobody for 1 hour at 4 degrees Celsius. Flowthrough was retained for Western blot analysis (FLOWTHROUGH). The affinity resin was then washed five times with lysis buffer. Western blot analysis was performed to assess NRF2 binding. The FN3 negative control peptide and KEAP1 monobody are detected by immunoblot for GST.

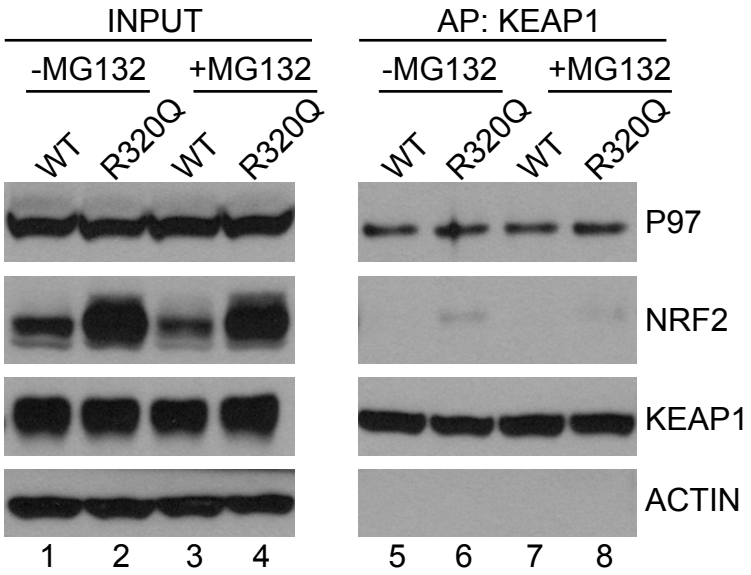

Figure 4.6. Wild-type KEAP1 and the R320Q superbinder mutant associate with p97 equally. HEK293T cells stably expressing wild-type KEAP1 (WT) or the R320Q superbinder mutant (R320Q) were affinity purified for KEAP1, followed by Western blot analysis to assess binding of p97.

REFERENCES

1. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response.* Mol Cell, 2009. **34**(6): p. 663-73.

2. Komatsu, M., et al., *The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1.* Nat Cell Biol, 2010. **12**(3): p. 213-23.

3. Hast, B.E., et al., *Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination.* Cancer Res, 2013. **73**(7): p. 2199-210.

4. Camp, N.D., et al., *Wilms tumor gene on X chromosome (WTX) inhibits degradation of NRF2 protein through competitive binding to KEAP1 protein.* J Biol Chem, 2012. **287**(9): p. 6539-50.

5. Simaga, S., et al., *Tumor cytosol dipeptidyl peptidase III activity is increased with histological aggressiveness of ovarian primary carcinomas.* Gynecol Oncol, 2003. **91**(1): p. 194-200.

6. Simaga, S., et al., *Dipeptidyl peptidase III in malignant and non-malignant gynaecological tissue.* Eur J Cancer, 1998. **34**(3): p. 399-405.

7. Konstantinopoulos, P.A., et al., *Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer.* Cancer Res, 2011. **71**(15): p. 5081-9.

8. Cancer Genome Atlas Research, N., *Comprehensive genomic characterization of squamous cell lung cancers.* Nature, 2012. **489**(7417): p. 519-25.

9. Katoh, Y., et al., *Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome.* Arch Biochem Biophys, 2005. **433**(2): p. 342-50.

10. Kobayashi, M., et al., *Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system.* Genes Cells, 2002. **7**(8): p. 807-20.

11. McMahon, M., et al., *Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron.* J Biol Chem, 2004. **279**(30): p. 31556-67.

12. Eggler, A.L., et al., *Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2.* Proc Natl Acad Sci U S A, 2005. **102**(29): p. 10070-5.

13. Kobayashi, A., et al., *Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1.* Mol Cell Biol, 2006. **26**(1): p. 221-9.

