PROTEOMIC DISSECTION OF KEAP1/NRF2 SIGNALING TO DETERMINE NEW PATHWAY INTERACTORS IN CANCER

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ABSTRACT

Kathleen Michelle Mulvaney: Proteomic dissection of KEAP1/NRF2 signaling to determine new pathway interactors in cancer
(Under the direction of Michael Benjamin Major)

KEAP1/NRF2 signaling regulates intracellular reactive oxygen species and protects cells from reactive oxygen-induced damage. KEAP1 serves as the substrate adaptor for a CULLIN3-based E3 ubiquitin ligase (KEAP1-CUL3-RBX1). Under homeostatic conditions, the KEAP1-CUL3-RBX1 ligase targets its well-established substrate NFE2L2/NRF2 for rapid proteasomal degradation. During oxidative stress conditions, KEAP1 is inactivated, and NRF2 protein levels increase. NRF2 then drives the transcription of a battery of cytoprotective genes that ultimately mitigate the cellular stress that was sensed by KEAP1. This elegant signaling pathway has long been thought to be the primary function of the redox-sensitive KEAP1 E3 ligase complex.

KEAP1/NRF2 signaling is the cell's primary defense against reactive oxygen stress. Therefore, perturbations in this pathway are associated with a number of human pathologies, including cancer. The KEAP1/NRF2 pathway is frequently mutated in cancer, where NRF2-activating mutations correlate with disease progression and poor patient outcomes. In addition to somatic gene mutations in KEAP1, NRF2 or CUL3, we have demonstrated that NRF2 is activated at the protein level in tumors by a

competitive binding method, underscoring the importance of understanding the protein-protein interactions within this pathway. Utilizing mass spectrometry-based approaches, we identified the KEAP1 protein interaction network under basal and proteasome-inhibited conditions. Coupling this screening with a candidate-based approach, MCM3 and NRF1 were identified as putative, novel KEAP1-CUL3-RBX1 substrates for ubiquitylation. MCM3, a subunit of the essential DNA replicative helicase, was validated as a KEAP1-CUL3-RBX1 substrate for ubiquitylation. We have characterized the binding and ubiquitylation of MCM3 by KEAP1 and determined that KEAP1 does not regulate MCM3 protein stability. Rather, we propose a model where KEAP1 ubiquitylates MCM3 to regulate its function within the replicative helicase. We demonstrate that KEAP1 associates with chromatin in a cell cycle-dependent fashion with kinetics similar to MCM3 and is thus poised to affect MCM3 function. We also demonstrate that loss of KEAP1 affects cell cycle progression and proliferation in normal cells. Therefore, we have found previously unappreciated roles for KEAP1 in cell cycle progression and chromatin dynamics.

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LIST OF ABBREVIATIONS

4NQO	Nitroquinoline 1-oxide
AD	Alzheimer's Disease
AP	Affinity purification
ARE	Antioxidant response element
BTB	Bric-a-brac, tramtrack domain
CDDO-me	Bardoxolone methyl
CRL	Cullin RING ligase
DME	Drug metabolizing enzyme
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
H_2O_2	Hydrogen peroxide
IP	Immunoprecipitation
IVR	Intervening region
mM	Millimolar
MS	Mass spectrometry
nM	Nanomolar
NaCl	Sodium chloride
NSCLC	Non-small cell lung cancer
O_2-	Superoxide
OH-	Hydroxyl radical
PCR	Polymerase chain reaction
PLA	Proximity ligation assay
qPCR	Quantitative real-time polymerase chain reaction

Redox	Oxidation-reduction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
tBHQ	Tert-butylhydroquinone
μM	Micromolar

CHAPTER I: INTRODUCTION

1.A Reactive Oxygen Species

Reactive oxygen species (ROS) are produced through many normal cellular pathways. ROS refer generally to any oxygen-derived free radical species including superoxide anions (O_2-), hydroxyl (HO), alkyoxyl (RO) and peroxyl (RO_2) radicals, and to molecules that are readily converted to radical species, such as hydrogen peroxide (H_2O_2) (1,2). The majority of intracellular ROS are produced by mitochondria during metabolic processes, particularly the oxidative phosphorylation pathway (3-6). Other organelle-based sources of intracellular ROS include enzymatic reactions within the endoplasmic reticulum (ER) and peroxisomes (7-9). ROS can also act as signaling molecules and can be produced focally at the plasma membrane to increase receptor-mediated signaling (10). For example, ROS have been shown to cooperate with the Fas ligand signal machinery to increase death receptor clustering and lipid raft formation upon receiving positive cues through NADPH oxidase (11). However, ROS signaling is not always detrimental. The transient production of ROS is required for cell proliferation in response to mitogens and H_2O_2 is produced in response to a number of pro-growth receptor tyrosine signaling cascades including epithelial growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGR) (12-16).

In addition to endogenously produced ROS, cells are exposed to environmental toxins and extracellular sources of electrophiles. These can either be converted to ROS upon interaction with cellular enzymes or can readily participate in reduction-oxidation (redox) reactions. The cell possesses means to maintain redox homeostasis (discussed in the next

section, Chapter 1B). However, rapid increases in intracellular ROS can lead to oxidative stress wherein macromolecules may be damaged through DNA adduct formation, lipid peroxidation, and protein oxidation and adduct formation (17-20). While the cell utilizes a host of cellular defense and detoxifying pathways to remove or neutralize ROS, when these cellular protection mechanisms are overwhelmed, the cell will undergo programmed cell death (21-23). Furthermore, if a damaged cell escapes cell death, it can contribute to disease. Therefore, it is essential for cell health and survival that the cell maintains redox balance and quickly mitigates any spikes in ROS.

1.B Cellular Defense Against Reactive Oxygen Stress

Several key genes responsible for ROS detoxification were found to have an AP-1 like enhancer sequence in their promoter regions (24,25). Nuclear factor, erythroid derived 2, like 2 (NFE2L2/NRF2) was first identified as a regulator of the cellular detoxification response when it was determined to be the transcription factor that recognized this motif in the NAD(P)H:quinone oxidoreductase (NQO1) and glutathione S-transferase (GST) genes (24-26). This AP-1 like enhancer is now known as the antioxidant response element (ARE) and has a well-defined consensus sequence of 5' TGA(C/T)nnnGCA 3' (27). NRF2 heterodimerizes with small Maf proteins and together they recognize and bind to the ARE in the promoter regions of NRF2 target genes (25,28,29). Global identification of NRF2 DNA binding sites by chromatin immunoprecipitation-sequencing (ChIP-Seq) as well as the NRF2-induced gene signatures measured by microarray have collectively identified hundreds of NRF2-responsive genes (27,30,31). In response to elevated ROS in the cell, NRF2 induces a battery of cytoprotective and antioxidant genes that act to clear ROS, mitigate cell stress and promote cell survival (27,32-34). Target genes of NRF2 fall into four major classes: 1) glutathione synthesis, 2) phase II detoxifying enzymes, 3) xenobiotic efflux pumps, and 4) protein degradation/proteotoxic

clearance (27,30,35). The role of each of these gene classes is discussed in the following sections (1.2A-1.2D).

The importance of NRF2 transcriptional activity in regulating the redox state of the cell is further confirmed by manipulations of NRF2 expression. This is demonstrated by the observation that *Nrf2* knockout mice, while viable, have a decreased threshold of stress tolerance and are thus more sensitive to ROS-mediated cell toxicity and carcinogenesis (36-40). Reciprocally, induction of NRF2 by pharmacological agents confers cellular protection against challenge with a host of toxicants and electrophiles (36-39). Collectively, these findings demonstrate that NRF2 is a master regulator of redox homeostasis and functions as the cell's primary defense against reactive oxygen stress.

1.B.1 The Glutathione System in Redox Homeostasis

Free radical scavengers act directly on reactive species and eliminate them by donating one or more electrons (41). The most abundant cellular thiol antioxidant and free radical scavenger is glutathione or γ-L-Glutamyl-L-cysteinylglycine (42). Other less abundant cellular free radical scavengers include ascorbic and uric acids, bilirubin and tocopherol. Glutathione (GSH) synthesis is enzymatically catalyzed in a two reaction process by glutamylcysteine synthetase and GSH synthetase (43,44). Glutathione exists in both a reduced (GSH) and an oxidized state (GSSG) and the ratio of GSH to GSSG provides a reasonable metric of the overall redox state of the cell. In the reduced GSH state, the sulfhydryl group on the cysteine residue can donate an electron and thereby act as a reducing equivalent for ROS. Following oxidation, GSSG can be readily converted back to GSH by glutathione reductase. Maintaining sufficient GSH in the cell is important for cell survival. NRF2 regulates the genes encoding proteins responsible for both glutathione synthesis (glutamate-cysteine ligase catalytic subunit*,* *Gclc,* and glutamate-cysteine ligase modifier subunit, *Gclm*) and for GSH recycling back into its reduced form (glutathione reductase) (45).

1.B.2 Electrophile Detoxifying Proteins and Enzymes

In addition to glutathione, the cell also activates redox-balancing antioxidant proteins and detoxifying enzymes that participate in and catalyze the reduction of free radicals. NRF2 induces a range of phase II detoxifying enzymes including NAD(P)H:quinone oxidoreductase (NQO1), superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CT) (46,47). These drug-metabolizing enzymes (DMEs) lead to the detoxification and elimination of various exogenous and endogenous chemicals. Specifically, NQO1 reduces reactive quinones. SOD reduces O_2- to H_2O_2 and GPX and CT each reduce H_2O_2 to H_2O. In the conversion of H_2O_2 to H_2O by GPX, glutathione is used as a reducing equivalent, yielding an oxidized GSSG molecule. The GPX-GSH system has been shown to be a major cellular means of repairing lipid peroxidation, protein adduction and DNA damage (48,49). Another class of detoxifying proteins activated by NRF2-mediated transcription is comprised of proteins involved in the conjugation of antioxidants to ROS. This class of NRF2 target genes includes glutathione S-transferase (GST), which conjugates GSH to electrophiles for detoxification, and glucuronosyltransferase (UGT), which conjugates glucuronic acid to xenobiotics to increase their excretion from the cell (31).

In addition to phase II DMEs, NRF2 also drives the transcription of several proteins that serve as antioxidants or that help to regenerate the pool of available antioxidants, but that do not directly reduce ROS. These include thioredoxin, thioredoxin reductase, ferritin, and heme oxygenase-1 (34,50,51) Thioredoxin primarily acts to form reduced disulfide bonds on proteins (51). In order to regenerate active thioredoxin, thioredoxin reductase reduces oxidized thioredoxin using NADPH as an electron donor. Similarly, as GSH is used as a reducing equivalent in reactions by GPX, the oxidized glutathione (GSSG) is returned to GSH by glutathione reductase using NADPH as an electron donor. Thus, many of the cellular redox reactions rely on nicotinamide pairs, NADP+/NADPH and NAD+/NADH. Therefore, the cell also activates proteins to replenish NADPH levels in the cell following elevated ROS (52). NADPH

regeneration is accomplished primarily through glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (31) and NRF2 also regulates these genes (53).

In the presence of transient metal ions, such as Fe^{2+}, hydrogen peroxide readily undergoes a Fenton reaction and generates the highly reactive hydroxyl free radical. Under elevated ROS, heme-carrying proteins can release their heme groups, driving Fenton reactions that can further amplify the ROS present in the cell. To combat this, cells employ NRF2-mediated transcription to activate heme oxygenase-1 (HMOX1), which catalyzes the pro-oxidant heme into bilirubin (54) and ferritin (Ftn), which sequesters free ferric ions in order to store and transfer them in a less toxic form (31,55).

1.B.3 Xenobiotic Efflux

In addition to genes required for the neutralization and catabolism of intracellular ROS, NRF2 also regulates genes involved in xenobiotic efflux. The transcription of the multidrug resistance protein (MDR) and multidrug resistance-associated protein (MRP) families of ATP binding cassette (ABC) transporters are also induced upon NRF2 activation (56,57). These plasma membrane-bound transporters are responsible for the efflux of normal intracellular metabolites, xenobiotics, and xenobiotic metabolites from the cell. These transporters relieve the potential for macromolecular damage by reactive xenobiotics or their metabolites. In addition to their role in relieving cellular stress, they also confer resistance to chemotherapeutic agents (58). NRF2-activated drug efflux through MRP and MDR transporters is thought to contribute to NRF2-mediated chemotherapeutic resistance (58).

1.B.4 Proteotoxic Response and Protein Degradation Pathways

ROS can reversibly or irreversibly modify proteins. These modifications can lead to altered protein structure and function, potentially contributing to aberrant signaling or protein aggregation. Thus, removing oxidatively damaged proteins following elevated ROS is an

important process to maintaining cellular fitness. NRF2 mitigates the removal of oxidatively damaged proteins by inducing the transcription of many proteasome subunits including Psma1, Psma4 and Psmb5 (59). The increase in antioxidant- and NRF2-mediated transcription of proteasome subunits increases proteasome activity and thereby increases protein turnover in the cell (59).

1.2E Summary of NRF2-Mediated Transcription in Redox Homeostasis

In summary, NRF2 activates the transcription of a broad network of genes that synergistically mitigate reactive oxygen stress. NRF2 transcriptionally activates genes responsible for synthesizing and regenerating glutathione--the most abundant cellular antioxidant. Additionally, NRF2 increases the levels of enzymes capable of utilizing glutathione as a reducing equivalent in the catabolism of ROS. Similarly, NRF2 coordinately activates expression of enzymes that utilize NADPH as reducing equivalents as well as the genes required to restore cellular NADPH levels. In parallel, NRF2 activates a host of phase II detoxifying enzymes and xenobiotic efflux transporters for the neutralization and elimination of ROS and xenobiotics. Finally, NRF2 repairs or removes proteins that have been damaged by ROS through driving the transcription of: 1) proteins that directly relieve the oxidation of cysteine residues within cellular proteins that have been modified by ROS (i.e, thioredoxin) and 2) proteasomal subunits that lead to increased protein turnover. Thus, NRF2 orchestrates an interconnected effort to eliminate reactive oxygen stress and return cells to redox homeostasis.

1.C NRF2 Regulation by the KEAP1-CUL3-RBX1 Ligase

Basal NRF2 proteins levels are kept low by sequestration and ubiquitylation by the KEAP1 E3 ligase complex (36,60). Kelch-Like ECH-Associated Protein 1 (KEAP1) is the substrate adaptor protein for a Cullin3-based E3 ubiquitin ligase complex, KEAP1-CUL3-RBX1

(61,62). Cullin-RING ligases (CRLs) are a family of enzymes that processively add ubiquitin molecules covalently to their substrates in conjunction with 1) a substrate adaptor protein (i.e, KEAP1) that recognizes substrates and places them in a favorable conformation for ubiquitylation, 2) an E2 conjugating enzyme (following activation of ubiquitin by an E1 enzyme), and 4) the RING-like protein RBX1 (63,64). Polyubiquitylation by E3 ubiquitin ligases can target substrates for proteolysis by the 26S proteasome—as is the case for NRF2, for lysosomal degradation or can affect the substrate's subcellular localization or function (65,66).

NRF2 is the most well established substrate of the KEAP1-CUL3-RBX1 complex (61,62,67). Under homeostatic conditions, NRF2 is rapidly targeted for ubiquitin-proteasome mediated degradation by the KEAP1-CUL3-RBX1 ligase, having a half-life of less than 15 minutes (60,68). Genetic deletion or pharmacological inhibition of KEAP1 or CUL3 leads to rapid stabilization of NRF2 protein levels and increased NRF2-driven transcription (62,69). KEAP1 acts as a sensor of cellular redox state through its many cysteine residues (discussed further in the following section). In response to ROS, or to the addition of KEAP1 antagonists, the reactive cysteine residues within KEAP1 undergo electrophilic attack, causing a conformational change in KEAP1 (70-75). NRF2 is no longer efficiently targeted for proteasome-mediated degradation and newly synthesized NRF2 is thus able to accumulate, translocate to the nucleus and drive transcription of the multitude of cytoprotective and antioxidant target genes that were discussed in the previous section (32,33,60,76). An illustration of the KEAP1-NRF2 pathway can be found in Figure 1.1. In response to ROS or KEAP1 antagonist treatment, NRF2 protein stabilization is detected within 15 minutes (60,69) and increased NRF2 target gene transcription is detectable within 2 hours after treatment with ROS mimetics/KEAP1 antagonists (unpublished data). Thus, tight regulation of NRF2 at the protein level allows for low basal levels of antioxidant gene transcription sufficient to handle physiological ROS and for a very rapid adaptive response to oxidative stress and electrophiles.

1.D KEAP1-NRF2 Protein Structures and Complex Formation

1.D.1 KEAP1 Domain Homology and Structure

KEAP1 was first identified as a NRF2 binding protein through a yeast two-hybrid assay baiting with the N-terminus of NRF2 (77). KEAP1 was later identified as a CUL3 binding protein and substrate adaptor protein for NRF2 because structurally related, BTB-containing proteins function had been shown to utilize this domain to bind CUL3 and form CRLs (61). KEAP1 is comprised of three protein domains: the N-terminal bric-a-brac, tramtrack, broad complex or BTB domain responsible for CUL3 binding and KEAP1 homodimerization (78,79), the cysteine-rich intervening region or IVR and the C-terminal KELCH domain that is required for substrate binding (77,80,81) (Figure 1.2). KEAP1 is a 624 amino acid protein that is uniquely cysteine-rich; it contains 27 cysteine residues and is thus poised to sense the redox state of the cell through ROS-mediated attack of its many thiol groups. These can be oxidized to form sulfenic acid, covalent adducts with the attacking electrophiles or new disulfide bonds with other cysteine residues (62,70,75,82,83).

A homodimer of KEAP1 binds to a single molecule of NRF2; the KELCH domains of each KEAP1 molecule form β-propeller structures that serve as substrate-binding pockets (84). KEAP1 binds to a high affinity motif (ETGE) and a second a lower affinity motif (DLG) within the NRF2 protein (79,84,85). Structural analysis by X-ray crystallography of the KELCH domain of KEAP1 co-complexed with the Neh2 domain of NRF2 demonstrated that the DLG and ETGE motifs form β-turns and bind through electrostatic interactions between their aspartate and glutamate residues and the arginine residues 380, 415 and 483 in the Kelch domain of KEAP1 (75,81,86). Site-directed mutagenesis of this ‘arginine-triad’ within KEAP1 demonstrated these amino acids are also critical for KEAP1 binding to NRF2 (79). Using cryo-electron microscopy (cryo-EM) and fluorescence resonance energy transfer (FRET) microscopy, a homodimer of KEAP1 was also visualized to form a ‘cherry-bob’ structure and interact with a single molecule

of NRF2 in the cell (84,87). While there have been several proposed models of how the KEAP1/NRF2 protein-protein interaction occurs and whether binding or ubiquitylation is disrupted under reactive oxygen stress, the prevailing model is the hinge-and-latch model where the ETGE forms the hinge and the DLG forms the latch for very tight binding between KEAP1 and NRF2 (85,88). Specifically, the affinity of KEAP1 for the ETGE motif is approximately 100-fold higher than that of the DLG motif (84). Under reactive oxygen stress or in the presence of a subset of KEAP1 tumor mutants, KEAP1 maintains the ability to associate with NRF2 yet NRF2 is not efficiently degraded (69,73,87). Indeed, we have observed similarly that treatment with the ROS inducer tBHQ or the ROS mimetic sulforaphane, which are known to adduct on cysteines within KEAP1, led to increases in NRF2 stability and activity, but ubiquitylation of NRF2 was not ablated (Figure 2.6B). Though, the proportion of ubiquitylated NRF2 to the proportion of total NRF2 was decreased (Figure 2.6B). Thus, KEAP1 attack by reactive oxygen species may be affecting the association between KEAP1 and the DLG/'latch' motif, or more likely it may be affecting the speed or processivity of the ubiquitylation reaction or the release/delivery of ubiquitylated NRF2 to the proteasome.

The binding of the DLG and ETGE motifs within NRF2 to the KELCH domains of KEAP1 places NRF2 in a sterically favorable orientation for ubiquitylation on the seven lysine residues spaced between the two binding motifs (79,85). The protein domain structures and pertinent amino acids for the KEAP1-NRF2 interface are illustrated in Figure 1.2. The IVR domain contains several of the redox-reactive cysteines that regulate its ability to degrade NRF2. While the majority of KEAP1 cysteine residues have been shown to be reactive with electrophiles *in vitro* (89), the three cysteines found to be the most reactive with intracellular ROS and electrophilies are Cys151, Cys273 and Cys288 (36,71,84,90,91).