14. Zhang, D.D., *Mechanistic studies of the Nrf2-Keap1 signaling pathway.* Drug Metab Rev, 2006. **38**(4): p. 769-89.

15. de Mol, N.J., *Surface plasmon resonance for proteomics.* Methods Mol Biol, 2012. **800**: p. 33-53.

16. Lewis, S.M., *Computational protein interface design and prediction with experimental constraints.* UNC Thesis Database, 2012.

17. Abramic, M., et al., *Highly reactive cysteine residues are part of the substrate binding site of mammalian dipeptidyl peptidases III.* Int J Biochem Cell Biol, 2004. **36**(3): p. 434-46.

18. Fukasawa, K., et al., *The HELLGH motif of rat liver dipeptidyl peptidase III is involved in zinc coordination and the catalytic activity of the enzyme.* Biochemistry, 1999. **38**(26): p. 8299-303.

19. Jones, T.H. and A. Kapralou, *A rapid assay for dipeptidyl aminopeptidase III in human erythrocytes.* Anal Biochem, 1982. **119**(2): p. 418-23.

20. Tong, K.I., et al., *Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response.* Mol Cell Biol, 2007. **27**(21): p. 7511-21.

21. Li, X., et al., *Crystal structure of the Kelch domain of human Keap1.* J Biol Chem, 2004. **279**(52): p. 54750-8.

22. Baral, P.K., et al., *The first structure of dipeptidyl-peptidase III provides insight into the catalytic mechanism and mode of substrate binding.* J Biol Chem, 2008. **283**(32): p. 22316-24.

23. Prajapati, S.C. and S.S. Chauhan, *Dipeptidyl peptidase III: a multifaceted oligopeptide N-end cutter.* FEBS J, 2011. **278**(18): p. 3256-76.

24. Jajcanin-Jozic, N. and M. Abramic, *Hydrolysis of dipeptide derivatives reveals the diversity in the M49 family.* Biol Chem, 2013. **394**(6): p. 767-71.

25. Gevaert, K., et al., *Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides.* Nat Biotechnol, 2003. **21**(5): p. 566-9.

26. Khaket, T.P., et al., *Enkephalin degrading enzymes: metalloproteases with high potential for drug development.* Curr Pharm Des, 2012. **18**(2): p. 220-30.

27. Lee, C.M. and S.H. Snyder, *Dipeptidyl-aminopeptidase III of rat brain. Selective affinity for enkephalin and angiotensin.* J Biol Chem, 1982. **257**(20): p. 12043-50.

28. Tomic, A., et al., *Human dipeptidyl peptidase III: insights into ligand binding from a combined experimental and computational approach.* J Mol Recognit, 2011. **24**(5): p. 804-14.

29. Bezerra, G.A., et al., *Entropy-driven binding of opioid peptides induces a large domain motion in human dipeptidyl peptidase III.* Proc Natl Acad Sci U S A, 2012. **109**(17): p. 6525-30.

30. Jozkowicz, A., H. Was, and J. Dulak, *Heme oxygenase-1 in tumors: is it a false friend?* Antioxid Redox Signal, 2007. **9**(12): p. 2099-117.

31. Lau, A., et al., *Dual roles of Nrf2 in cancer.* Pharmacol Res, 2008. **58**(5-6): p. 262-70.

32. Wang, X.J., et al., *Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2.* Carcinogenesis, 2008. **29**(6): p. 1235-43.

33. Vogelstein, B., et al., *Cancer genome landscapes.* Science, 2013. **339**(6127): p. 1546-58.

34. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends Biochem Sci, 2009. **34**(4): p. 176-88.

35. Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes Dev, 1999. **13**(1): p. 76-86.

36. Shibata, T., et al., *Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy.* Proc Natl Acad Sci U S A, 2008. **105**(36): p. 13568-73.