1.D.2 NRF2 Domain Homology and Structure

NRF2 is a member of the cap 'n' collar (CNC)-b-zip transcription factor family and consists of 7 NRF2-ECH homology (Neh) domains (Figure 1.2). NRF2 associates with KEAP1

through its N-terminal Neh2 domain, which contains the DLG and ETGE binding motifs that regulate NRF2 protein stability (79,85,88). The Neh2 domain is largely responsible for NRF2 protein stability and contains seven lysine residues that are ubiquitylated by the KEAP1-CUL3-RBX1 E3 ligase (36,61,62,67,77). The Neh 3, Neh4 and Neh5 serve as the trans-activation domains required for NRF2-mediated transcription. Neh3 is important for the association of NRF2 with the DNA helicase CHD6 (92). While Neh4 and Neh5 interact with the transcriptional co-activator and histone acetyltransferase CREB-binding protein (CBP) (93,94). The Neh1 domain contains the basic region leucine zipper motif that is essential for both NRF2 heterodimerization with small maf proteins (Maf F, Maf G, Maf K) as well as for DNA binding (24,32). The Neh6 and Neh7 domains are less well characterized, but Neh6 is also responsible for the stability of the NRF2 protein and contains the binding sites/degron motifs for $SCF^{\beta TRCP}$ (DSGIS, DSAPGS) (80,95). In addition to KEAP1, NRF2 has been reported to be ubiquitylated and targeted for proteasomal degradation by a second E3 ubiquitin ligase, $SCF^{\beta TRCP}$. While KEAP1 is the primary regulator of NRF2 stability, a second level of NRF2 regulation lies in the $SCF^{\beta TRCP}$ ubiquitylation of NRF2 in the Neh6 domain that is increased by NRF2 phosphorylation by GSK3β (80,95,96). The Neh7 domain has been shown to associate with the retinoic acid receptor (RXRα), a nuclear receptor and transcription factor, and this interaction represses NRF2-mediated transcription (97).

1.E A Balance of KEAP1-NRF2 is Necessary for Effective Disease Prevention

Perturbations in KEAP1-NRF2 signaling have been reported in a number of human diseases. Paradoxically, while NRF2 hyperactivation promotes tumor cell survival and chemotherapeutic resistance, NRF2 is also thought to be chemopreventive against cancer initiating events such as oxidative DNA damage (98). Several antioxidants with chemopreventive activity act through induction of NRF2-mediated transcription (37,99). These include plant-based phytopharmaceuticals, such as sulforaphane found in broccoli (100).

Furthermore, NRF2 knockout mice show increased susceptibility to chemically-induced cellular toxicity. For example, NRF2 null mice show increased DNA adduct formation in response to diesel exhaust exposure (101). Similarly, oxidative DNA damage was measured in response to treatment with the hepatocarcinogen, pentachlorophenol (PCP). Therein, hydroxydeoxyguanosine (8-OH-dG) DNA damage was significantly increased in the NRF2 knockout mice compared to wildtype mice (102). Inversely, the efficacy of the chemopreventive agent oltipraz was greatly diminished in the absence of NRF2 (103). Thus, Nrf2 null mice exhibit increased sensitivity to several chemical toxicants and carcinogens and are refractory to the cytoprotective activity of chemopreventive agents. Taken together, these data suggest NRF2 prevents initiation, but promotes progression of cancer. This underscores the need for balance in the KEAP1-NRF2 system, which allows for successful ROS scavenging and effective disease prevention (illustrated in Figure 1.3). Further highlighting the need for redox balance is the observation that elevated ROS leads to increased inflammation, cell death and overall exacerbated disease phenotypes in a number of inflammatory and neurodegenerative diseases (104,105).

1.F KEAP1-NRF2 Signaling in Cancer

KEAP1 and NRF2 Mutations in Cancer

A role for KEAP1-NRF2 signaling in cancer was initially posited due to the high mutational frequency of KEAP1 and NRF2 observed in non-small cell lung cancer (NSCLC). Sequencing efforts performed by The Cancer Genome Atlas (TCGA) determined that KEAP1, NRF2 and CUL3 are mutated in 34% of squamous NSCLC patient tumors (106). These data confirmed the results of smaller studies that also detected mutations in KEAP1 and NRF2 in NSCLC (107). This high mutational frequency suggested a role for KEAP1-NRF2 signaling in lung cancer development or progression. The missense mutations in NRF2 occur almost exclusively in or very near the DLG and ETGE motifs, inhibiting the two-site binding of NRF2 to

KEAP1 (106,108-110). Further characterization of these mutations in cells demonstrated that indeed these mutations conferred loss of KEAP1 regulation and constitutive hyperactivation of NRF2 target genes (111-113). Reciprocally, mutations in KEAP1 span the entire length of the KEAP1 gene/protein and mutations within the arginine triad required for association with NRF2 and other substrates were quite rare (106,108). Detailed biochemical and functional characterization of 18 patient-derived NSCLC mutations in KEAP1 demonstrated that most KEAP1 mutants lost the ability to completely repress NRF2, though many of these were hypomorphic—meaning that they partially inhibited NRF2 (69). NRF2 hyperactivation is thought to confer a growth advantage by allowing tumors to cope with the elevated ROS, which is sustained due to the increased and aberrant metabolism, and by activating xenobiotic efflux pumps that efflux chemotherapeutic agents from the tumor cells (56,112,114-116). In addition to NSCLC, KEAP1/NRF2 mutations have been observed in a range of other tumor types including breast (117), gallbladder (112), ovarian (118), and esophageal cancers (114). Gene alterations in other tumor types are also largely NRF2-activating mutations. Constitutive NRF2 activation is associated with disease malignancy and poor patient outcomes (110-112,114,119). Taken together, KEAP1/NRF2 are frequently mutated in cancer and these mutations are associated with disease progression in NSCLC and many other types of cancer.

1.F.1 Alternative Mechanisms of NRF2 activation in cancer

In addition to somatic point mutations in KEAP1/NRF2, a number of other NRF2-activating mechanisms in cancer have been identified. These mechanisms are particularly pertinent because NRF2 mRNA levels and the mRNA levels of the NRF2 target gene signature were found to be elevated in tumors where the canonical KEAP1/NRF2 components were all wildtype (106,108), indicating that tumor cells have evolved other mechanisms to activate the pro-survival action of NRF2. One means of activating NRF2 is through inhibition of KEAP1 expression. KEAP1 mRNA and thereby protein levels are repressed through KEAP1 promoter

methylation in a subset of colorectal cancers and gliomas (120,121). This leads to NRF2 activation due to the increased ratio of NRF2 to KEAP1 in the cell, allowing for increased NRF2 protein levels and transcriptional activity.

A second means of non-mutagenic NRF2 activation is through posttranslational modifications of KEAP1 and NRF2, which have also been shown to regulate NRF2 stability and activity. For example, cysteines within KEAP1 have also been shown to undergo modifications such as s-glutathionylation and succination by cellular enzymes (122,123). Succination of KEAP1, and thereby activation of NRF2, was found in papillary renal carcinoma to be due to a loss of fumarate hydratase (FH) in these tumors (123). Using mass spectrometry, the succinated cysteines within KEAP1 were identified: Cys38, Cys151, Cys241, Cys288, Cys319 and Cys613 (123). Cysteines 151 and 288 have previously been shown to be regulated by oxidative stress and to regulate the activity of NRF2 (75,90,124). Additionally, NRF2 is also activated by PKC-mediated phosphorylation of NRF2 on Serine 40 (125). This is a notable example of crosstalk between KEAP1/NRF2 signaling and other tumorigenic signaling pathways to increase NRF2 signaling.

NRF2 expression is also increased in a number of tumors due to NRF2 gene amplification, which generates higher NRF2 mRNA and protein levels conferring increased NRF2 activity (126). We, and others, have also shown that competitive binders can displace NRF2 from KEAP1 and allow NRF2 to accumulate and drive transcription of its target genes. Proteins that are able to activate NRF2 through ETGE-dependent competitive binding to KEAP1 include DPP3, WTX, and PALB2 (127-129). Congruently, p21 is able to bind to the ETGE motif within NRF2 and thus inhibit its association with KEAP1 (130). Each of these competitive binders induces NRF2 stability and NRF2-mediated transcription when overexpressed (128,130). We demonstrated that DPP3 is overexpressed in cancers, confirming this is a protein-level mechanism utilized by cancer cells to activate NRF2 (128).

In summation, tumors activate NRF2 through many different mechanisms including genetic mutations of NRF2, KEAP1 or CUL3, silencing of KEAP1 gene expression, posttranslational modification of KEAP1 or NRF2, gene amplification of NRF2, and competitive binding by other proteins that disrupts the KEAP1/NRF2 binding interface.

1.G NRF2 in Chronic Inflammatory Conditions

NRF2 has been demonstrated to be protective from the development of and restorative in the treatment of a number of chronic inflammatory conditions. For example, NRF2 knockout mice spontaneously develop chronic inflammatory diseases at an incidence rate higher than that of their wild type littermates and are more sensitive to certain inflammatory insults (40,131,132). Furthermore, NRF2 knockout mice showed increased cell death as well as increased animal mortality in response to LPS-induced sepsis, compared to wildtype control mice (133). Conversely, pharmacological activation of NRF2 with CDDO-me was also preventative in a mouse model of ischemic tissue injury (133,134)

Neurodegenerative diseases are characterized by the presence of heightened inflammation and neuronal damage. Alzheimer's Disease (AD) is a progressive neurodegenerative condition reported to have elevated levels of ROS, oxidized DNA and oxidized misfolded proteins (104,135). In line with elevated ROS, NRF2 levels were decreased in AD patients' brains (136). Little data are available about NRF2 therapies in AD, but these observations suggest NRF2 activators could provide therapeutic benefits. Loss of NRF2 exacerbates mouse models of several human neuropathologies including multiple sclerosis. In kind, pharmacologic activation of NRF2 is beneficial in a mouse model of traumatic brain injury (137). Due to the elevated ROS, stress-mediated cell death, and inflammation observed in neurodegenerative disorders, NRF2 has been suggested as a putative therapeutic target therein (138).

1.H Therapeutically Targeting the KEAP1-NRF2 Pathway

1.H.1 NRF2 activators

Due to the increased ROS and cell death prevalent in a number of neurodegenerative and autoimmune diseases, NRF2 activating drugs have become attractive therapeutics in the treatment of these diseases. A number of natural and synthetic chemical NRF2 activators have been identified. The most notable example is the NRF2 activating molecule dimethyl fumarate (BG-12) that is currently used in the clinic to treat multiple sclerosis—a disease characterized by inflammation and neuronal cell death (139). In addition to chemical activators of NRF2, synthetic peptide and protein inhibitors of the KEAP1-NRF2 binding interface have been shown to have positive effects (140,141). The protein-based activators of NRF2 have been shown to dampen the neuronal cell death in murine models of ischemic brain injury and Parkinson's disease (142-144). Therefore, NRF2 activation is a feasible and attractive therapeutic option for the treatment of neurodegenerative diseases.

1.H.2 NRF2 Repressors

In light of the importance of NRF2 activation in tumor progression and chemotherapeutic resistance, identifying and developing potent NRF2 inhibitors is of great interest. Unfortunately, this is a largely unmet need in our field. One molecule, Brusatol, was found to inhibit NRF2 protein levels and showed synergy with chemotherapeutic treatment of tumor cell models (145,146). However, this molecule was later found to be less specific; brusatol inhibited general protein synthesis rather than NRF2 specifically (147). Notably, the brusatol-related compound bruceantin was tested in clinical trials for advanced breast cancer and melanoma (148). Unfortunately, neither trial yielded a significant benefit from the bruceantin treatment (148,149).

Recently, another chemical NRF2 inhibitor was identified: luteolin. The flavonoid luteolin decreases the stability of the mRNA encoding NRF2 and thereby decreases its protein levels and activity in cells. Luteolin showed synergistic activity in the chemotherapeutic treatment of A549 NSCLC cells with a known NRF2-activating mutation in KEAP1 (150). Furthermore, luteolin also decreased NSCLC tumor growth in an in vivo xenograft mouse model (151). While no clinical cancer trials using luteolin have been published to date, luteolin was shown to be non-toxic and have beneficial effects on the behavioral metrics of children with autism (152). Collectively, these findings demonstrate a potential therapeutic benefit to targeting KEAP1 and NRF2 in cancer and neurodegenerative diseases.

1.J Alternative KEAP1 Substrates and NRF2-independent functions for KEAP1

Degradation of NRF2 has been considered the primary function of the KEAP1-CUL3-RBX1 ligase for some time. However, our recent mass spectrometry-based studies have shown that KEAP1 associates with a number of proteins with interesting and diverse functions, which suggests KEAP1 may possess additional cellular functions (128,153). A large subset of the identified high-confidence KEAP1 interactors (17/40) contain the high affinity KEAP1-binding motif and degron sequence: E(T/S)GE (128). In fact, we determined that the KEAP1 protein interaction network was statistically significantly enriched for proteins possessing this motif (Figure 1.4) and of the E(T/S)GE-containing interactors we tested, all showed KEAP1 binding that was dependent on the E(T/S)GE motif (128).

In agreement with the notion of alternative KEAP1 functions, several other KEAP1 substrates have been independently reported. An illustration of these substrates and the functional outcome of their modification by KEAP1-CUL3-RBX1 can be found in Figure 1.5. The mitochondrial membrane phosphatase PGAM5 was identified as a KEAP1 substrate that tethers KEAP1 to the cytoplasmic side of the mitochondrial membrane (154,155). Furthermore, the

KEAP1 ligase complex was shown to target PGAM5 for proteasome-mediated degradation through its ESGE motif. Notably, the half-life of PGAM5 is much longer than that of the stress-responsive substrate NRF2. The half-life of PGAM5 was calculated to be approximately 75 hours and this was decreased to 6 hours upon ectopic expression of KEAP1 (154). Another recently reported ETGE-containing KEAP1 substrate is the NF-kappaB signaling regulator, IkappaB kinase beta referred to as IKKβ/IKBKB (156). By targeting IKBKB for lysosome-mediated degradation, KEAP1 represses NF-kappaB signaling (157). KEAP1 regulates IKBKB protein stability in a reactive-cysteine and ETGE-dependent fashion (157).

A fourth KEAP1 substrate was recently identified in PALB2. The KEAP1 ubiquitylation of PALB2 is different than that of its other known substrates (NRF2, PGAM5, IKBKB) because it is a functional and seemingly non-degradative modification (158). The KEAP1-CUL3-RBX1 ligase ubiquitylates lysines within the N-terminal region of PALB2. This inhibits the association of PALB2 with BRCA1 and thereby inhibits homologous recombination in the G1 phase of the cell cycle (158). Another study had previously demonstrated that neither siRNA knockdown of KEAP1 nor treatment with the ROS mimetic/KEAP1 antagonist tert-butylhydroquinone (tBHQ) stabilized PALB2 protein levels (127). Therefore, PALB2 provides the first evidence that the KEAP1-CUL3-RBX1 ligase can regulate substrate function. In summary, the four known substrates of the KEAP1-CUL3-RBX1 demonstrate that it is capable of ubiquitylating its substrates to regulate substrate stability through either proteasome- or autophagy-mediated degradation or to regulate substrate function by controlling protein-protein interactions. Each of the KEAP1 substrates identified to-date possess an E(T/S)GE motif required for their association with and ubiquitylation by KEAP1. Therefore, we tested whether MCM3, a member of the hexameric DNA replicative helicase, was a KEAP1 substrate. We previously determined that MCM3 bound to KEAP1 through its an ETGE motif (128). We found that MCM3 is a KEAP1-CUL3-RBX1 substrate for ubiquitylation (these findings are shown in Chapter 2), but it is

not targeted for rapid degradation by either the proteasome or autophagy. We therefore hypothesize that the KEAP1-CUL3-RBX1 ligase regulates MCM3 function in the DNA replicative helicase (See chapter 2). While little is known about NRF2-independent functions for KEAP1, this recent body of work suggests that KEAP1 may link cellular redox-sensing to a number of cellular pathways including NFkappaB, DNA damage responses and cell cycle progression.

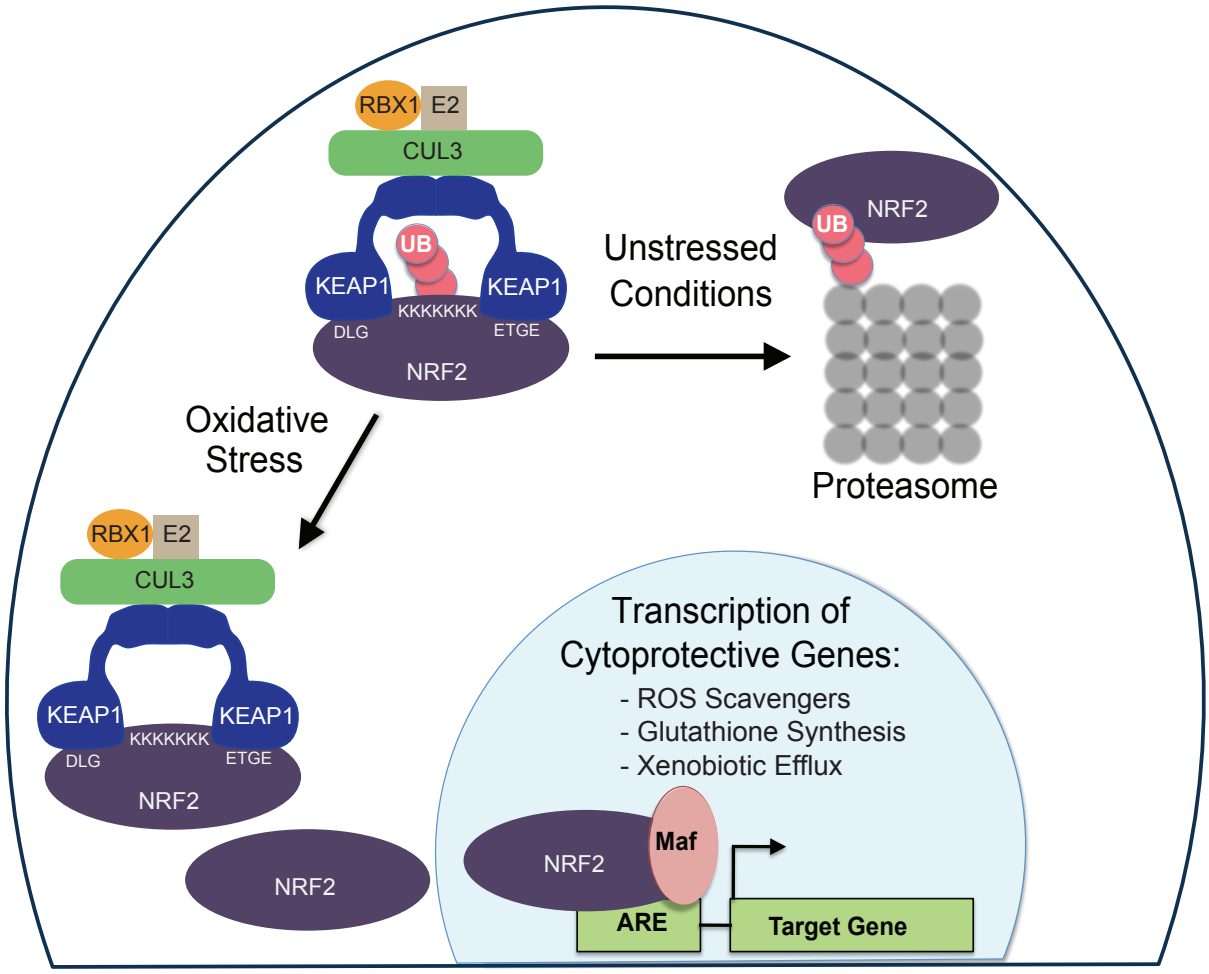

Figure 1.1 KEAP1-CUL3-RBX1 targets NRF2 for degradation by the ubiquitin proteasome system. Under homeostatic conditions, NRF2 is rapidly degraded by the proteasome in a KEAP1-dependent fashion. Following exposure to elevated cellular ROS or oxidative stress, reactive cysteine residues within KEAP1 are modified and KEAP1 undergoes a conformational change. NRF2 is therefore no longer efficiently targeted for degradation. Nascent NRF2 accumulates, translocates to the nucleus and drives a battery of cytoprotective and antioxidant genes that ultimately mitigate the oxidative stress sensed by KEAP1.