37. Hanada, N., et al., *Methylation of the KEAP1 gene promoter region in human colorectal cancer.* BMC Cancer, 2012. **12**: p. 66.

38. Muscarella, L.A., et al., *Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome.* Epigenetics, 2011. **6**(3): p. 317-25.

39. Muscarella, L.A., et al., *Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer.* Epigenetics, 2011. **6**(6): p. 710-9.

40. Sasaki, H., et al., *Increased NRF2 gene (NFE2L2) copy number correlates with mutations in lung squamous cell carcinomas.* Mol Med Rep, 2012. **6**(2): p. 391-4.

41. Wang, R., et al., *Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues.* Biochem Biophys Res Commun, 2008. **373**(1): p. 151-4.

42. Zhang, P., et al., *Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth.* Mol Cancer Ther, 2010. **9**(2): p. 336-46.

43. Govindan, R., et al., *Genomic landscape of non-small cell lung cancer in smokers and never-smokers.* Cell, 2012. **150**(6): p. 1121-34.

44. Ji, L., et al., *Nrf2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer.* PLoS One, 2013. **8**(5): p. e63404.

45. Merk, J., et al., *Chemoresistance in non-small-cell lung cancer: can multidrug resistance markers predict the response of xenograft lung cancer models to chemotherapy?* Eur J Cardiothorac Surg, 2011. **40**(1): p. e29-33.

46. Yamadori, T., et al., *Molecular mechanisms for the regulation of Nrf2-mediated cell proliferation in non-small-cell lung cancers.* Oncogene, 2012. **31**(45): p. 4768-77.

47. DeNicola, G.M., et al., *Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis.* Nature, 2011. **475**(7354): p. 106-9.

48. Shelton, P. and A.K. Jaiswal, *The transcription factor NF-E2-related factor 2 (Nrf2): a protooncogene?* FASEB J, 2013. **27**(2): p. 414-23.

49. Niture, S.K. and A.K. Jaiswal, *INrf2 (Keap1) targets Bcl-2 degradation and controls cellular apoptosis.* Cell Death Differ, 2011. **18**(3): p. 439-51.

50. Lo, S.C. and M. Hannink, *PGAM5, a Bcl-XL-interacting protein, is a novel substrate for the redox-regulated Keap1-dependent ubiquitin ligase complex.* J Biol Chem, 2006. **281**(49): p. 37893-903.

51. Soundararajan, R., et al., *Differential activities of glucocorticoid-induced leucine zipper protein isoforms.* J Biol Chem, 2007. **282**(50): p. 36303-13.

52. Canterini, S., et al., *THG-1pit moves to nucleus at the onset of cerebellar granule neurons apoptosis.* Mol Cell Neurosci, 2009. **40**(2): p. 249-57.

53. Jones, A., et al., *TSC22D4 is a molecular output of hepatic wasting metabolism.* EMBO Mol Med, 2013. **5**(2): p. 294-308.

54. Wagner, S., et al., *Association of the Ste20-like kinase (SLK) with the microtubule. Role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading.* J Biol Chem, 2002. **277**(40): p. 37685-92.

55. Quizi, J.L., et al., *SLK-mediated phosphorylation of paxillin is required for focal adhesion turnover and cell migration.* Oncogene, 2012.

56. Wagner, S., et al., *FAK/src-family dependent activation of the Ste20-like kinase SLK is required for microtubule-dependent focal adhesion turnover and cell migration.* PLoS One, 2008. **3**(4): p. e1868.

57. Cybulsky, A.V., et al., *The Ste20-like kinase SLK promotes p53 transactivation and apoptosis.* Am J Physiol Renal Physiol, 2009. **297**(4): p. F971-80.

58. Sabourin, L.A. and M.A. Rudnicki, *Induction of apoptosis by SLK, a Ste20-related kinase.* Oncogene, 1999. **18**(52): p. 7566-75.

59. Cybulsky, A.V., et al., *Renal expression and activity of the germinal center kinase SK2.* Am J Physiol Renal Physiol, 2004. **286**(1): p. F16-25.

60. Zhang, Y.H., et al., *Expression of the Ste20-like kinase SLK during embryonic development and in the murine adult central nervous system.* Brain Res Dev Brain Res, 2002. **139**(2): p. 205-15.

61. Ellinger-Ziegelbauer, H., et al., *Ste20-like kinase (SLK), a regulatory kinase for polo-like kinase (Plk) during the G2/M transition in somatic cells.* Genes Cells, 2000. **5**(6): p. 491-8.

62. O'Reilly, P.G., et al., *The Ste20-like kinase SLK is required for cell cycle progression through G2.* J Biol Chem, 2005. **280**(51): p. 42383-90.

63. Hao, W., et al., *Induction of apoptosis by the Ste20-like kinase SLK, a germinal center kinase that activates apoptosis signal-regulating kinase and p38.* J Biol Chem, 2006. **281**(6): p. 3075-84.

64. Jeong, G.S., et al., *Protective effect of sauchinone by upregulating heme oxygenase-1 via the P38 MAPK and Nrf2/ARE pathways in HepG2 cells.* Planta Med, 2010. **76**(1): p. 41-7.

65. Naidu, S., et al., *Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2.* J Immunol, 2009. **182**(11): p. 7048-57.

66. Zipper, L.M. and R.T. Mulcahy, *Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes.* Biochem Biophys Res Commun, 2000. **278**(2): p. 484-92.

67. Cheng, I.H., L.A. Roberts, and B.K. Tye, *Mcm3 is polyubiquitinated during mitosis before establishment of the pre-replication complex.* J Biol Chem, 2002. **277**(44): p. 41706-14.

68. Maiorano, D., J.M. Lemaitre, and M. Mechali, *Stepwise regulated chromatin assembly of MCM2-7 proteins.* J Biol Chem, 2000. **275**(12): p. 8426-31.

69. Okuno, Y., et al., *Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle.* EMBO J, 2001. **20**(15): p. 4263-77.

70. Nguyen, T., et al., *Interactions of the human MCM-BP protein with MCM complex components and Dbf4.* PLoS One, 2012. **7**(4): p. e35931.

71. Nishiyama, A., L. Frappier, and M. Mechali, *MCM-BP regulates unloading of the MCM2-7 helicase in late S phase.* Genes Dev, 2011. **25**(2): p. 165-75.

72. Fava, L.L., et al., *Probing the in vivo function of Mad1:C-Mad2 in the spindle assembly checkpoint.* EMBO J, 2011. **30**(16): p. 3322-36.

73. Tipton, A.R., et al., *Closed MAD2 (C-MAD2) is selectively incorporated into the mitotic checkpoint complex (MCC).* Cell Cycle, 2011. **10**(21): p. 3740-50.

74. Tipton, A.R., et al., *BUBR1 and closed MAD2 (C-MAD2) interact directly to assemble a functional mitotic checkpoint complex.* J Biol Chem, 2011. **286**(24): p. 21173-9.

75. Suzuki, T., J. Maher, and M. Yamamoto, *Select heterozygous Keap1 mutations have a dominant-negative effect on wild-type Keap1 in vivo.* Cancer Res, 2011. **71**(5): p. 1700-9.

76. Hayes, J.D., et al., *Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway.* Antioxid Redox Signal, 2010. **13**(11): p. 1713-48.

77. Kensler, T.W., N. Wakabayashi, and S. Biswal, *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 89-116.

78. Lee, J.M. and J.A. Johnson, *An important role of Nrf2-ARE pathway in the cellular defense mechanism.* J Biochem Mol Biol, 2004. **37**(2): p. 139-43.

79. Rangasamy, T., et al., *Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice.* J Clin Invest, 2004. **114**(9): p. 1248-59.

80. Satoh, H., et al., *Nrf2 prevents initiation but accelerates progression through the Kras signaling pathway during lung carcinogenesis.* Cancer Res, 2013.