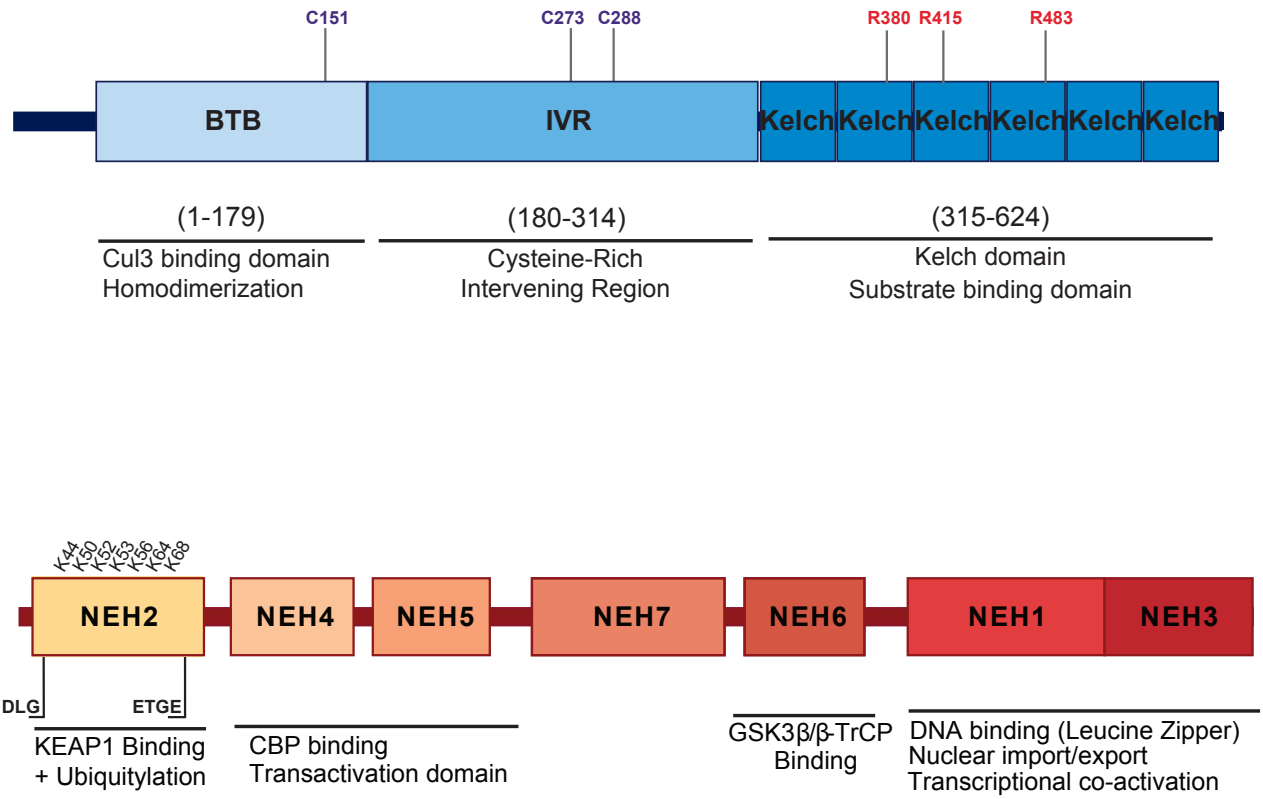

Figure 1.2 KEAP1 and NRF2 protein domain structures. KEAP1 contains 3 major protein domains: the BTB domain associated with CUL3 and is required for KEAP1 dimerization, the cysteine-rich intervening region (IVR), and the KELCH domain essential for substrate binding. The arginine triad within KEAP1 (shown in red) forms electrostatic interactions with the ETGE motif within NRF2. The most redox-reactive cysteines (C151, C273, C288) within KEAP1 are shown in blue. NRF2 contains 7 Neh domains. The Neh2 domain associates with KEAP1 through the DLG and ETGE motifs. The 7 lysines of NRF2 that KEAP1 ubiquitylates are shown above the NRF2 structure; these lie between the two KEAP1 degrons (DLG, ETGE) in NRF2. The Neh1 domain is critical for small Maf dimerization and NRF2-mediated transcription.

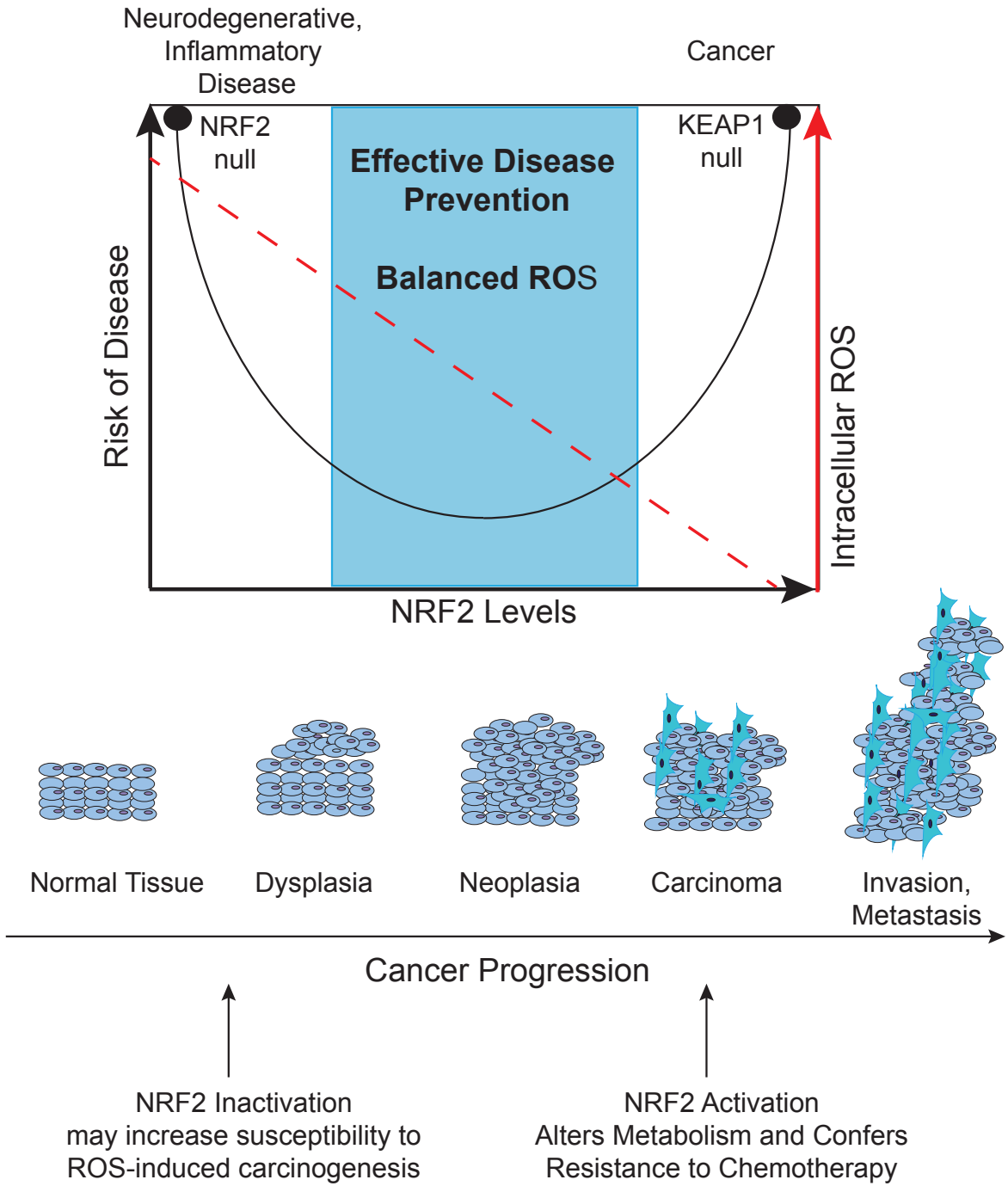

Figure 1.3 Perturbations in KEAP1/NRF2 are associated with human disease. NRF2 null settings are associated with increased reactive oxygen stress, inflammation and cell death. Decreased NRF2 activity is observed in neurodegenerative and chronic inflammatory diseases. Conversely, NRF2 hyperactivation (i.e, KEAP1 null setting) contributes to the progression of cancer through augmentation of metabolism and transcription of pro-survival genes. NRF2 inactivation may also increase susceptibility of certain chemical and ROS-induced carcinogenesis through ROS-mediated cellular damage.

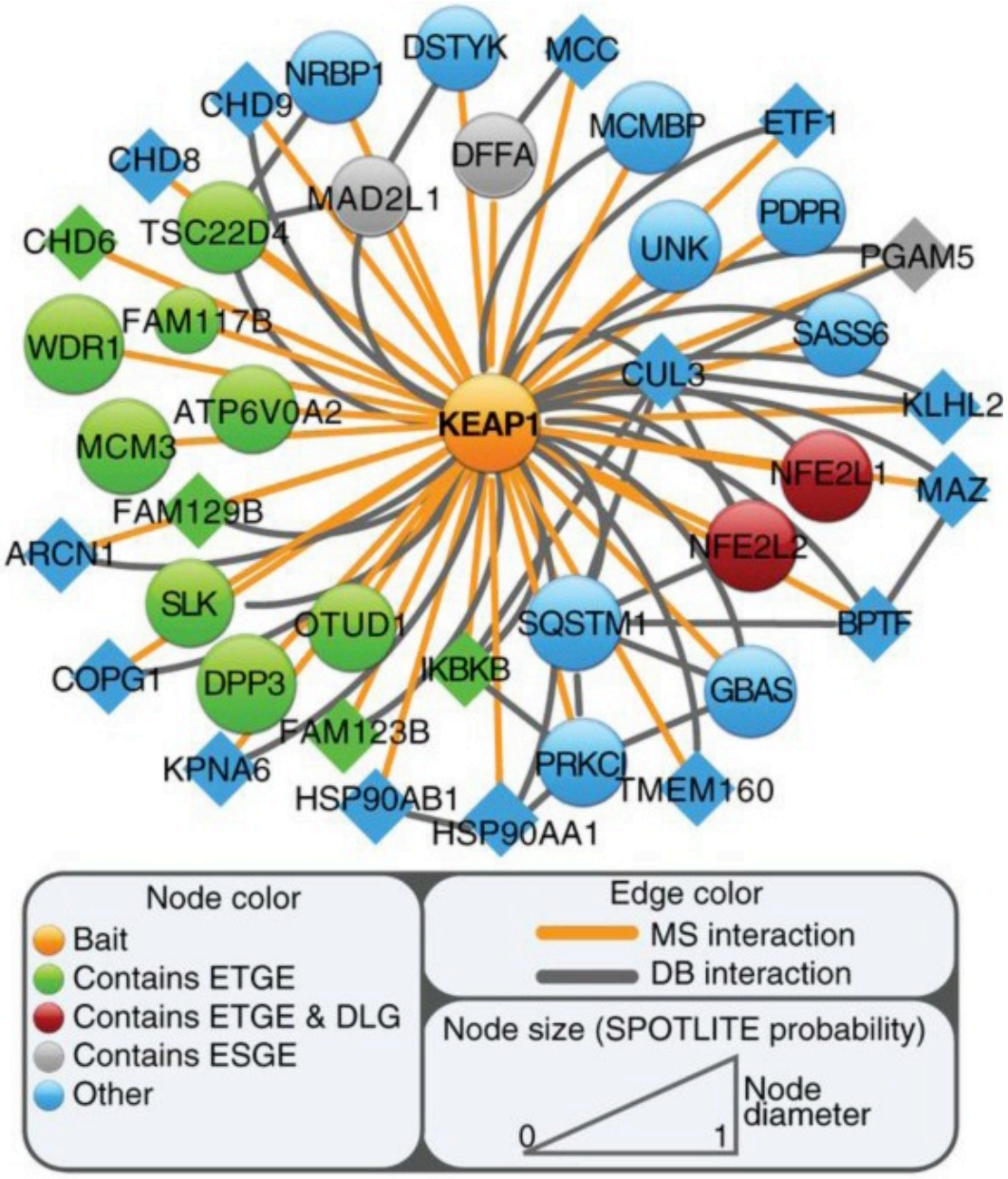

Figure 1.4[1] The KEAP1 Protein Interaction Network is Enriched for E(T/S)GE-Containing Proteins. The high-confidence KEAP1 protein interaction network identified by KEAP1 affinity purification mass spectrometry (AP/MS) is shown here. The AP/MS network contains known interactors (Database curated interactor-DB) and novel ones. The interactors that contain a putative KEAP1 degron E(T/S)GE are noted. The network includes 17 E(T/S)GE proteins. Of these, NFE2L2/NRF2, PGAM5, IKBKB and MCM3 are known substrates. The substrate status and biological function of the remaining ETGE-containing KEAP1 binding proteins are largely unknown.

[1] The following figure has been previously published in the journal Cancer Research and 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.

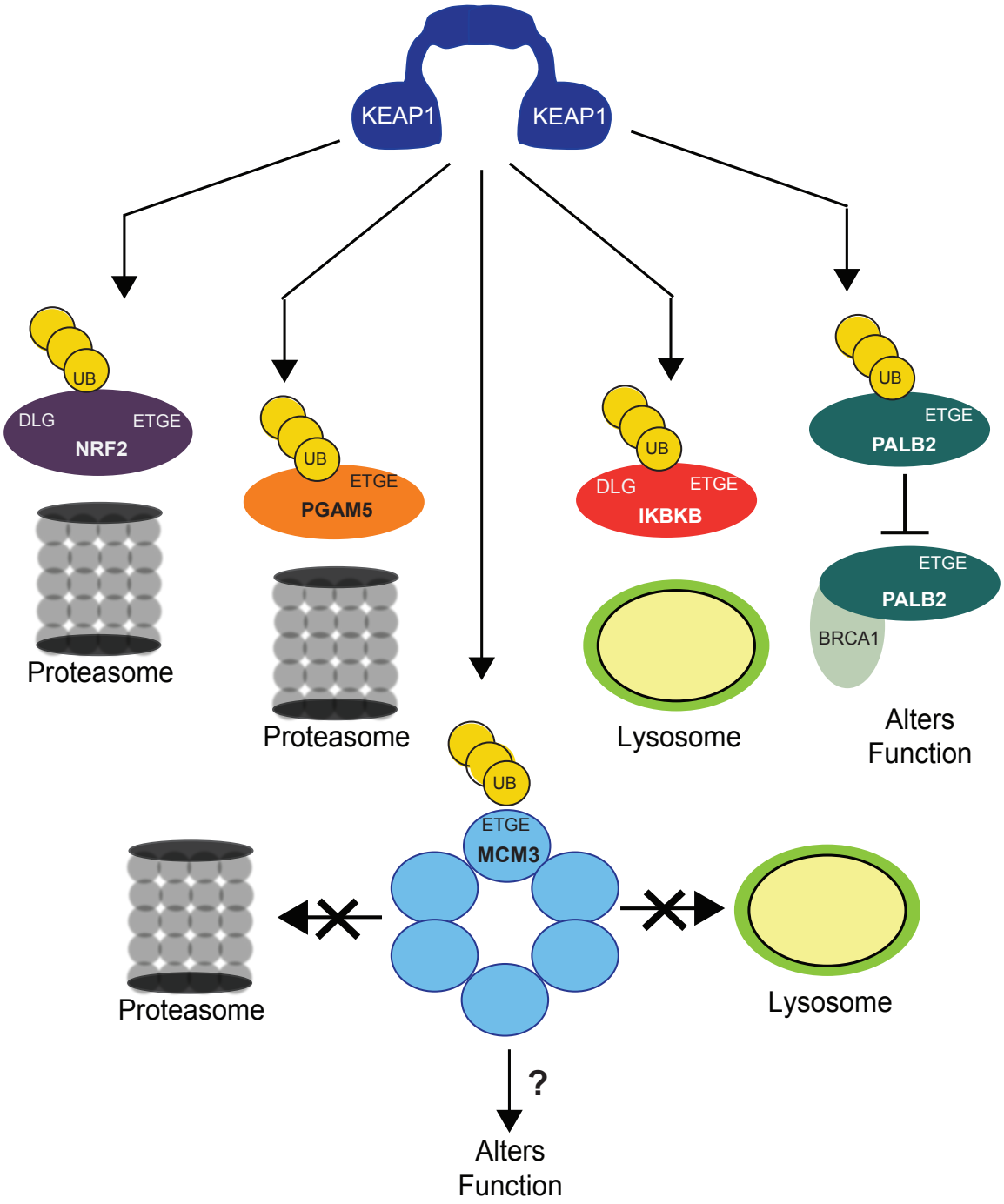

Figure 1.5 Substrates of the KEAP1-CUL3-RBX1 E3 ligase. The KEAP1 E3 complex targets NRF2 and PGAM5 for degradation by the proteasome. Ubiquitylated IKBKB is targeted to lysosomes for autophagy-mediated degradation by the KEAP1-CUL3-RBX1 ligase. When PALB2 is modified by KEAP1, this inhibits its association with BRCA1, thus affecting PALB2 function. MCM3 is a KEAP1 substrate for ubiquitylation that is not rapidly turned over by either the proteasome or the lysosome. Thus, KEAP1 may ubiquitylate MCM3 to regulate DNA replication and cell cycle progression.

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II. CHAPTER TWO: IDENTIFICATION AND CHARACTERIZATION OF MCM3 AS A KEAP1 SUBSTRATE[1]

2.A. OVERVIEW

KEAP1 is a substrate adaptor protein for a CUL3-based E3 ubiquitin ligase. Ubiquitylation and degradation of the antioxidant transcription factor NRF2 is considered the primary function of KEAP1; however, few other KEAP1 substrates have been identified. Because KEAP1 is altered in a number of human pathologies and has been proposed as a potential therapeutic target therein, we sought to better understand KEAP1 through systematic identification of its substrates. Towards this goal, we combined parallel affinity capture proteomics and candidate-based approaches. Substrate-trapping proteomics yielded NRF2 and the related transcription factor NRF1 as KEAP1 substrates. Our targeted investigation of KEAP1 interacting proteins revealed MCM3, an essential subunit of the replicative DNA helicase, as a new substrate. We show that MCM3 is ubiquitylated by the KEAP1-CUL3-RBX1 complex in cells and *in vitro*. Using ubiquitin remnant profiling, we identify the sites of KEAP1-dependent ubiquitylation in MCM3, and these sites are on predicted exposed surfaces of the MCM2-7 complex. Unexpectedly, we determined that KEAP1 does not regulate total MCM3 protein stability or subcellular localization. Our analysis of a KEAP1 targeting motif in MCM3 suggests MCM3 is a point of direct contact between KEAP1 and the MCM hexamer. Moreover, KEAP1 associates with chromatin in a cell cycle dependent fashion with kinetics similar to the MCM2-7 complex. KEAP1 is thus poised to affect MCM2-7 dynamics or function rather than MCM3

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abundance. Together, these data establish new functions for KEAP1 within the nucleus and identify MCM3 as a novel substrate of the KEAP1-CUL3-RBX1 E3 ligase.

2.B. INTRODUCTION

Kelch-Like ECH-Associated Protein 1 (KEAP1) is a substrate adaptor protein for a Cullin3 (CUL3)-RBX1 E3 ubiquitin ligase complex (1-3). Recent studies have described the molecular architecture and mechanism for the KEAP1-CUL3-RBX1 ubiquitylation machine (4,5). The most well-studied and established substrate of the KEAP1 complex is the NFE2L2 transcription factor (henceforth referred to as NRF2) (1-3,6,7). A homodimer of KEAP1 tethered to CUL3 via its amino-terminal BTB domains binds to a single molecule of NRF2; the C-terminal kelch domains of a KEAP1 homodimer bind to a high affinity motif (ETGE) and a lower affinity motif (DLG) within the NRF2 protein (2,8,9). Under homeostatic conditions, ubiquitylated NRF2 is rapidly degraded by the proteasome, having a half-life of less than 30 minutes (10). KEAP1 acts as a sensor of cellular reduction-oxidation (redox) state through its 27 cysteine residues (6,11,12). The reactive cysteine residues within KEAP1 can be modified by reactive oxygen species (ROS), which is thought to trigger a conformational change in the KEAP1 complex (3,6,11). As a result, NRF2 is no longer efficiently degraded and thus accumulates, translocates to the nucleus and promotes the transcription of antioxidant and cytoprotective genes (10,13,14). Specifically, nuclear NRF2 forms heterodimers with small Maf proteins and together they bind to the antioxidant response elements (ARE) within the promoter region of NRF2 target genes, which include free radical scavengers, glutathione synthesis genes, and xenobiotic efflux proteins (7,10,15,16). The upregulation of NRF2 target genes mitigates oxidative stress and confers resistance to a number of toxins, including chemotherapeutics (7,13,17,18). The KEAP1-NRF2 signaling pathway serves as the cell's primary defense against oxidative stress (13,14).

While NRF2 degradation has long been thought to be KEAP1's primary function, we have shown that KEAP1 associates with a number of interesting and diverse proteins, suggesting previously unknown roles for KEAP1 (19,20). In support of this concept, three substrates have recently been reported for the KEAP1 E3 ligase: IKBKB (21), PGAM5 (22) and PALB2 (23). All three substrates contain an ETGE or ESGE motif that is essential for their interactions with and ubiquitylation by KEAP1. While we have a strong understanding of the dynamics and regulation of NRF2 as a KEAP1 substrate, these other substrates are less well studied. IKBKB is reported to be a KEAP1 substrate targeted for autophagy-mediated degradation; PGAM5 is thought to be ubiquitylated and targeted to the proteasome; KEAP1-mediated PALB2 ubiquitylation regulates its function by blocking its interaction with BRCA1 (21-24). Thus, the KEAP1-CUL3-RBX1 ligase is capable of ubiquitylating its substrates to regulate substrate stability through either proteasome-mediated or autophagy-mediated degradation or to regulate substrate function by directing protein-protein interactions.

In addition to the vital role the pathway plays in normal physiology, perturbations in KEAP1-NRF2 signaling have been reported in a variety of diseases, including cancer, and inflammatory, cardiovascular and neurodegenerative diseases (25-34). Most notably, sequencing efforts have determined that approximately 30% of non-small cell lung cancer (NSCLC) patient tumors harbor mutations in the KEAP1-NRF2 pathway; 12-15% of NSCLC tumors have mutations within KEAP1 (20,35-38). The high mutation frequency suggests a role for KEAP1-NRF2 in cancer progression. KEAP1 loss is thought to promote tumorigenesis through hyperactivation of NRF2, though little is known about what other effects KEAP1 mutation or loss has. A better understanding of KEAP1 substrates would enhance our understanding of both normal KEAP1 function and of KEAP1-mutant tumors. We sought to define new KEAP1 substrates and to determine the function of their ubiquitylation by KEAP1.

Here we identify a subunit of the replicative DNA helicase, MCM3, as a KEAP1 substrate; we selected it from our set of potential KEAP1 substrates for further study based on

its important role in cell cycle regulation. Interestingly, human MCM subunits do not undergo ubiquitin-mediated proteolysis during normal proliferation but instead, the chromatin-loading of the MCM complex is tightly controlled during the cell cycle to ensure once per cell cycle genome duplication. MCM complexes are chromatin loaded strictly during G1 phase, activated in S phase, and progressively unloaded as DNA replication forks terminate (reviewed in (39-41)). The MCM2-7 complex is extensively modified by posttranslational modifications (42-48) and in particular, recent studies linked polyubiquitylation of the MCM7 subunit to MCM unloading in both *S. cerevisiae* and *X. laevis* (49-52). In *S. cerevisiae*, the SCF^{Dia2} ligase ubiquitylates MCM7 and in *X. laevis* MCM7 is ubiquitylated by an unidentified cullin family member (49,50). Thus, interaction with and polyubiquitylation by KEAP1 represents a potentially novel form of MCM regulation. We suggest that our discovery of KEAP1-mediated MCM3 ubiquitylation establishes a physical link between a key player in the oxidative stress response and chromosome replication.

2.C. EXPERIMENTAL PROCEDURES

Tissue Culture, Treatments, Transfections, and Small Interfering RNAs—HEK293T, HDF-Tert and HeLa cells were obtained from the American Tissue and Culture Collection. The cell lines were passaged for no more than 3 continuous months after resuscitation. HDF-Tert, HEK293T and HeLa cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS and 1% Pen/Strep in a 37°C humidified incubator with 5% CO_2. Mouse embryo fibroblasts (MEFs) were cultured in IMDM supplemented with 10% FBS and 1% Pen/Strep. The KEAP1 and NRF2 knockout MEFs were kindly provided by Thomas Kensler and Nobunao Wakabayshi. Drugs used for cell treatments were acquired as follows: MG132 (Calbiochem), bortezomib (SelleckChem), tert-butyl hydroquinone (Sigma), sulforaphane (Sigma), etoposide (Sigma), chloroquine (Sigma) and gemcitabine (Sigma). For transient transfections, cDNA expression constructs were transfected in HEK293T cells using

Lipofectamine 2000 (Life Technologies) for 24 h before harvest. Transfection of siRNA (20 nM) in HEK293T cells was performed with Lipofectamine RNAiMAX (Life Technologies). Transfection of siRNA in Hela cells was done with either Dharmafect (50 nM) or Lipofectamine RNAiMAX (20nM). siRNA sequences were as follows: Control: CGUACGCGGAAUACUUCGATT ; KEAP1-A: GGGCGUGGCUGUCCUCAAU; KEAP1-B: CAUGUGAUUUAUUCUUGGAUACCUG; KEAP1-C: UGGCUGUCCUCAAUCGUCUCCUUUA; CUL3-A: GGUCUCCUGAAUACCUCUCAUUAUU; CUL3-B: GAAUGUGGAUGUCAGUUCACGUCAA.

Immunoprecipitations, Affinity Pulldowns, and Western Blotting—These experiments were performed as previously described (53), with minor modifications. Briefly, for Streptavidin and FLAG affinity and immune purification, cells were lysed in 0.1% NP-40 lysis buffer (10% glycerol, 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, supplemented with protease inhibitor mixture (Thermo Scientific) and phosphatase inhibitor (Thermo Scientific), 10 mM N-ethylmaleimide and 250 U Benzonase (Sigma)), then passed through a 26 ½ gauge needle 3 times. The 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 4X SDS loading buffer (Life Technologies). For siRNA knockdown, HEK293T cells were transiently transfected and lysed in RIPA buffer (1% NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 10% glycerol, 25 mM Tris, 2mM EDTA supplemented with protease inhibitor mixture, phosphatase inhibitor and N-ethylmaleimide) 60-72 h post-transfection. For BirA* affinity purification, HEK293T cells were pretreated with 50 μM biotin for 2-4 h, lysed in supplemented RIPA buffer and cleared lysates were subjected to streptavidin AP as above, and eluted with a 1:1:1:1 mixture of 1M dTT; 4X SDS loading buffer; 50 μM biotin; RIPA. For endogenous IP (Fig. 2*B*,*C*), HEK293T cells were lysed in co-IP buffer (50 mM HEPES pH 7.2, 33 mM Potassium Acetate, 1 mM $MgCl_2$, 1 mM ATP, 0.1% NP-40, 5 mM $CaCl_2$,

10% Glycerol, with protease and phosphatase inhibitors) then treated with 10U of S7 micrococcal nuclease (Roche), sonicated, and cleared by centrifugation. Samples were rotated with MCM2 antibody or control rabbit IgG, followed by rotation with Protein A-sepharose (Roche), then washed 3 times with co-IP buffer and eluted with 2X SDS loading buffer, 5% 2-Mercaptoethanol (Sigma) and boiling for 10 minutes. Lysates were resolved on 4-12% SDS-PAGE gradient gels (Invitrogen), transferred to nitrocellulose or PVDF membranes and probed using the following antibodies: anti-MCM3 (Bethyl, A300-192A), anti-KEAP1 polyclonal (ProteinTech, Chicago IL), anti-FLAG M2 monoclonal (Sigma), anti-HA monoclonal (Roche), anti-MAD2L1 (Bethyl, Montgomery TX, A300-301A), anti-SLK (Bethyl, A300-499A), anti-βactin polyclonal (Sigma, A2066), anti-btubulin monoclonal (Sigma, T7816), anti-DPP3 polyclonal (abcam, Cambridge MA, 97437), anti-GFP (abcam, ab290), anti-NRF2 H300 polyclonal (Santa Cruz, Santa Cruz CA), anti-PGAM5 polyclonal (abcam, 126534), anti-NRF1 polyclonal (Santa Cruz, D5B10), anti-MCM2 polyclonal (Bethyl, A300-191A), anti-Pan MCM (a kind gift from D. MacAlpine, Duke University) and anti-VSV polyclonal (Bethyl, A190-131A). Biotinylated proteins associated with BirA* AP blots were detected using a fluorescently labeled streptavidin (IRDye 680CW-LI-COR). Protein quantification was performed in the LI-COR imaging suite (Image Studio Lite) where all blots were determined to be in the linear range by the software.

Cell Fractionations and Cell Synchronization—HeLa cells were synchronized with a double thymidine block in early S phase by treating with 2 mM thymidine (Sigma) for 18 h, washing out for 9 h, and re-addition of 2 mM thymidine for an additional 17 h. HeLa cells were synchronized in M phase by double thymidine, nocodazole block: first, the double thymidine block (as above; with 24 h 2mM thymidine, release 6 h, re-addition of thymidine 18 h) and then cells were released into fresh DMEM containing 100 ng/mL nocodazole for 8-12 h. In each case, cells were washed twice with warm media and released into fresh DMEM. Chromatin fractionation was performed by gentle lysis with CSK buffer (0.5% Triton-X 100, 300 mM

sucrose, 10 mM PIPES, 100 mM NaCl, 2 mM EDTA) supplemented with protease and phosphatase inhibitors for 20 mins on ice. Lysates were centrifuged at 900 x g for 5 min at 4^{o}C to pellet the nuclei. Soluble fractions were transferred to new tubes. The nuclei were resuspended in CSK buffer containing 10 U DNAse (RQ1, Promega) for 10 mins at room temperature, pelleted at 900 x g for 5 mins at 4°C. Remaining nuclei were washed 1x with CSK buffer and the first DNAse digest and the wash were pooled. Nuclear proteins solubilized by DNAse digest are the chromatin fraction.

Plasmids, Expression Vectors, and Site-directed Mutagenesis—Expression constructs in the SBPHA backbone were generated with standard PCR techniques, as previously described (20). The expression constructs for MCM3 and NRF2 were obtained from Open Biosystems and cloned into a custom gateway lentiviral vector (pHAGE-CMV-FLAG-DEST). MCM3 and NRF2 were obtained as orfeome entry clones and gateway cloned into the pHAGE-CMV-FLAG-DEST. The MCM3 EAAE alanine mutant was created using PCR-based mutagenesis (Q5 site-directed mutagenesis kit; NEB) and sequence verified before use.

Cell-based ubiquitylation experiments—These experiments were performed as previously reported (20), with few modifications. Briefly, HEK293T cells were transfected with VSV-UB, FLAG-NRF2, or FLAG MCM3, and either SBPHA-KEAP1 or SBP-GFP as a control such that each condition received the same mass of DNA. The cells were lysed under near-denaturing conditions in 1% SDS lysis buffer (1% SDS, 150 mM NaCl, 2 mM EDTA) and boiled at 90°C for 10 minutes. SDS lysis buffer was diluted 1:10 in cold 0.5% NP-40 lysis buffer supplemented with protease, phosphatase and deubiquitylase inhibitors, and the lysates were cleared by centrifugation. FLAG IPs were carried out by incubating lysates with FLAG resin for 1-4 h at 4°C, followed by washing 3X in lysis buffer and eluting with 4X SDS loading buffer and DTT.

In vitro ubiquitylation experiments—For *in vitro* ubiquitylation studies, SBPHA-KEAP1 was generated using a TNT assay (Promega) and MCM3 was purified from HEK293T cells stably expressing FLAG-MCM3 using 1% Triton-X Lysis buffer (as decribed above for immunoprecipitations, but with 500 mM NaCl). For the *in vitro* ubiquitylation assay, KEAP1 was mixed with recombinant human E1 (Ube1, Boston Biochem), UbcH5B (E2, Boston Biochem), CUL3-RBX1 (Co-expressed in E. coli as GST-Rbx1 and His-CUL3 fusions and purified sequentially over HiTRAP NiNTA (GE Health Care), glutathione sepherose (GE Health Care), and proteolytically cleaved off the resin using thrombin (Sigma) followed by size exclusion chromatography over a superdex-200 16/60 column (GE Health Care)), ubiquitin (Boston Biochem) and FLAG-tagged MCM3 in buffer containing 25 mM Hepes pH 8.0, 5 mM $MgCl_2$, 2 mM DTT and 4 mM ATP. Ubiquitylation was carried out for 20 mins at 30°C and the products were analyzed by western blot with anti-FLAG antibody.

Protein Structural Modeling— The crystal structure of an archaeal MCM from *Sulfolobus solfataricus* (PDB ID 4FDG) was identified as a template for predicting the atomic structure of human MCM3 by HHpred (http://toolkit.tuebingen.mpg.de/hhpred) [PMID 15531603], and the homology model was generated using MODELLER [PMID 25199792]. In the structural model, 94 residues at the C-terminus as well as the residues between 510-562 were omitted due to poor homology to the archaeal MCM. The MCM3 model was superimposed onto a published model of the MCM2-7 heterohexamer from yeast (54) using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). PyMOL was used to prepare the images used in Figure 4*B,C*).

Affinity Purification and Mass Spectrometry—These experiments were performed as previously described (19,53,55), with minor modifications. Briefly, HEK293T cells (3-5x15cm

plates) stably expressing either FLAG-MCM3 or SBPHA-KEAP1 were lysed in 0.1% NP-40 lysis buffer for FLAG immunoprecipitations (IP) or streptavidin affinity purifications (AP), respectively. Cell lysates were incubated, rotating with FLAG or Streptavidin resin for 1 hr at 4^{o} C, then washed 3X with lysis buffer. The precipitated proteins were next trypsinized (Promega) on beads at 37°C overnight (12-18 h) using the FASP Protein Digestion Kit (Protein Discovery). For the KEAP1 substrate-trapping experiment (Fig 1*A*,*B*), SBPHA-KEAP1 expressing HEK293T cells were grown in SILAC media (light: K_0R_0; heavy: K_6R_{10}) for at least 10 cell divisions prior to harvesting for lysis. Tryptic peptides were cleaned up using a C18 Spin Column (Thermo Scientific), then separated by reverse phase nano-HPLC using a nanoAquity UPLC system (Waters Corp.). Chromatographic separation and mass spectrometry analysis of peptides from FLAG-MCM3 experiments (Fig. 2*A*) was performed using the same methods as our previous work (55). Peptides from SBPHA-KEAP1 and ubiquitin remnant experiments were first trapped in a 2 cm trapping column (Acclaim® PepMap 100, C18 beads of 3.0 µm particle size, 100-Å pore size) and a 25 cm EASY-spray analytical column (75-µm ID, C18 beads of 2.0 µm particle size, 100-Å pore size) at 35°C. The flow rate was 250 nl/min over a gradient of 1% buffer B (0.1% formic acid in acetonitrile) to 30% buffer B in 180 min and an in-line Orbitrap Elite mass spectrometer (Thermo Scientific) performed mass spectral analysis. The ion source was operated at 2.4–2.8 kV with ion transfer tube temperature set at 300°C. Full MS scan (300–2000 m/z) was acquired in Orbitrap with 120,000 resolution setting, data-dependent MS2 spectra were acquired in the linear ion trap by collision induced dissociation (CID) using the 15 most intense ions. Precursor ions were selected based on charge states (^{3}+2) and intensity thresholds (above 1e5) from the full scan, dynamic exclusion (one repeat during 30 s, a 60 s exclusion time window) was also used. The polysiloxane lock mass of 445.120030 was used throughout spectral acquisition.

Protein Identification, Filtering and Bioinformatics— For the MCM3 protein interaction network (Fig. 2*A*), the raw mass spectrometry data was searched with MaxQuant (1.5.2.6) along

with an internal lab FLAG APMS dataset of an additional 17 baits and 35 experiments. Search parameters were as follows: specific tryptic digestion, up to 2 missed cleavages, a static carbamidomethyl cysteine modification, variable protein N-term acetylation, and variable methionine oxidation using the human UniProtKB/Swiss-Prot sequence database (Release 2013_07). Proteins were then filtered for a 1% protein level false discovery rate (FDR). Filtering of false interactions was then accomplished using Spotlite (19), with a 10% FDR for the entire dataset including the additional baits. These results were then imported into Cytoscape v3.2.1 for network visualization. Spotlite results are provided in Table S2. For the SBPHA-KEAP1 SILAC experiments, the additional MaxQuant parameters were a variable GlyGly modification on lysines, and K6 and R10 heavy SILAC labels. Maxquant results are provided in Table S1.

For ubiquitin remnant profiling, raw files were searched using Sorcerer™-SEQUEST® (build 5.0.1, Sage N Research) and the Transproteomic Pipeline (TPP v4.7.1). Search parameters used were a precursor mass between 400 and 4,500 amu, a maximum of 2 missed cleavages, a precursor-ion tolerance of 3 amu, accurate mass binning within PeptideProphet, semi-tryptic digestion, a static carbamidomethyl cysteine modification (57.021465), and variable methionine oxidation (+15.99492), ubiquitylated lysine (114.042931), and STY phosphorylation (79.966331). A 1% peptide-level FDR was determined by PeptideProphet. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the dataset identifier PXD003929.

Ubiquitin Remnant Profiling—For mapping the ubiquitylated lysines within MCM3, immunoprecipitations for FLAG-MCM3 were done under near-denaturing conditions in the presence or absence of SBPHA-KEAP1. Then, the proteins were subject to tryptic digest and the peptides were flowed over beads conjugated to the ubiquitin remnant specific antibody (Cell Signaling) for 4 h at 4°C in IAP buffer supplied by the manufacturer. Beads were washed twice with IAP buffer and once with PBS. Peptides were eluted with 0.15% TFA for 5 minutes at room

temperature and dried at room temperature by speed vacuuming. Peptides were shot in the mass spectrometer as described above. Ubiquitylated peptides identified by MS/MS were then quantified in Skyline v3.5. For each peptide, the MS1 intensity of its extracted ion chromatogram was integrated for each of 3 label-free replicate experiments (3 runs with KEAP1, 3 runs without KEAP1). Any peptide identified by MS/MS or which was aligned by m/z, retention time, and had an isotope dot product >.8 was included in the analysis. The mean total area of each MS1 belonging to a ubiquitylated MCM3 peptide was taken from the 3 replicate experiments and a ratio was created for the +KEAP1/-KEAP1 conditions. For any peptide found to be true in one condition, but that was missing in the other, the background at the same mass to charge window was quantified to calculate a fold change. The complete results are provided in Table S3. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the dataset identifier PXD003929.

Immunostaining—These experiments were performed as previously reported, with minor modifications (55). To determine the subcellular distribution of KEAP1 and MCM3 proteins, HEK293T cells stably expressing venus-KEAP1 were plated on 10ug/ml fibronectin-coated coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and subjected to staining. Cells were fixed in 4% paraformaldehyde (PFA) in cytoskeletal buffer (5 mM PIPES, pH 6, 137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.4 mM MgCl2, 0.4 mM $NaHCO_3$, 2 mM EGTA, 50 mM glucose) for 15 minutes and permeabilized with 0.1% Triton X-100/PBS solution for 5 minutes. After blocking with 1% BSA/PBS, cells were incubated overnight with a rabbit polyclonal antibody against MCM3 (Bethyl; 1:250 dilution), followed by incubation with TRITC conjugated donkey secondary antibody (1:300 dilution) against Rabbit IgG. Coverslips were mounted to slides using the Prolong Gold antifade reagent (Invitrogen Molecular Probes) and images were acquired using a Zeiss LSM710 I Confocal Laser Scanning Microscope equipped with a 40X/1.3 Oil Plan Neo and a 63X/1.4 Oil Plan Apo

objective lenses.

Proximity Ligation Assays—For detection of KEAP1 and MCM3 interactions, proximity ligation was performed with the Duolink II proximity ligation assay kit according to the manufacturer's protocol (Sigma, DUO092101). Briefly, HEK293T cells stably expressing venus-KEAP1 were plated, fixed and permeabilized as described above. Next, cells were incubated in blocking solution in a humidified chamber at 37°C for 30 minutes, followed by overnight co-incubation with rabbit polyclonal antibodies against MCM3 (Bethyl, 1:250) and mouse monoclonal antibody against KEAP1 (Origene, 1:100) in antibody diluent solution at 4°C. Cells were washed in Buffer A and incubated with PLA probes (PLA probe minus and plus in antibody diluent, 1:5 dilution) for 1h at 37°C, washed in Buffer A, and incubated with ligation solution (1:5 dilution of ligation buffer and 1:40 dilution of ligase in pure water) for 30 minutes at 37°C. After ligation, cell were washed in Buffer A and subjected to amplification with Detection Reagents Red from (1:5 dilution amplification stock and 1:80 dilution of polymerase in water) for 100 min at 37°C. Amplified samples were washed in Buffer B then mounted with Duolink II Mounting Medium with DAPI. Confocal Z-stack images were acquired using a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope equipped with a 63X/1.42 Oil PlanApo objective lenses. Image J software was used to process the images to a two-dimensional illustration of all dots in each cell.