81. Shen, H., S. Zhou, and J. Wang, *The paradoxical role of Nrf2 in tumor biology.* Crit Rev Eukaryot Gene Expr, 2013. **23**(1): p. 37-47.

82. Zhang, D.D. and M. Hannink, *Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress.* Mol Cell Biol, 2003. **23**(22): p. 8137-51.

83. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008. **132**(3): p. 344-62.

84. Lee, J.M., et al., *Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis.* J Biol Chem, 2003. **278**(14): p. 12029-38.

85. Mauro, C., et al., *The NF-kappaB transcription factor pathway as a therapeutic target in cancer: methods for detection of NF-kappaB activity.* Methods Mol Biol, 2009. **512**: p. 169-207.

86. Lee, D.F., et al., *KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta.* Mol Cell, 2009. **36**(1): p. 131-40.

87. Niture, S.K. and A.K. Jaiswal, *Inhibitor of Nrf2 (INrf2 or Keap1) protein degrades Bcl-xL via phosphoglycerate mutase 5 and controls cellular apoptosis.* J Biol Chem, 2011. **286**(52): p. 44542-56.

88. Tian, H., et al., *Keap1: one stone kills three birds Nrf2, IKKbeta and Bcl-2/Bcl-xL.* Cancer Lett, 2012. **325**(1): p. 26-34.

89. Sporn, M.B. and K.T. Liby, *NRF2 and cancer: the good, the bad and the importance of context.* Nat Rev Cancer, 2012. **12**(8): p. 564-71.

90. Hu, R., et al., *Regulation of NF-E2-related factor 2 signaling for cancer chemoprevention: antioxidant coupled with antiinflammatory.* Antioxid Redox Signal, 2010. **13**(11): p. 1679-98.

91. Eades, G., et al., *miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells.* J Biol Chem, 2011. **286**(47): p. 40725-33.

92. Majem, M. and C. Pallares, *An update on molecularly targeted therapies in second- and third-line treatment in non-small cell lung cancer: focus on EGFR inhibitors and anti-angiogenic agents.* Clin Transl Oncol, 2013. **15**(5): p. 343-57.

93. Gerber, D.E., et al., *Stromal platelet-derived growth factor receptor alpha (PDGFRalpha) provides a therapeutic target independent of tumor cell PDGFRalpha expression in lung cancer xenografts.* Mol Cancer Ther, 2012. **11**(11): p. 2473-82.

94. Shepherd, F.A., P.A. Bunn, and L. Paz-Ares, *Lung cancer in 2013.* Am Soc Clin Oncol Educ Book, 2013. **2013**: p. 339-46.

95. Taguchi, K., et al., *Genetic analysis of cytoprotective functions supported by graded expression of Keap1.* Mol Cell Biol, 2010. **30**(12): p. 3016-26.

96. Elsasser, S. and D. Finley, *Delivery of ubiquitinated substrates to protein-unfolding machines.* Nat Cell Biol, 2005. **7**(8): p. 742-9.

97. Finley, D., *Recognition and processing of ubiquitin-protein conjugates by the proteasome.* Annu Rev Biochem, 2009. **78**: p. 477-513.

98. Meyer, H., M. Bug, and S. Bremer, *Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system.* Nat Cell Biol, 2012. **14**(2): p. 117-23.

99. Zhang, D.D., et al., *Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway.* J Biol Chem, 2005. **280**(34): p. 30091-9.

100. Villeneuve, N.F., et al., *USP15 Negatively Regulates Nrf2 through Deubiquitination of Keap1.* Mol Cell, 2013.

101. Cayli, S., et al., *COP9 signalosome interacts ATP-dependently with p97/valosin-containing protein (VCP) and controls the ubiquitination status of proteins bound to p97/VCP.* J Biol Chem, 2009. **284**(50): p. 34944-53.