2.D. RESULTS

Identification of MCM3 as a KEAP1 substrate

We used two complementary strategies to identify proteins ubiquitylated by the KEAP1-CUL3-RBX1 complex. First, we employed parallel affinity capture (PAC) mass spectrometry (Fig. 1*A*). E3 ubiquitin ligases are processive in action, binding, ubiquitylating and releasing substrates. As such, traditional purification of E3 ligases often fails to identify the transient

interactions of co-complexed substrates. The PAC approach uses genetic or pharmacological tools to block substrate degradation, which results in stabilization of the E3-substrate interaction. HEK293T cells engineered for stable expression of KEAP1 fused with streptavidin binding peptide (SBP) and hemagglutinin (HA) epitope were grown in SILAC-light medium or SILAC-heavy medium before the addition of vehicle or MG132 proteasome inhibitor (Fig.1*A*). Mass spectrometry analysis of streptavidin-purified protein complexes from these cells revealed SILAC ratios for KEAP1 and KEAP1-associated proteins (Fig.1*B* and Table S1-S2). As expected, NRF2 abundance increased within the KEAP1 complex following MG132 treatment (SILAC ratio ~4). The NRF2 related transcription factor NFE2L1 (henceforth referred to as NRF1) similarly increased within the KEAP1 complex following proteasome inhibition, suggesting that it is also a KEAP1 substrate (SILAC ratio ~2). NRF1 is an established KEAP1 associated protein, but surprisingly has not previously been reported to be a KEAP1 substrate (57,58). With the exception of NRF2 and NRF1, PAC-based analysis of the KEAP1 protein complex did not reveal new putative substrates. PGAM5 is ubiquitylated by KEAP1 and targeted for proteasome-dependent degradation (22). Unexpectedly, PGAM5 did not accumulate in cell lysates or on KEAP1 following proteasome inhibition. Additionally, other high confidence KEAP1 interacting proteins that contain an E(T/S)GE motif also did not show increased binding to KEAP1 with proteasome inhibition.

We hypothesized that although successful for identifying rapidly catalyzed substrates, the PAC-based method may fail to reveal KEAP1 substrates with slower rates of ubiquitylation. Additionally, by design the PAC-method will not identify E3 substrates that are not bound for the proteasome. Therefore, in a second approach, we interrogated KEAP1-interacting proteins that contain an ETGE or ESGE motif. Specifically, SBPHA-KEAP1 was affinity purified from HEK293T cells over a time course of MG132 treatment. Co-complexed proteins were quantified by LI-COR-based immunoblotting. Two patterns were observed. Class 1 proteins NRF1 and NRF2 increased rapidly in whole cell lysates and within the purified KEAP1 protein complex (~5

fold and ~18 fold, respectively) (Fig. 1*C,D*). Class 2 proteins include the known KEAP1 substrate PGAM5, as well as MCM3, SLK and MAD2L1. Although the steady-state abundances of these proteins were not affected by MG132 treatment, they reproducibly demonstrate a modest increase in KEAP1 binding (<2 fold) between 4h and 6h of MG132 treatment. DPP3, another ETGE-containing KEAP1 interactor, did not fall into either class. Rather, it decreased modestly within the KEAP1 complex during proteasome inhibition.

While both NRF1 and NRF2 respond rapidly to proteasome inhibition, NRF2 is the only KEAP1 substrate that robustly accumulated in response to treatment with ROS mimetic (sulforaphane) or KEAP1-CUL3 antagonist (MLN4924, CDDO) (Figure 5*E*). Collectively, these results suggest two distinct classes of KEAP1 putative substrates: NRF1 and NRF2 are short-lived, stress-responsive proteins that are rapidly turned over by the proteasome, and a second, more stable and higher abundance class of KEAP1 substrates comprised of PGAM5, MCM3, SLK and MAD2L1. We chose MCM3, a member of the essential DNA replicative helicase, for further study based on its important role in cell cycle regulation.

Biochemical Analysis of the KEAP1-MCM3 Complex

Having previously identified MCM3 as a high-confidence KEAP1 interacting protein by affinity-purification/mass spectrometry (AP/MS) (19,20), and now as a putative substrate (Fig. 1*C*,*D*), we sought to validate and determine the localization of this interaction. We first conducted a reciprocal MCM3 IP/MS experiment with ectopically expressed FLAG epitope tagged MCM3 and detected endogenous KEAP1, in addition to all the expected MCM3 associated proteins, largely those important for DNA replication (Fig. 2*A*). We also detected KEAP1 interaction with the MCM complex by endogenous co-immunoprecipitation using antibodies to MCM3 or MCM2 (another subunit of the MCM2-7 heterohexameric complex) (Fig 2*B*,*C*). Next, we expressed a KEAP1 fusion to a biotin ligase proximity detector, BirA* and tested MCM subunits for in vivo biotinylation. We detected biotin-stimulated modification of both

endogenous MCM3 and MCM2 only in cells expressing the KEAP1-BirA* fusion demonstrating its close proximity to the MCM hexamer (Fig. 2*D*). We next assessed where in the cell KEAP1 and MCM3 interact. Immunofluorescence analysis using antibodies against MCM3 in cells stably expressing VENUS-KEAP1 revealed that MCM3 was mainly nuclear, but a small fraction of MCM3 antibody reactivity diffusely localized to the cytosol, in contrast to VENUS-KEAP1, which was mainly cytoplasmic, with a small pool in the nucleus (Fig. 2*E*). To test in which compartment(s) KEAP1 and MCM3 associate, we performed an *in situ* proximity ligation assay (PLA) using primary antibodies for KEAP1 and MCM3. Figure 2*F* shows representative images for this assay, demonstrating that KEAP1 and MCM3 are in close proximity to one another in both the nucleus and cytoplasm. Using subcellular fractionation followed by western blotting, we observed that a small fraction of KEAP1 was indeed in the nucleus, in agreement with our microscopy analysis and other reports that ~5% of KEAP1 is nuclear (Fig. 2*G*)(59).

MCM3 is a KEAP1 substrate for ubiquitylation in vivo and in vitro

Next, we tested whether KEAP1 directly ubiquitylates MCM3. Under near-denaturing conditions, FLAG-MCM3 or FLAG-NRF2 was immunoprecipitated from HEK293T cells expressing control GFP or KEAP1. Western blot analysis showed strong induction of MCM3 ubiquitylation by KEAP1, similarly to the positive control NRF2 (Fig.3*A,B*). Reciprocally, siRNA-mediated silencing of KEAP1 suppressed ubiquitylation of FLAG-MCM3 (Fig.3*C*). To evaluate ubiquitylation of endogenous MCM3, we immunoprecipitated MCM3 under near-denaturing conditions from cells transfected with siRNAs targeting KEAP1 or CUL3. Both KEAP1 and CUL3 silencing suppressed ubiquitylation of endogenous MCM3 (Fig.3*D*). These data demonstrate that the KEAP1-CUL3-RBX1 ligase is responsible for the majority of MCM3 ubiquitylation in proliferating cells. As expected, ubiquitylation of MCM3 by KEAP1 required the MCM3-KEAP1 physical interaction. Specifically, we mutated the ETGE motif within MCM3 to EAAE and found that this mutant was not ubiquitylated by KEAP1, and did not bind KEAP1 (Fig. 3*E,F*). Together,

these data suggest that the KEAP1-CUL3 complex directly ubiquitylates MCM3. To confirm this, an *in vitro* ubiquitylation assay was performed. The KEAP1-CUL3-RBX1 complex was sufficient to ubiquitylate MCM3 (Fig.*G*).

To identify the sites of ubiquitylation within MCM3, and specifically those that respond to KEAP1, we performed ubiquitin remnant profiling on immunopurified MCM3 complexes from control cells or cells overexpressing KEAP1. Specifically, tryptic peptides from FLAG-MCM3 complexes were subjected to ubiquitin remnant IP followed by LC/MS/MS. This method uses an antibody specific for the ubiquitin remnant left on the ubiquitylated lysine following tryptic digest. The results (shown in Fig.4*A*) further support our western blot data that KEAP1 indeed ubiquitylates MCM3. Using a three-fold arbitrary threshold, six lysine residues were identified as responsive to KEAP1-dependent ubiquitylation: K229, K270, K283, K351, K435, K748 (Fig.4*A*). Of the ubiquitylated lysines mapped, K435 showed the greatest fold increase by KEAP1. This site was also found to be differentially ubiquitylated in an unbiased screen for cullin ring ligase substrates (60). To visualize these lysines on the structure of the MCM2-7 complex, we used protein structural modeling. Human MCM3 was threaded around a homologous archaeal MCM protein (61) and superimposed over a published model of the yeast MCM2-7 heterohexamer (54). The lysines observed to be most ubiquitylated in response to ectopic KEAP1 were found to be on predicted exposed surfaces of the C-terminal domain (CTD) in MCM3 (Fig.4*B*,*C*).

KEAP1 does not regulate MCM3 levels, subcellular localization, or MCM2-7 complex formation

After identifying MCM3 as a novel KEAP1 substrate, we sought to determine the function of this ubiquitylation. First, we tested whether KEAP1 targets MCM3 for proteasome-mediated degradation as it does its well-known substrate NRF2. KEAP1 manipulation did not affect steady-state levels of total cellular MCM3. Specifically, KEAP1 knockdown, deletion, overexpression, or chemical antagonist caused no changes in total MCM3 protein levels, while all of these perturbations affected NRF2 levels (Fig. 5*A*-*D*). Attempts to determine whether

KEAP1 loss affects MCM3 half-life were hampered by the extremely long MCM3 half-life. A very long (30 hour) chase with the protein synthesis inhibitor cycloheximide did not yield an appreciable change in MCM3 levels (Fig. 5*F*), in agreement with a report that showed the MCM complex had a half-life of approximately 24 hours in vivo (62). Treating with the proteasome inhibitor bortezomib also did not stabilize MCM3 over the course of 8 hr, in agreement with KEAP1-CUL3-RBX1 not targeting MCM3 for proteasome mediated degradation (Fig. 5*D*). Similarly, treating with the lysosomal inhibitor chloroquine did not stabilize MCM3 over an 8 h time course, supporting that the KEAP1-CUL3-RBX1 ligase is not targeting MCM3 for lysosome-mediated degradation (Fig. 5*E*).

Next, we tested whether KEAP1 could be ubiquitylating MCM3 to affect its subcellular localization. Using immunofluorescence in HEK293T cells transiently transfected with KEAP1, we found no difference in the localization of endogenous MCM3, which remains largely diffuse in the nucleus (Fig. 5*H*, compare cells expressing KEAP1 in red to those not expressing). We also expressed increasing amount of exogenous KEAP1 and assayed the amount of MCM3 in the nuclear and cytoplasmic compartments and found no difference in the amount of MCM3 in either compartment, suggesting KEAP1 does not regulate total MCM3 subcellular localization (Fig. 5*G*).

The ability of MCM3 to associate with the other members of the MCM2-7 heterohexamer was evaluated by immunoprecipitating either WT FLAG-MCM3 or the KEAP1-deficient binding mutant (FLAG-MCM3 EAAE) and probing with anti-MCM2. A comparable amount of MCM2 associated with both forms of MCM3, suggesting KEAP1 binding and ubiquitylation do not regulate MCM3 incorporation into the MCM2-7 hexamer (Fig. 3*E*). Furthermore, MCM3 was associated with the other MCM proteins at similar levels in the presence or absence of KEAP1 siRNA knock down (Data not shown).

KEAP1-dependent ubiquitylation is not responsive to treatment with DNA damage, ROS mimetics or Autophagy

As DNA damage by etoposide was recently reported to lead to increased phosphorylation and ubiquitylation of a number of sites in subunits of the MCM2-7 complex (63), including three ubiquitylation sites within MCM3, we examined whether KEAP1 could be one of the E3 ligases that ubiquitylate MCM3 in response to DNA-damage. We found however that overnight treatment with a panel of DNA damaging agents (etoposide, gemcitabine and 4NQO) did not strongly affect the MCM3-KEAP1 interaction (Fig. 6*A*) or KEAP1-dependent ubiquitylation of MCM3 but did activate phospho-Chk1, a marker of DNA damage (Fig. 6*B*). These data suggest that KEAP1 is not the ligase modifying MCM3 in response to etoposide-mediated DNA damage.

The ability of KEAP1 to act an as efficient substrate adaptor for NRF2, its well-known substrate, relies on the REDOX state of the cell because during oxidative stress KEAP1 undergoes electrophilic attack by ROS and is placed in a conformation no longer favorable to target NRF2 (3,6,11,12,64-66). To test whether MCM3 is also a ROS-dependent substrate of KEAP1, we employed surrogate compounds (sulforaphane and tert-butylhydroquinone (tBHQ)) that mimic ROS by attacking the reactive cysteines within KEAP1. These drugs are widely used as NRF2 agonists, though whether these inhibit NRF2 ubiquitylation or block release of ubiquitylated NRF2 is debated. Here, we find that treatment with these compounds stabilizes NRF2 but does not ablate KEAP1-dependent ubiquitylation of either NRF2 or MCM3 (Fig.6*C,D*). Together, these data suggest that the ubiquitylation of MCM3, like NRF2, is not inhibited by sulforaphane or tBHQ.

As MCM3, along with other important DNA replication factors, was recently reported to undergo autophagy-mediated degradation, we tested whether KEAP1 could be ubiquitylating MCM3 to target it for lysosomal degradation (67).

We found that treatment with a lysosome-mediated degradation inhibitor (chloroquine, 100 μM for 8 h) or an autophagy activator (rapamycin, 10 μM for 8 h) did not affect KEAP1-dependent ubiquitylation of MCM3 but did augment LC3 I/II conversion, a marker of autophagy (Fig. 6*E*). These data suggest that KEAP1 is not targeting MCM3 for autophagy-mediated degradation.

KEAP1 associates with chromatin in a cell cycle-dependent fashion

Our detection of KEAP1-MCM3 association in the nuclei of actively proliferating cells suggested that KEAP1 may associate with MCM3 during a normal cell cycle. MCM3 is chromatin-loaded as part of the MCM2-7 complex during G1 phase and unloaded as DNA replication completes throughout S phase. To examine whether KEAP1 associates with chromatin in a cell cycle-dependent fashion, HeLa cells were synchronized, and lysates were collected during G1 and early S phase. The lysates were fractionated into chromatin and soluble fractions and immunoblotted for KEAP1, MCM3 and fractionation and loading controls. Strikingly, we found that KEAP1 loaded onto chromatin during G1, as MCM3 did (Fig. 7*A*, loading is seen 4-10 h time points, chromatin fraction). To investigate when KEAP1 also unloads from the chromatin, HeLa cells were synchronized in early S phase and lysates were collected from S to M phase. These lysates were fractionated into chromatin and whole cell fractions and probed for KEAP1, MCM3 and fractionation and loading controls. KEAP1 unloaded in G2, similar to MCM3, but the unloading of KEAP1 was slightly behind that of MCM3 (Fig. 7*B*, unloading is seen 6-10 h time points, chromatin fraction). Thus, KEAP1 associates with DNA in a cell cycle-dependent fashion, and KEAP1 is at the right place at the right time during the cell cycle to regulate the MCM complex.

In an asynchronous population, KEAP1 bound MCM3 predominantly in the soluble fraction, with a weaker association on chromatin (Fig. 7*C*). This observation is consistent with the fact that most of the cellular MCM is soluble, and only a fraction is chromatin loaded. To test where in the cell this KEAP1-dependent ubiquitylation occurs, cells expressing FLAG-MCM3

and either GFP or KEAP1 were fractionated into chromatin and soluble fractions, subjected to FLAG (MCM3) IPs in both fractions and probed by western blot for ubiquitin on MCM3. Most ubiquitylation of MCM3 by KEAP1 was seen in the soluble fraction. Ubiquitylation of MCM3 in the chromatin bound fraction was detectable, though only during proteasome inhibition (Fig.7*D*). Therefore, KEAP1-dependent ubiquitylation of MCM3 occurs in both the chromatin and soluble fractions.

2.E. DISCUSSION

Through its ubiquitin ligase activity, KEAP1 serves as a sensor and molecular switch for the cellular response to oxidative stress. Here, we provide data that support the emerging concept of NRF2-independent functions for KEAP1. To date, in addition to NRF2 signaling and the coordinated antioxidant response, KEAP1 has been shown to regulate NF-KB signaling through its degradation of IKBKB, to target the mitochondrial membrane phosphatase PGAM5 for proteasome-mediated degradation and to regulate DNA break repair through a degradation-independent ubiquitylation of PALB2 (21-23). Our analyses here establish a fourth NRF2-independent function for KEAP1 and raise the additional possibility that KEAP1 regulates cell cycle progression and/or genome stability through ubiquitylation of MCM3.

Here, we focused on a biochemical assessment of KEAP1 substrates in general and on defining MCM3 as a KEAP1 substrate in particular. Our future studies will delve into the role of KEAP1 in MCM3 function or dynamics. Our present data suggest that KEAP1 interacts with MCM3 when it is in the MCM2-7 hexameric complex. We detect KEAP1 in MCM2 immunoprecipitates, and further, we observe biotinylation of MCM2 by the BirA* tagged KEAP1. Together, these data suggest that KEAP1 interacts with the full MCM2-7 hexamer (e.g. Fig. 2*C*). Moreover, we detect KEAP1-mediated MCM3 ubiquitylation in both the soluble and chromatin-bound fractions (Fig. 7*D*). The association of KEAP1 with chromatin during S phase in a pattern that closely follows MCM loading and unloading is also consistent with KEAP1 associating with

the MCM complex rather than monomeric MCM3 since only the MCM complex is loaded for replication and not individual subunits (68). We thus favor the notion that KEAP1 impacts the replication function of the MCM complex though we cannot yet rule out KEAP1 involvement in a novel non-replication role for MCM3 as has been reported for other individual MCM subunits (69,70).

The high affinity KEAP1-targeting motif (ETGE) is uniquely found in MCM3 and not in the other five MCM subunits, suggesting that MCM3 is at least one direct point of contact between the MCM complex and KEAP1. MCM4 contains the lower affinity DLG binding motif for KEAP1, raising the possibility for a second point of contact. All six subunits of the human MCM2-7 heterohexamer have been reported to be ubiquitylated in cells (71). It is thus possible that KEAP1 ubiquitylates additional MCM proteins. Based on the *S. cerevisiae* and *D. melanogaster* MCM complexes, we presume that human MCM3 is adjacent to both MCM5 and MCM7 in the hexamer (72,73). MCM5 constitutes one side of the MCM2/5 "gate" where the MCM ring opens to allow double-stranded DNA to pass during MCM loading in G1 (74,75); KEAP1 could modulate MCM loading by regulating conformational changes at the MCM2/5 interface. MCM3 is the subunit that directly contacts the helicase activator complex GINS which only associates with MCM during helicase activation and fork progression (73); KEAP1-mediated MCM3 ubiquitylation could impact helicase activation either globally or at a subset of origins. Interestingly, polyubiquitylation of the MCM7 subunit in both *S. cerevisiae* and *X. laevis* is associated with replication termination and MCM unloading (49,50,52) but not changes in MCM7 stability; the KEAP1 interaction with MCM3 could also impact MCM unloading. KEAP1 could thus link ROS sensing to the control of MCM chromatin loading, to activation of MCM-dependent DNA unwinding, or to MCM unloading during S phase as a means to preserve DNA integrity and genome stability.

Human MCM complexes undergo cell cycle-dependent phosphorylation and sumoylation (46), and it is certainly possible that additional E3 ubiquitin ligases participate with KEAP1 in

MCM control. Nonetheless, KEAP1 is clearly the major MCM3 E3 ubiquitin ligase in actively proliferating cells (Fig 3). Our data thus far suggest KEAP1 binds and ubiquitylates only a subset of the total MCM3 molecules and likely regulate their activity through altering MCM2-7 protein-protein interactions or helicase activity. Future work will explore not only the molecular consequences of KEAP1-mediated MCM ubiquitylation, but also under what cellular circumstances KEAP1 may be stimulated to ubiquitylate MCM3. Our discovery that KEAP1 associates with chromatin during S phase may reflect a novel nuclear role for KEAP1 in monitoring replication fork progression and perhaps coordinating origin firing or replisome activity with cellular redox state. If so, then the KEAP1-MCM interaction represents a novel, nuclear role for KEAP1 outside of NRF2 regulation, emphasizing the breadth of KEAP1-regulated cellular events.

We have previously defined a static KEAP1 protein interaction network and demonstrated that it is enriched for proteins containing the KEAP1 binding motif, E(T/S)GE (19,20). While the ETGE motif in NRF2 has been established as a KEAP1 degron therein, and several ETGE-containing proteins have been shown to activate NRF2 through competitive binding to KEAP1 (53,76), the function of the interactions between KEAP1 and these other E(T/S)GE containing proteins remained largely unknown. In the present study, we have combined PAC proteomics to capture substrates and annotated them against what we identified in our prior studies to be high-confidence KEAP1 interactors. Our data suggest that in addition to MCM3, which we validated as a KEAP1-CUL3-RBX1 substrate here, we may have also discovered NRF1 as a novel KEAP1 substrate. NRF1 has previously been shown to bind KEAP1, but KEAP1 was shown not to be responsible for targeting NRF1 to the ER membrane or regulating an artificial reporter of NRF1-driven transcription (57,58). To the best of our knowledge, our study is the first report that NRF1 is a putative KEAP1 substrate for proteasome-mediated degradation. We observe NRF1 accumulating with proteasome inhibition in both whole cell lysates and in the KEAP1 complex by both AP/MS and AP-western blotting. In

cell and *in vitro* ubiquitylation assays will be important to confirm the substrate status of NRF1. Our findings neither confirm nor rule out whether MAD2L1, SLK, DPP3, TSC22D4, FAM117B, or other ETGE-containing KEAP1 interactors are bona fide substrates. Since we, and others, have now demonstrated that KEAP1-CUL3-RBX1 substrates may not be turned over by the proteasome (23), the substrate status of each of these interactors will need to be evaluated individually.

Herein, we broadly defined two classes of KEAP1 substrates: class I comprised of NRF2, and potentially NRF1 and IKBKB and class II: comprised of PGAM5, MCM3, and potentially PALB2, MAD2L1 and SLK (though not detected in our MS data, IKBKB and PALB2 have been validated by others in the field). These classes of substrates can likely be further characterized by whether or not the KEAP1 complex targets them for degradation or not and by whether that degradation occurs via the proteasome or the lysosome. Additionally, the presence of both the DLG and ETGE motifs in the rapidly degraded class I substrates (NRF2, IKBKB and NRF1) suggest KEAP1-CUL3-RBX1 substrate dynamics may be governed by the number and/or types of degrons within a given substrate. This pattern is in contrast to the class II substrates PGAM5, PALB2 and MCM3, which contain only the E(T/S)GE motif. These proteins are generally longer-lived than class I proteins, and their ubiquitylation by KEAP1 may be unrelated to their stability. Thus, whether a KEAP1-CUL3-RBX1 substrate is marked for degradation by ubiquitylation and the dynamics of that degradation may be in part dependent on the presence of a DLG motif. The stoichiometry of the class II substrates PGAM5, PALB2 and MCM3 within the KEAP1 complex is not known. Whether these may contain a second KEAP1 binding motif that acts similarly to the DLG and positions these substrates for ubiquityation by the KEAP-CUL3-RBX1 machinery merits further study.

While our approaches were successful in identifying a cohort of KEAP1 substrates, an interesting complementary study would be to perform KEAP1 AP/MS using the NEDD8 inhibitor that dampens CUL3 activity, MLN4924, to trap substrates in complex with KEAP1. In this way,

substrates that may or may not be targeted for proteasome-mediated degradation could be identified without the need for follow-up, targeted studies. Given that IKBKB has been shown to be targeted for lysosomal degradation by KEAP1, another important experiment will be to perform substrate-trapping experiments for lysosome-mediated degradation by KEAP1, where substrates may be 'trapped' on KEAP1 with lysosomal inhibitors, such as chloroquine. Alternatively, another means to explore the full complement of KEAP1 substrates would be to look at the global ubiquitylation status of the cell in the presence or absence of KEAP1 using whole cell ubiquitin remnant proteomics. Further proteomics studies could provide a better understanding of the many diverse functions of this important E3 ligase complex.

This work is important from a basic biology standpoint because little is known about NRF2-independent functions for this REDOX-sensitive E3 ligase. Additionally, this is clinically relevant because a more complete understanding of this pathway is essential to treating patients harboring KEAP1 mutations and to fully grasping the impact of chemically altering the activity or substrate interface of KEAP1. Furthermore, this work provides a previously unappreciated link between KEAP1, genome stability and cell cycle progression.

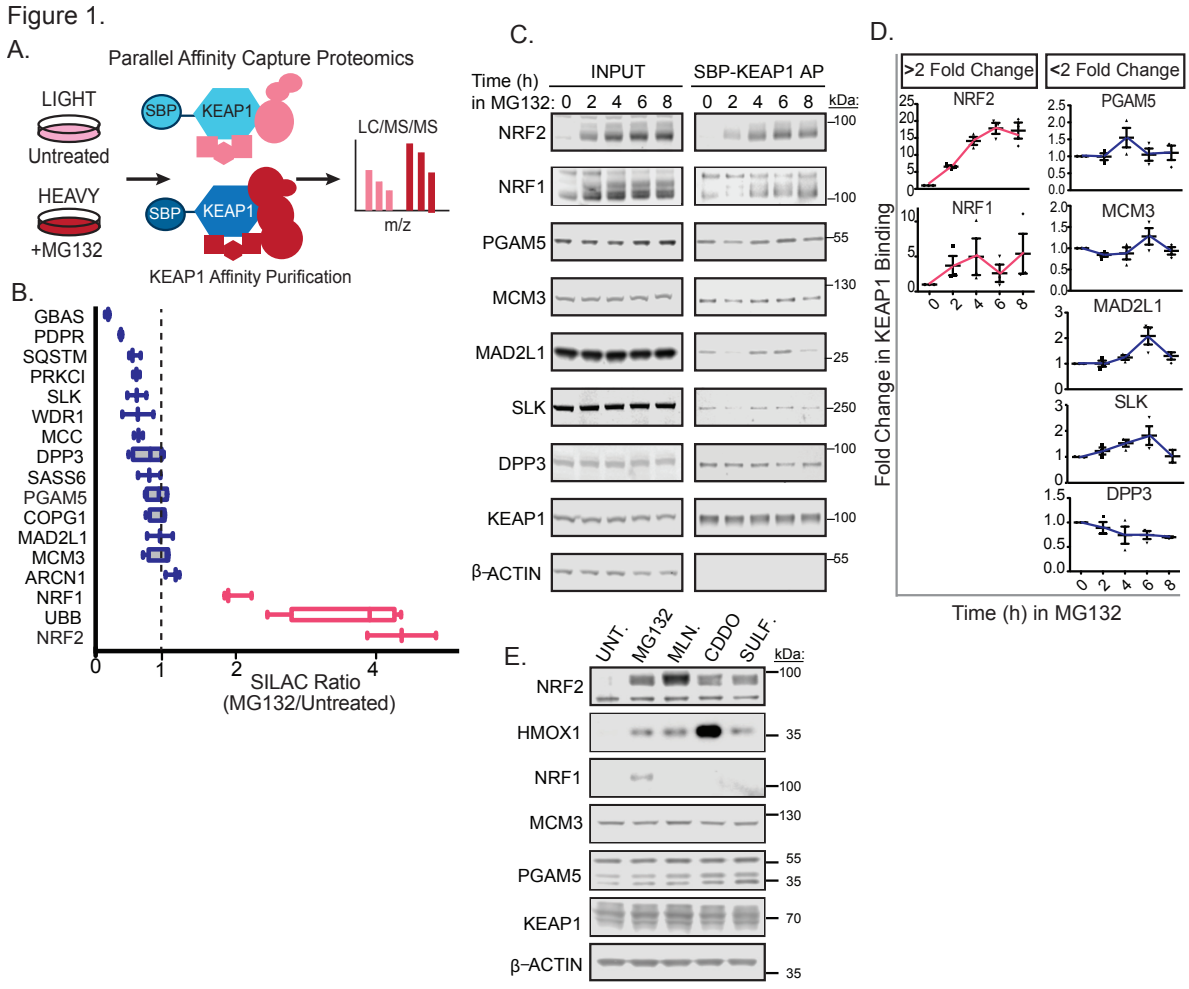

Figure 2.1. Parallel adaptor capture (PAC) proteomics and a candidate-based approach reveal putative KEAP1 substrates. A) Experimental schematic for the KEAP1 PAC-proteomics. Putative substrates increase in association with KEAP1 following proteasome inhibition (red circles). B) The mean SILAC ratios (Heavy/Light) of high confidence KEAP1 interactors detected by streptavidin affinity purification (AP) of streptavidin binding peptide (SBP) tagged KEAP1 (SBPHA-KEAP1) followed by LC/MS/MS in the presence or absence of 2-4 h proteasome inhibitor (MG132) are plotted (High confidence interactors determined by Spotlite-scored KEAP1 interaction network (53)). Proteins plotted were detected in at least two experimental replicates. Pink box-and-whisker plots show proteins with increased association with KEAP1 under proteasome inhibition (increased>50%) (Tables S1-2). C) Western blot analysis of streptavidin affinity purified KEAP1 protein complexes across a MG132 time course (0-8 h) are shown. The horizontal line represents the mean of the 3 biological replicates of each time point and the trend line connects the mean level of each protein bound to KEAP1 at of each the time points. D) LICOR-based quantification of data shown in (C). The horizontal line represents the mean of the 3 biological replicates at each time point and the trend line connects the mean level of each protein bound to KEAP1 at of each the time points. E) Western blot analysis of HDF-Tert cell lysates treated with the indicated proteasome inhibitor or KEAP1-CUL3 antagonist for 6h. Each experiment (B-E) was performed 3-5 times.

Figure 2.

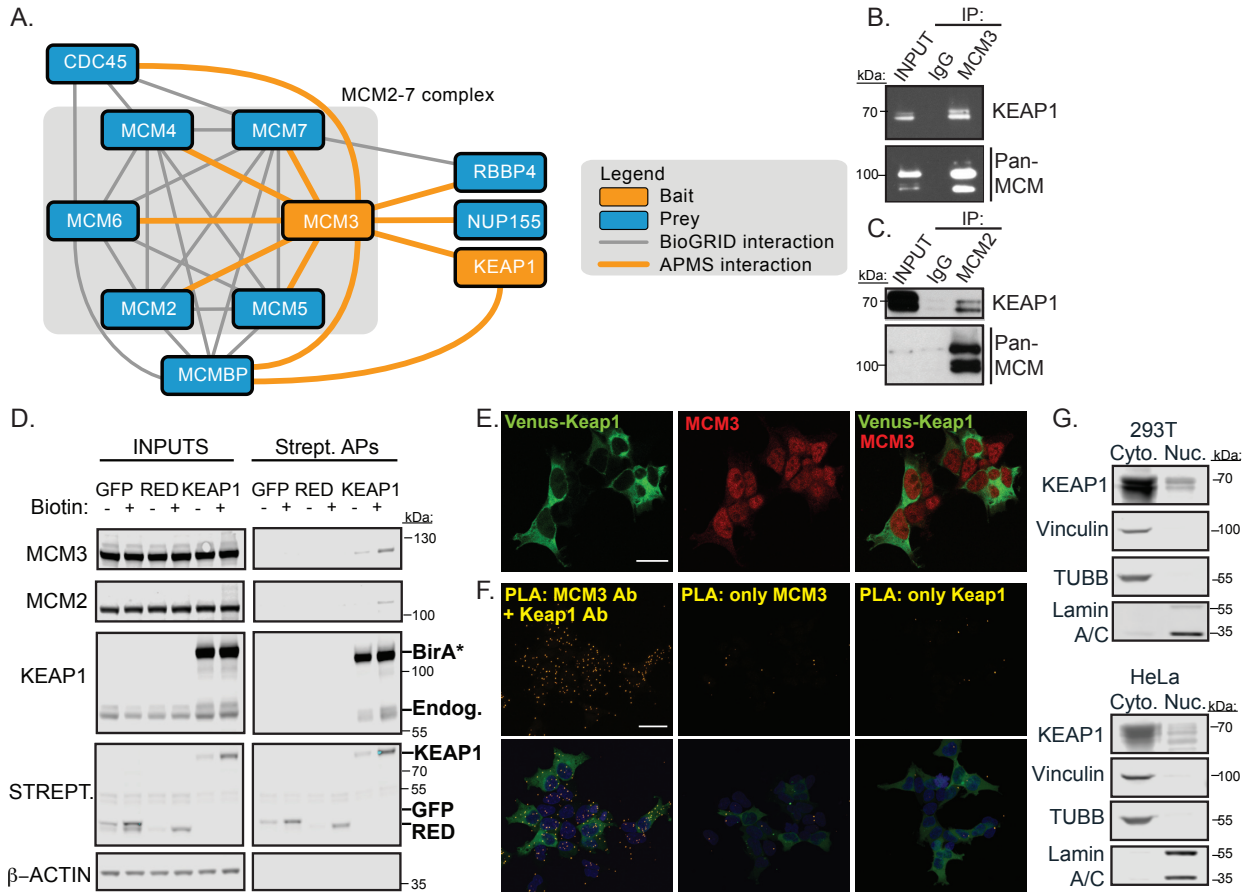

Figure 2.2 KEAP1 associates with MCM3 in the MCM2-7 complex in both the nucleus and cytoplasm. A) FLAG-KEAP1 and FLAG-MCM3 protein interaction networks were determined by FLAG IP/MS. Spotlite-scored high confidence interactors are shown (Table S3). B) Endogenous MCM3 IP was probed for KEAP1 and MCM proteins using an antibody to an epitope common to multiple MCM subunits. C) Endogenous MCM2 IP was probed for KEAP1 and MCM proteins. D) HEK293T cells stably expressing BirA*-KEAP1 (a biotin ligase proximity detector), or cells stably expressing controls (BirA*-GFP or BirA*-HC Red (denoted "RED")) were subjected to streptavidin affinity purification and probed for the indicated proteins. Biotinylated proteins were detected using fluorescently labeled streptavidin (strept.). E) HEK293T immunofluorescence of VENUS-KEAP1 and endogenous MCM3. Scale bar is 20 μm. F) Duo-Link *in situ* proximity ligation assay of KEAP1 and MCM3. Images represent maximum intensity projections of z-stacks. Each yellow fluorescent dot represents a single interaction between KEAP1 and MCM3 (Left panel). VENUS-KEAP1 is shown in green. DAPI stain for nuclei is shown in blue. The middle and right panels are the negative controls. For clarity, the yellow PLA puncta are shown alone in the upper panel. Images were acquired using a confocal microscope. Scale bar is 20 μm. G) Western blot analysis of cytoplasmic and nuclear fractions of HEK293T and HeLa cells to determine the localization of KEAP1. b-Tubulin, Vinculin and Lamin A/C serve as controls for cell fractionation. Each experiment (A-G) is representative of 2-3 biological replicates.

Figure 3.

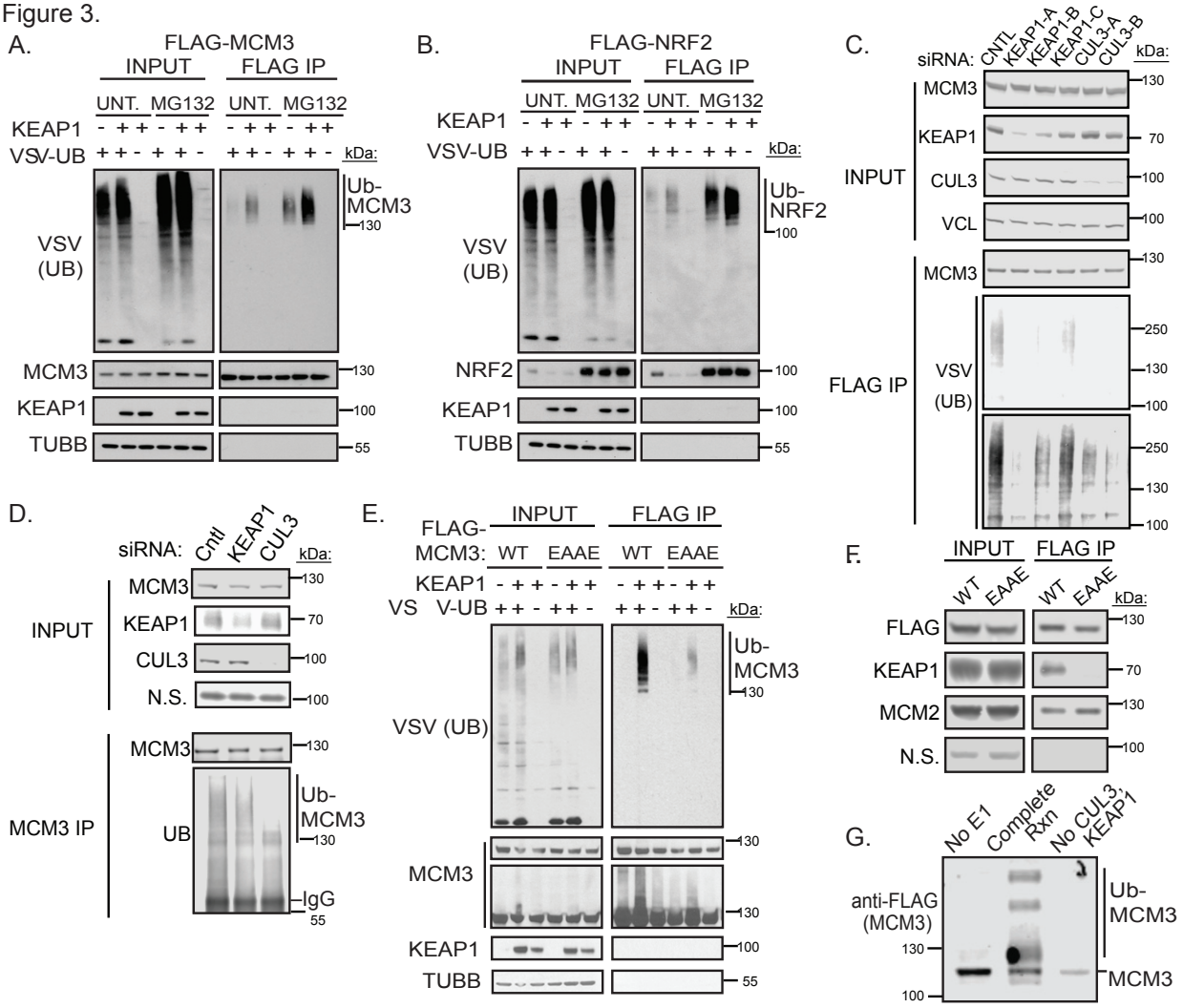

Figure 2.3. MCM3 is a KEAP1-CUL3 substrate for ubiquitylation. A) HEK293T cells were co-transfected with plasmids encoding SBPHA-KEAP1, FLAG-MCM3 and VSV-tagged ubiquitin (UB). Ubiquitylated MCM3 was detected by immunoblot analysis of immunopurified FLAG-MCM3 protein complexes. The IP was performed under near-naturing conditions. B) HEK293T cells were co-transfected with plasmids encoding SBPHA-KEAP1, FLAG-NRF2 (positive control) and VSV-tagged ubiquitin (UB). Ubiquitylated NRF2 was assessed by near-denaturing FLAG-NRF2 IP, as in (A). C) HEK293T cells stably expressing FLAG-MCM3 were transfected with control or KEAP1 siRNA for 72 h, and the amount of ubiquitylated FLAG-MCM3 was determined as in (A). D) Ubiquitylation of endogenous MCM3 was determined by an anti-MCM3 IP after control, KEAP1 or CUL3 siRNA transfection. N.S. is a nonspecific band shown as loading control. E) HEK293T cells were transfected with plasmids encoding FLAG-MCM3 or FLAG-EAAE MCM3, and ubiquitylation was assayed as in (A). F) HEK293T cells were transfected with plasmids encoding FLAG-MCM3 or FLAG-EAAE MCM3 and assessed for binding to KEAP1 by FLAG IP and western blot. G) *In vitro* ubiquitylation assay using KEAP1, CUL3-RBX1, UB, Ube1 (E1), UbcH5B (E2), and FLAG-MCM3. No E1 and no CUL3/no KEAP1 serve as negative controls. UB-MCM3 was detected by anti-FLAG (MCM3). These data are representative of 2-5 biological replicates of each (A-G).

Figure 4.

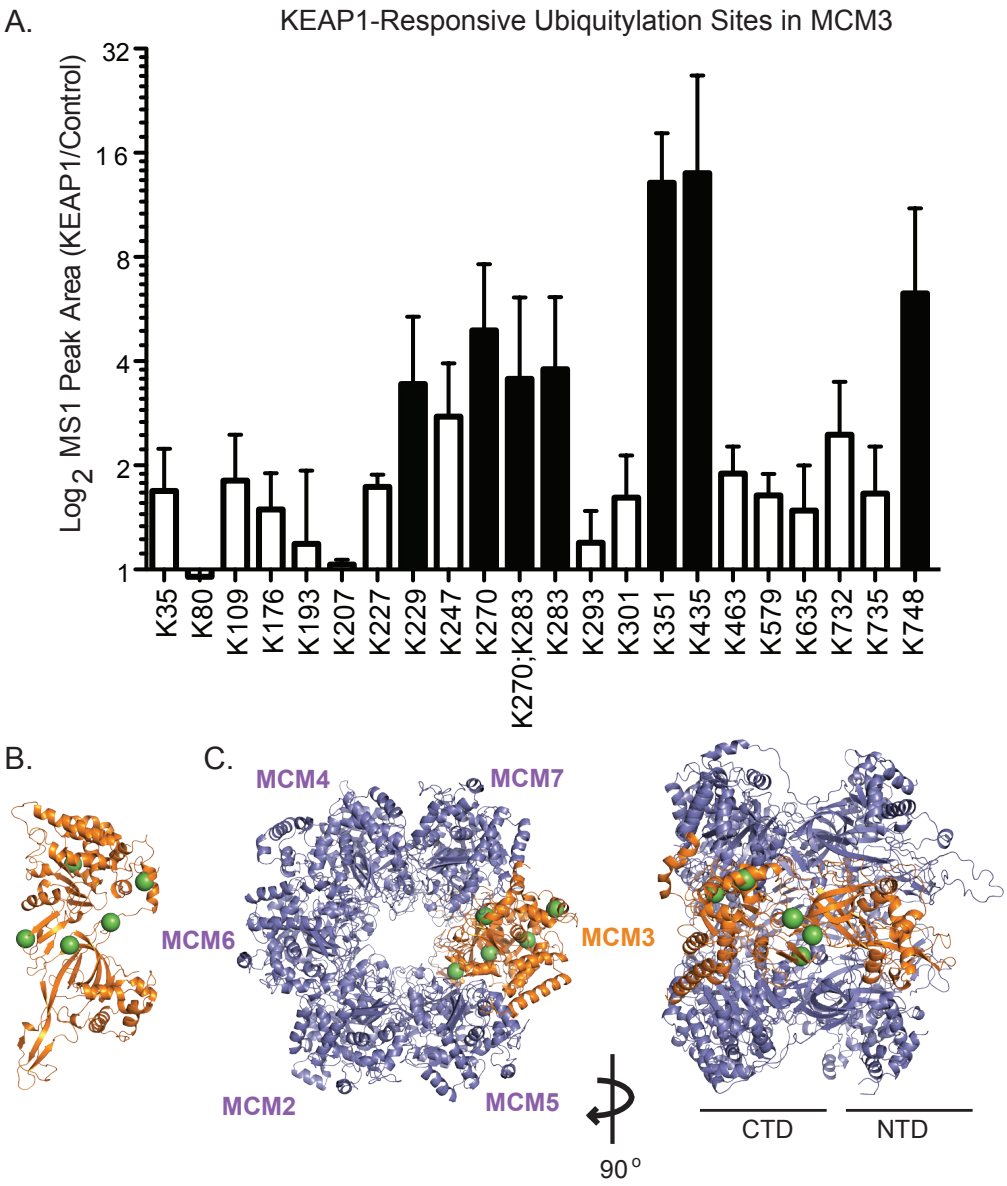

Figure 2.4. Mapping the KEAP1-dependent ubiquitylation sites in MCM3. A) HEK293T cells were transfected with plasmids encoding FLAG-MCM3 +/- SBPHA-KEAP1 plasmid, and a near-denaturing FLAG IP was performed as in Figure 3. A tryptic digest and ubiquitin remnant IP were then performed followed by LC/MS/MS on the resultant peptides. Ubiquitylated peptides of MCM3 detected are plotted as mean (+/-SEM) MS1 peak areas of three biological replicate experiments. Black bars are lysine residues that increased beyond an arbitrary threshold of three fold increase in the presence of SBPHA-KEAP1 (Table S4). B) Protein structural modeling of human MCM3 (Uniprot ID: P25205-1) threaded around an archaeal MCM structure (PDB ID: 3F9V). KEAP1 modified lysines detected in (A) are shown as green spheres. C) Protein structural modeling of human MCM3 from (B) superimposed over the published model of the yeast MCM2-7 complex (54). KEAP1 modified lysines detected in (A) are shown as green spheres. A top-down view (left) and a side view (right) are shown.

Figure 5.

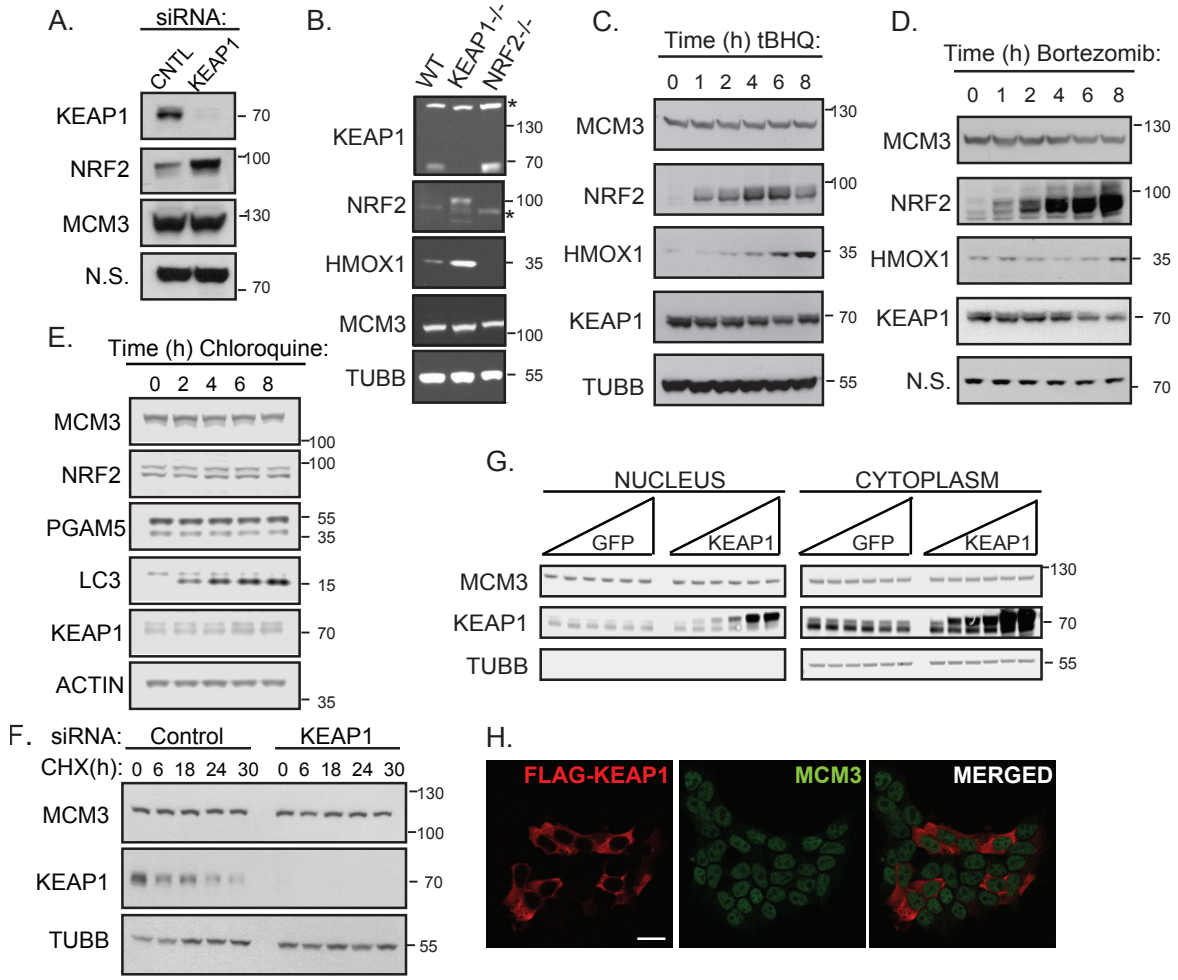

Figure 2.5. MCM3 levels and subcellular localization are not regulated by KEAP1. A) HEK293T cells were transfected with control or KEAP1 siRNA (20 nM, 72 h), lysed and probed for KEAP1, NRF2 and MCM3 protein levels. B) WT, KEAP1-/- or NRF-/- MEFs were lysed and probed for KEAP1, NRF2, MCM3, HMOX1 protein levels by western blot. C) HEK293T cells were treated with the KEAP1 antagonist/ROS mimetic compound tert-butyl hydroquionone (tBHQ) at 50 μM for 0-8 h. Whole cell lysates were subjected to western blotting for MCM3, KEAP1, NRF2 (positive control), HMOX1 and TUBB as a loading control. D) HEK293T cells were treated with 40 nM bortezomib (proteasome inhibitor) for 0-8 h before western blot. N.s. is a non-specific band that serves as a loading control. E) HEK293T cells were treated with the lysosomal-degradation inhibitor chloroquine at 100 μM for 0-8 h. Whole cell lysates were subjected to western blotting for MCM3, KEAP1, NRF2, LC3 (positive control) and Actin as a loading control. F) HEK293T cells were transfected with control or KEAP1 siRNA for 48 h, followed by a 30 h cycloheximide treatment (10ug/mL) and lysates were probed for MCM3, TUBB and KEAP1 protein levels. G) HEK293T cells were transfected with increasing amounts of SBPHA-GFP or SBPHA-KEAP1 plasmid, lysed by fractionation into nuclei and cytoplasm, and blots were probed for MCM3, KEAP1 and loading and fractionation controls. H) HEK293T cells were transfected with FLAG-KEAP1 plasmid and stained for endogenous MCM3 and anti-FLAG to determine MCM3 localization in the presence or absence of KEAP1.

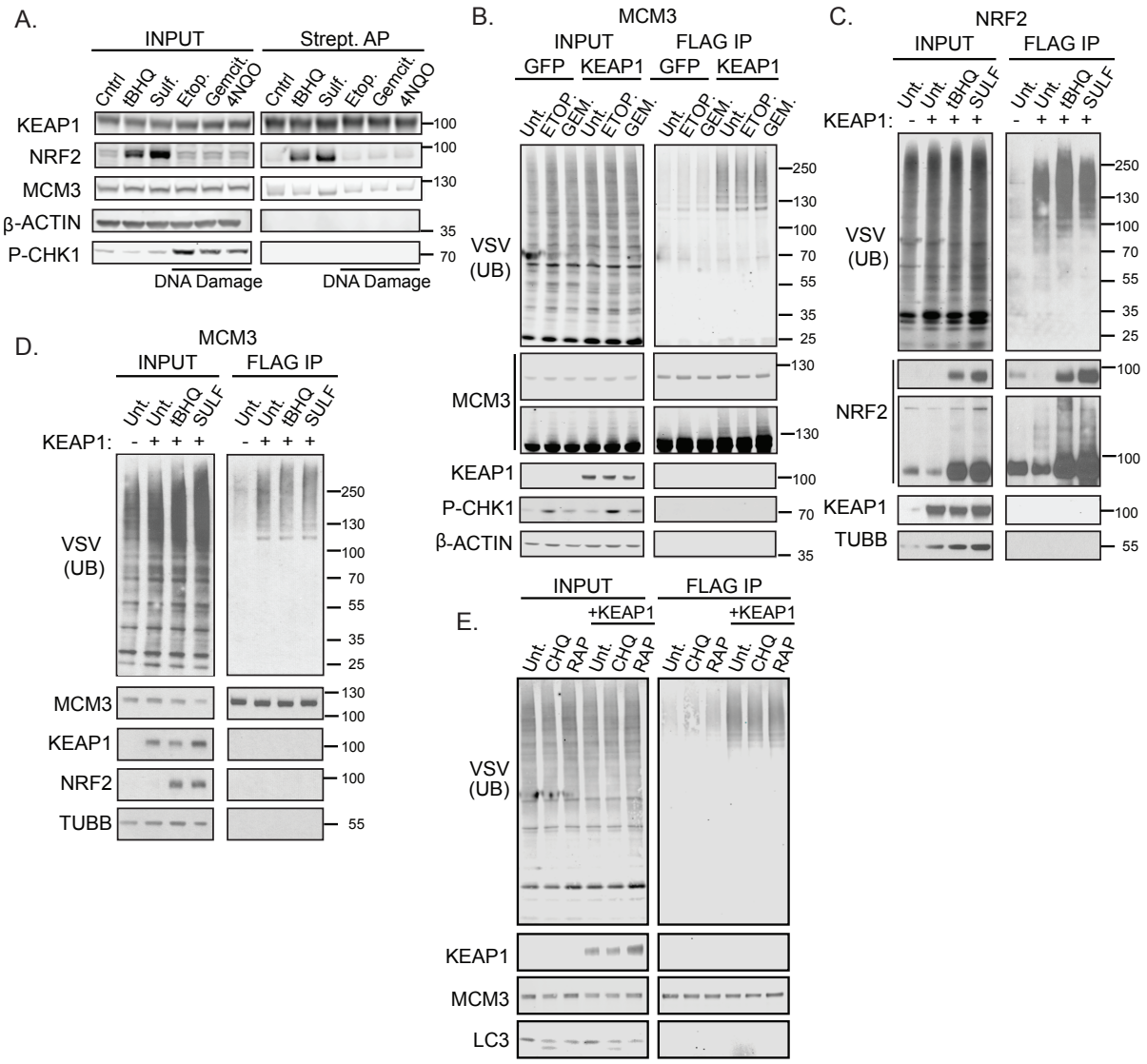

Figure 2.6. MCM3 ubiquitylation by KEAP1 is not affected by DNA damage, ROS mimetics or Autophagy. A) HEK293T cells stably expressing SBPHA-KEAP1 were treated overnight with a panel of DNA damaging agents (etoposide, 20 mM; gemcitabine, 1 mM; 4NQO, 100 nM) or KEAP1 antagonists (tBHQ, 50 mM; sulforaphane, 20 mM). Western blot analysis of KEAP1 affinity purified protein complexes are shown. Phospho-Chk1 serves as a marker of DNA damage. B) HEK293T were transfected with plasmids encoding FLAG-MCM3 and VSV-UB +/- KEAP1 and were treated with DNA damaging agents as in (B). MCM3 ubiquitylation assays were performed as in Fig 3. D) FLAG-NRF2 and VSV-UB +/- KEAP1 were transfected into HEK293T cells and treated with or without ROS mimetics/KEAP1 antagonists for 6h as indicated (tBHQ, 50 μM; sulforaphane, 20 μM). Ubiquitylation of NRF2 was assayed as in Fig 3. E) HEK293T were transfected with plasmids encoding FLAG-MCM3 and VSV-UB +/- KEAP1 and treated with or without ROS mimetics/KEAP1 antagonists as in (C). MCM3 ubiquitylation was assayed as in Fig. 3. E) HEK293T were transfected with plasmids encoding FLAG-MCM3 and VSV-UB +/- KEAP1 and treated with or without chloroquine (lysosomal degradation inhibitor; 100 mM) or rapamycin (autophagy inducer; 10 mM) for 6 h. MCM3 ubiquitylation was assayed as in Fig. 3. LC3 I-II conversion serves as a control for treatment. Each experiment (A-E) is representative of 2-3 biological replicates.

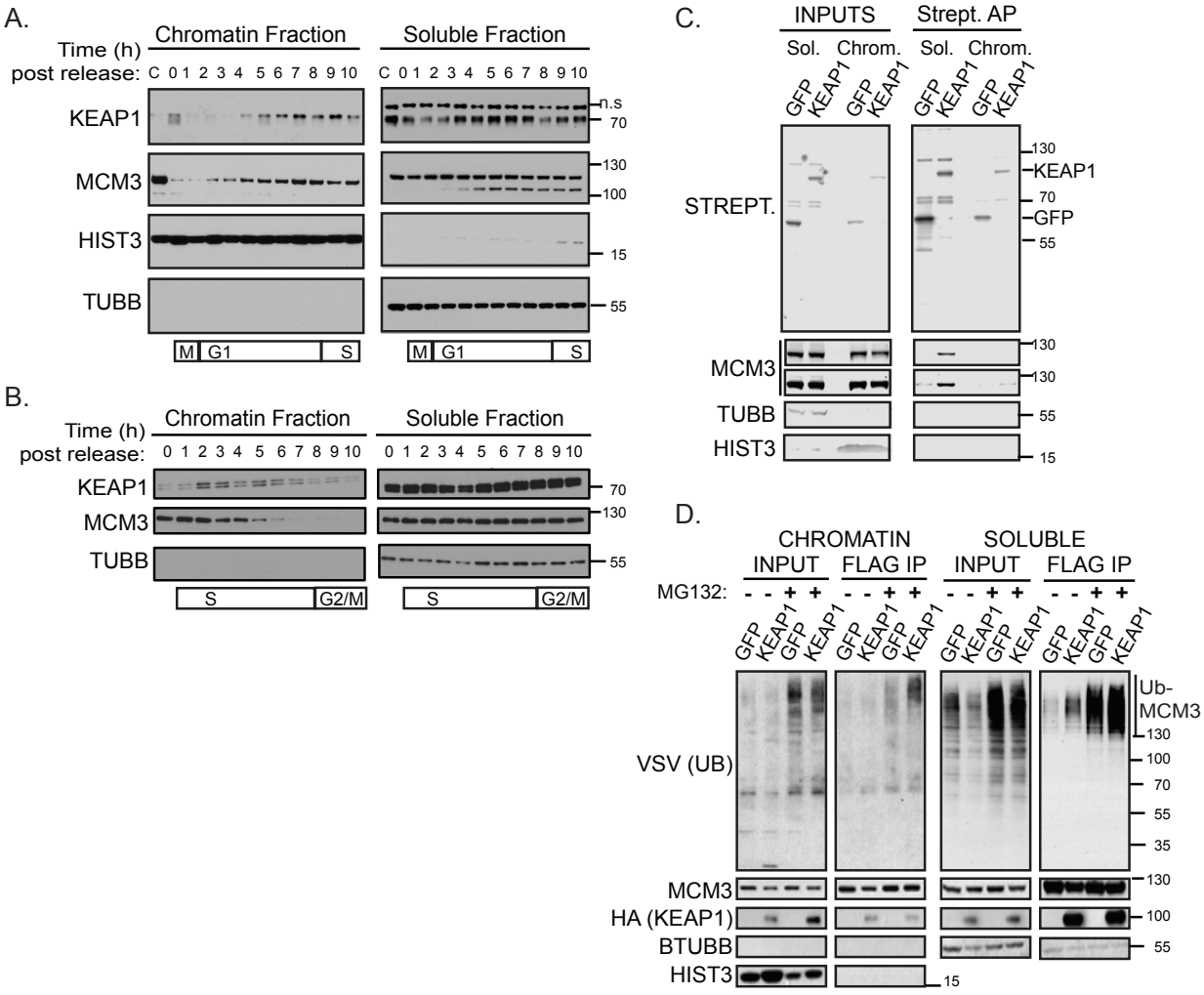

Figure 2.7. KEAP1 associates with chromatin. A) HeLa cells were transfected with control or KEAP1 siRNA and synchronized by double thymidine-nocodazole block. Lysates were collected during G1 and S phases, separated into soluble and chromatin fractions, and probed for KEAP1, MCM3, and loading and fractionation controls. B) HeLa cells were transfected as in (A), synchronized by double thymidine block and collected during S and G2 phases, fractionated and blotted as in (A). C) HEK293T cells stably expressing BirA*-KEAP1 or BirA*-GFP were separated into chromatin and soluble fractions and the amount of MCM3 biotinylated by KEAP1 in each fraction was assessed by streptavidin AP. D) HEK293T cells were transfected with the indicated plasmids and separated into chromatin and soluble fractions. Ubiquitylated MCM3 in each fraction was evaluated by IP/western blot. Each experiment is representative of 2-3 biological replicates.

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CHAPTER III: DISCUSSION

3.A KEAP1 in Cell Cycle Regulation

KEAP1 regulates normal cell proliferation

We identified a subunit of the replicative DNA helicase, MCM3, as a KEAP1 substrate. MCM3, within the MCM2-7 heterohexamer complex, controls the progression of cell cycle through its role in unwinding DNA to allow for DNA synthesis and S phase progression. To address whether KEAP1 was regulating cell cycle and more specifically the MCM3-dependent process of DNA synthesis, we measured DNA replication by tritiated thymidine incorporation in human fibroblasts (Figure 3.1). Loss of KEAP1 was found to decrease DNA synthesis by ~30% in 3 different experiments using 2 different KEAP1 targeting siRNA sequences. However, these data did not reach statistical significance (KEAP1-A versus CNTL p=0.124; KEAP1-B versus CNTL p=0.068). This implies KEAP1 may have an effect on MCM3 function in the replicative helicase, but further experimentation would be required to conclude this. These data are in agreement with the model we are putting forward regarding MCM3 ubiquitylation being functional rather than degradative.

Given that transient KEAP1 loss led to effects on the cell cycle in human fibroblasts, we sought to characterize the cell cycle in a KEAP1 genetically null system. We derived and characterized WT, KEAP1 +/- and KEAP1-/- mouse embryonic fibroblasts (MEF) from a heterozygous cross. KEAP1 knockout mice are early neonatal lethal due to an esophageal defect (1). Utilizing these cell lines, we observed that the KEAP1-/- cells showed a gross defect

in cell division (Figure 3.2). The KEAP1-/- MEFs stop growing at a very early passage number, possibly indicative of an early senescence phenotype. Additional experiments will be required to conclude whether this is indeed a senescence-mediated halt in cell division.

To capture whether the cell cycle or DNA synthesis were affected in the KEAP1-/- MEF cells, DNA content analysis using propidium iodide was performed on very early passage cells (passage 1-passage 4), a time before the decrease in proliferation was observed. These data showed a subtle but reproducible increase in G0/G1 and a decrease in S and G2/M phases. Overall, these data support the KEAP1 -/- cells being less proliferative as was observed in the 3T3 immortalization/proliferation assay. These data could indicate that KEAP1 plays a role in the cell's decision whether to enter the cell cycle or to become dormant.

Determining the KEAP1 substrate(s) responsible for KEAP1 loss-of-function phenotypes

We have identified a role for KEAP1 in proliferation using KEAP1 null murine fibroblasts as well as human fibroblasts. While the proliferation phenotype is quite robust (Figure 3.2), it is very difficult to attribute this phenotype to one or more KEAP1 substrate(s). This is in part because a KEAP1 null system will alter all of the KEAP1 substrates, rather than any particular substrate. Additionally, traditional mutagenesis on KEAP1 would be complicated by the observation that all of these substrates associate with the same region of the Kelch domain in KEAP1. Similarly, chemical antagonism of KEAP1 or CUL3 robustly increases NRF2 stability and activity. Thus, attributing a KEAP1 loss-of-function phenotype to a particular KEAP1 substrate will require utilizing mutations within the particular substrate(s) of interest. To do this, we made an alanine mutant in the ETGE motif within MCM3 (MCM3-EAAE) that could no longer associate with or be ubiquitylated by KEAP1 (Figure 2.3). We created a knockdown-rescue system using siRNA sequences targeting the 3'-untranslated region of the MCM3 gene coupled

with ectopic expression of either WT or EAAE MCM3. Then, we tested the ability of WT-MCM3 versus EAAE-MCM3 to 'rescue' DNA synthesis. These experiments had unforeseen technical challenges with expressing a single subunit of a hexameric complex; WT-MCM3 was unable to recover DNA synthesis in the absence of endogenous MCM3. Thus, this system could not be used to assess the directness of the KEAP1 loss-of-function cell cycle effects. An interesting experiment to circumvent these technical challenges would be to use CRISPR technology to introduce this EAAE mutation directly into the endogenous MCM3 gene and assay its functionality in the MCM2-7 hexamer.

Understanding when and why KEAP1 ubiquitylates MCM3

Though we demonstrate that MCM3 is a bona fide KEAP1 substrate in cells and *in vitro* (Chapter 2), the functional outcome of this remains unresolved. We found that KEAP1 does not regulate steady-state levels of the protein and does not target MCM3 for proteolysis by the proteasome or the lysosome. Thus, we favor a model where KEAP1 regulates MCM3 function. As MCM2-7 associates with tightly wound DNA or chromatin, we tested whether KEAP1 was in the right place, at the right time during cell cycle. Notably, we found that KEAP1 loads onto chromatin in a cell cycle-dependent fashion with kinetics similar to the MCM2-7 complex. This demonstrates that KEAP1 is poised to affect MCM3 function. However, further experiments will be required to address whether KEAP1 affects: 1) the ability of the helicase to load onto DNA, 2) its ability to recruit other replication factors to DNA (i.e, Cdc45/GINS and DNA Polymerase), 3) helicase activity (either rate or processivity) or 4) its ability to unload from DNA at late S phase. A key first step in future studies would be to determine whether KEAP1 binds and ubiquitylates MCM3 preferentially at a specific point during the cell cycle.

3.B KEAP1 Substrate Identification

Mechanics of the KEAP1-CUL3-RBX1 Ligase

The identification of KEAP1-CUL3-RBX1 substrates with very different half-lives and disparate functional consequences suggests that this ligase may have previously unknown regulatory capabilities. This elicits many exciting questions about the ligase's activity.

1) How does KEAP1 accomplish targeting substrates differentially to the proteasome, lysosome and for functional, non-degradative ubiquitylation?

2) Is this driven by the presence of one degron (ETGE only) versus two (DLG and ETGE) within the substrate?

3) Does the KEAP1-CUL3-RBX1 complex have alternative components or subunits that dictate which type of ubiquitylation occurs?

4) Does KEAP1-CUL3-RBX1 use different ubiquitin chain linkages, i.e, K48 for proteasome-mediated and K63 for autophagy-mediated degradation?

5) Are the functional ubiquitylation events on PALB2 and MCM3 poly-monoubiquitylation or poly-ubiquitylation on individual lysine residues?

6) Does KEAP1 recognize or participate with other posttranslational modifications on its substrates?

To address the overarching question about how KEAP1-CUL3-RBX1 targets its substrates to the proteasome versus autophagy, domain swapping between known KEAP1 substrates that are targeted for each form of degradation could be applied. For example, mutagenesis and domain-swapping between IKBKB and NRF2 might provide the identification of residues, motifs or domains that promote proteasomal degradation versus autophagy-mediated degradation resulting from KEAP1-mediated ubiquitylation.

Are there classes of KEAP1 substrates defined by the presence of one or two KEAP1 degrons?

In light of identifying MCM3, a very stable, highly abundant protein as a KEAP1 substrate, we propose that KEAP1 may have multiple classes of substrates. We postulate that NRF2, IKBKB and potentially NRF1 are rapidly turned over, stress-responsive 'Class I' KEAP1 substrates. Each of these short-lived proteins (NRF2, IKBKB and NRF1) contains both the ETGE and the DLG motif, indicating that they can form the 'hinge and latch' conformation with KEAP1 and associate with KEAP1 in a 2:1 stoichiometry. We find that MCM3, like PGAM5 and PALB2, is not targeted for rapid degradation by the proteasome or lysosome. These ETGE-only proteins (PGAM5, PALB2 and MCM3) constitute our 'Class II' substrates. PGAM5 is a much more stable protein than NRF2, it is also targeted for proteasome-mediated degradation by KEAP1, but with much slower dynamics than those of NRF2. Specifically, NRF2 has a half-life of less than 15 minutes, which is further diminished by addition of ectopic KEAP1 (2,3). PGAM5 has a half-life of 75 hours in the cell, which is shortened to 6 hours with ectopic KEAP1 expression (4). The half-life of MCM3 is reported to be over 24 hours (5). In line with this, PALB2 and MCM3 are also highly stable proteins and their protein abundances/stability are not affected by overexpression or depletion of KEAP1. Thus, addressing whether having only the ETGE versus having both a DLG and an ETGE motif confers speed of ubiquitylation and protein half-life. These notions could be addressed by generating a green fluorescent protein (GFP)

containing: 1) the ETGE only or 2) both the DLG and the ETGE. Studying the dynamics of the binding, ubiquitylation and degradation of these GFP pseudo KEAP1 substrates could prove useful in addressing whether KEAP1 substrate classes are dictated by the presence or absence of the second KEAP1 degron (DLG).

Does KEAP1-CUL3-RBX1 utilize different ubiquitin chain linkages or other posttranslational modifications on substrates to alter the functional outcome of substrate ubiquitylation?

Questions of ubiquitin chain linkages could be addressed by quantitative mass spectrometry-based analysis of each of the known KEAP1 substrates. The question of poly- mono versus polyubiquitylation could be resolved by future studies assaying the levels of MCM3 and PALB2 ubiquitylation in the presence of wildtype ubiquitin or a ubiquitin mutant that cannot form chains. Addressing such questions would be beneficial in our understanding of KEAP1/NRF2 signaling as well as in answering some of the unknowns about the regulation of substrates by the CRL family. Question (6) is particularly interesting because the KEAP1-CUL3-RBX1 ligase has never been shown to require a priming posttranslational modification, but other E3 ligases, such as $SCF^{\beta TRCP}$ do require a preliminary modification (i.e, phosphorylation) in order to recognize and degrade substrates (6). Thus, if KEAP1 requires either a posttranslational modification or a subcellular localization on a particular subset of its substrates and utilizes this modification or location in order to distinguish how and when to place ubiquitin molecules onto those substrates, then this could greatly enhance our understanding of how E3 ubiquitin ligases target substrates for differential cellular outcomes.

Determining the effects of KEAP1-targeting therapeutics on substrates beyond NRF2

Understanding the mechanics of the KEAP1-CUL3-RBX1 ligase also has therapeutic value because KEAP1 and NRF2 have been proposed as drug targets in a number of human diseases including multiple sclerosis, Parkinson's Disease, Alzheimer's Disease and cancer. Therefore, furthering our grasp of how this complex regulates different substrates may inform strategies for drug development and aid in the anticipation of potential off-target effects. For example, because all known KEAP1 substrates associate with the same binding interphase in the KELCH domain of KEAP1, pharmacologic inhibitors of the KEAP1-NRF2 interface will likely also activate NF-kappaB signaling through loss of the KEAP1-IKBKB substrate interaction. Similarly, as roles for KEAP1 in DNA damage pathways have been established through PALB2, loss of KEAP1 may alter how cells repair DNA (7). Therefore, a better understanding of the mechanics of this ligase and its substrate interactions will be important for appreciating the cellular consequences of therapeutically targeting this pathway.

Are other KEAP1 substrates altered in the context of KEAP1-mutant tumors?

Our lab has previously demonstrated that tumor-derived KEAP1 mutants have decreased abilities to regulate NRF2 protein stability (8). However, it is unknown whether these other substrates (IKBKB, PGAM5, PALB2, MCM3) are also affected by the somatic KEAP1 mutations observed in cancer. Future studies should address whether these cancer-derived KEAP1 mutations maintain the ability to ubiquitylate these other four KEAP1 substrates and whether these KEAP1 substrates have altered stability and/or activity functions in the presence of mutant forms of KEAP1. Preliminary data from our lab suggests that at least one KEAP1 tumor-derived mutant (S224Y) has diminished MCM3 binding when expressed ectopically in HEK293T cells (8). Therefore, the S224Y KEAP1 mutant should be tested for its ability to ubiquitylate MCM3.

3.C KEAP1 is frequently mutated, but rarely lost in cancer

An interesting observation from the analysis of the KEAP1 mutational frequency and expression in cancer is that, compared to other tumor suppressor genes, KEAP1 is rarely lost in cancer. Specifically, while KEAP1 is mutated in 15% of squamous NSCLC tumors, KEAP1 homozygotic deletion is rare among patient tumors (9). Furthermore, previous analyses from our lab demonstrated that cancer-derived KEAP1 mutants are largely still expressed in the cell and they retain their ability to associate with CUL3 (8). The KEAP1 mutations that were tested were also largely hypomorphic in their ability to repress NRF2, suggesting that many KEAP1 tumor mutations retain some functionality (8). In contrast, NRF2 activating mutations in cancer are thought to confer a maximally active NRF2 pathway. In this way, NRF2 and KEAP1 mutations while mutually exclusive in NSCLC are not functionally equivalent. These findings suggest that KEAP1 may possess alternative functions that are important for cell survival and thus KEAP1 expression or activity is diminished sufficiently to activate NRF2, but some KEAP1 activity remains. This hypothesis will require further experimentation to test.

3.D Establishing a nuclear role for KEAP1

KEAP1 is a predominantly cytoplasmic protein, with a reported 5% of total cellular KEAP1 being located in the nucleus (10). However, here we define a role for the minority population of KEAP1 in binding and ubiquitylating MCM3 in the nucleus. KEAP1 has been reported to shuttle in and out of the nucleus to regulate NRF2 (11). However, the majority of studies focus on regulation of NRF2 by KEAP1 occurring in the cytoplasm. Therefore, the role of KEAP1 in the nucleus is much less well established. Interestingly, ours is the first study to demonstrate a role for the cell cycle in regulating KEAP1 subcellular localization and the first

report that KEAP1 associates with chromatin in general. Thus, our findings further support a model of KEAP1 cycling into the nucleus.

KEAP1 contains a nuclear export sequence (NES) and when mutated KEAP1 was shown to persist in the nucleus (11). The nuclear export of KEAP1 requires CRM-1 and the intact NES (11,12). However, how KEAP1 is entering the nucleus is somewhat unresolved. The import of KEAP1 requires the importin alpha family member KPNA6, but KEAP1 does not possess a known nuclear import sequence. Thus, KEAP1 may be entering the nucleus bound to one of its interacting proteins that does contain a nuclear import signal. It would be interesting to determine whether the primary recruitment of KEAP1 into the nucleus is through NRF2, MCM3 or another factor. Additionally, while MCM3 appears to recruit and/or tether KEAP1 to chromatin, KEAP1 may be regulating multiple chromatin-bound substrates once it is there. For example, a curation of all the proteins in the proteome that contain the KEAP1 E(T/S)GE degron yielded the chromatin and MCM3-associated protein MCM10 as having both the high affinity E(T/S)GE degron as well as the lower affinity DLG degron. Interestingly, MCM10 interacts with a double hexamer of MCM2-7 that is loaded onto chromatin and this association is required for helicase splitting and therefore required for functional DNA replication (13). MCM10 was not detected strongly enough in our KEAP1 AP/MS to pass a high-confidence threshold, but MCM10 has been detected in 2 unique KEAP1 AP/MS runs (data not shown). This suggests KEAP1 may interact with and even ubiquitylate MCM10, potentially through its MCM3-dependent recruitment onto chromatin. It would be interesting to address whether MCM10 is also a KEAP1 substrate and more broadly to identify and examine the nuclear and even chromatin-bound KEAP1 interaction network in the future.

3.E Proteomic Dissection of KEAP1/NRF2 Interacting Proteins—Are other E(T/S)GE-containing proteins KEAP1 substrates?

In addition to MCM3 and the other 4 known KEAP1 substrates, our KEAP1 protein interaction network (PIN) contains 12 other proteins that possess the KEAP1 degron, E(T/S)GE, but whose substrate statuses are not known. Several of these E(T/S)GE proteins (i.e, DPP3, WTX and PALB2) have been shown to activate NRF2 through competitively binding to KEAP1 (14,15). However, whether DPP3 or WTX are ubiquitylated by KEAP1 is unknown.

Of particular interest within the KEAP1 PIN is the relatively uncharacterized protein TSC22D4, which contains the KEAP1 E(T/S)GE degron. Very little is known about its cellular functions beyond the study where it was first cloned and identified in 1999 (16). TSC22D4, transforming growth factor-beta-stimulated clone-22, is a leucine zipper family transcription factor that is named for being identified in a screen for TGF-β-responsive genes (16). This initial study also found that TSC22D4 functions as a transcriptional repressor that can homodimerize or heterodimerize with related transcription factors (16). Our preliminary studies on this protein demonstrate that TSC22D4 is primarily localized to the nucleus (observed by immunofluorescence, data not shown) and that it heterodimerizes with the related transcription factor TSC22D2 (data not shown). Together, our data are congruent with the small amount of information about this protein in the literature. In the future, it would be interesting to examine the transcriptional target genes that are repressed or activated by TSC22D4. The original paper on TSC22D4 also describes this family as inducible by a number of different growth factors (i.e, fibroblast growth factor 2, epidermal growth factor) (16). Therefore, it will be interesting to study the protein interaction network of TSC22D4 under basal and induced conditions and to determine by microarray the genes/pathways that are regulated by this putative KEAP1

substrate. Once the functionality of TSC22D4 is established, logical future studies would include determining how KEAP1 and NRF2 regulate the levels or functions of TSC22D4.

3.F Summary and perspective

Overall, we have defined novel KEAP1-interacting proteins that associate with KEAP1 through the ET/SGE binding motif and have identified one such ETGE-containing protein, MCM3, as a KEAP1-CUL3-RBX1 substrate for ubiquitylation. We have demonstrated that the KEAP1 complex can bind and ubiquitylate MCM3 within the nucleus and on chromatin, suggesting that KEAP1 has more nuclear roles than previously thought. Lastly, we find that loss of KEAP1 leads to decreased proliferation and decreased S phase entry in normal cells. While these phenotypes may be tied to the discovery that KEAP1 ubiquitylates MCM3, a member of the DNA replicative helicase, future work will be necessary to address the specific contributions of NRF2, MCM3 and other KEAP1 interactors/substrates to this newly revealed role for KEAP1 in regulating the cell cycle (illustrated in Figure 3.4). Particularly, future studies will be required to first define the role of MCM3 in these phenotypes by expressing a form that cannot associate with KEAP1 and assessing MCM complex function by measuring DNA synthesis, DNA damage and rates of proliferation. Future work should also focus on identifying and characterizing other KEAP1 substrates and assessing their roles in cell cycle and DNA damage response as well as in other cellular pathways. As is shown in the high confidence KEAP1 interaction network in Figure 1.4, KEAP1 associates with seventeen proteins that possess its ET/SGE degron (including the putative substrates NRF1 and TSC22D4 discussed in sections 2.D-E and 3.E, respectively), but only five have been extensively studied as KEAP1 substrates for ubiquitylation. These known substrates have linked KEAP1 to the antioxidant pathway (NRF2) (3,17,18), mitochondrial phosphatase dynamics (PGAM5) (4), the regulation of DNA damage responses (PALB2) (7), NFkappaB signaling (IKBKB) (19,20) and DNA replication (MCM3) (21), and this growing list suggests that KEAP1, which is frequently mutated in cancer, may have

many more presently unknown cellular functions. Thus, an exhaustive identification and functional characterization of the substrates of KEAP1 will further our understanding of the KEAP1 E3 ubiquitin ligase complex in both normal biology and cancer, and the work presented herein moves us toward that goal.

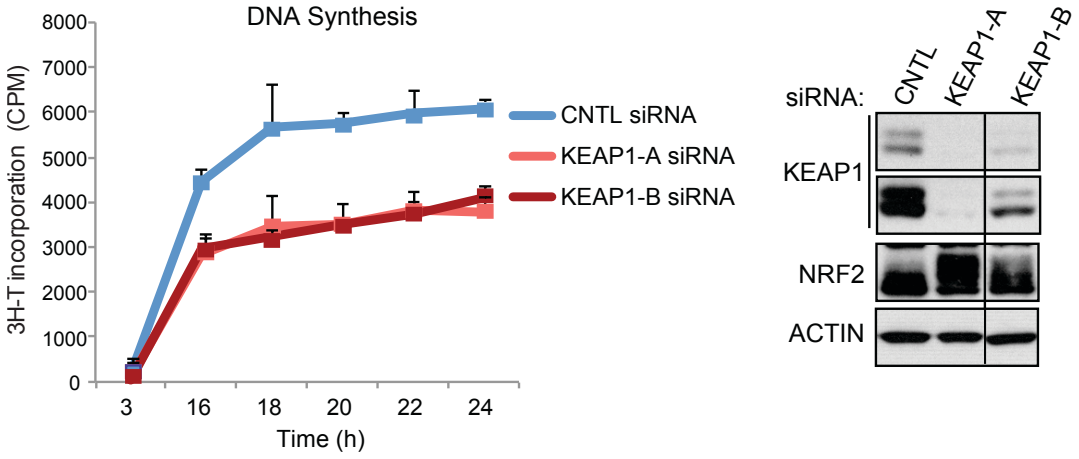

Figure 3.1 KEAP1 knockdown decreases DNA Synthesis in Human Fibroblasts. Left panel: HDF cells were transfected with Control (CNTL) or 1 of 2 KEAP1 targeting siRNA sequences (KEAP1-A; KEAP1-B), synchronized in G0 and released. Cells were harvested across G1 and S phase and the amount of 3H-thymidine incorporated into nascently synthesized DNA was measured by a scintillation counter. Right panel: western blots measuring the amount of KEAP1 and NRF2 in the cells to confirm knockdown, ACTIN serves as a loading control.

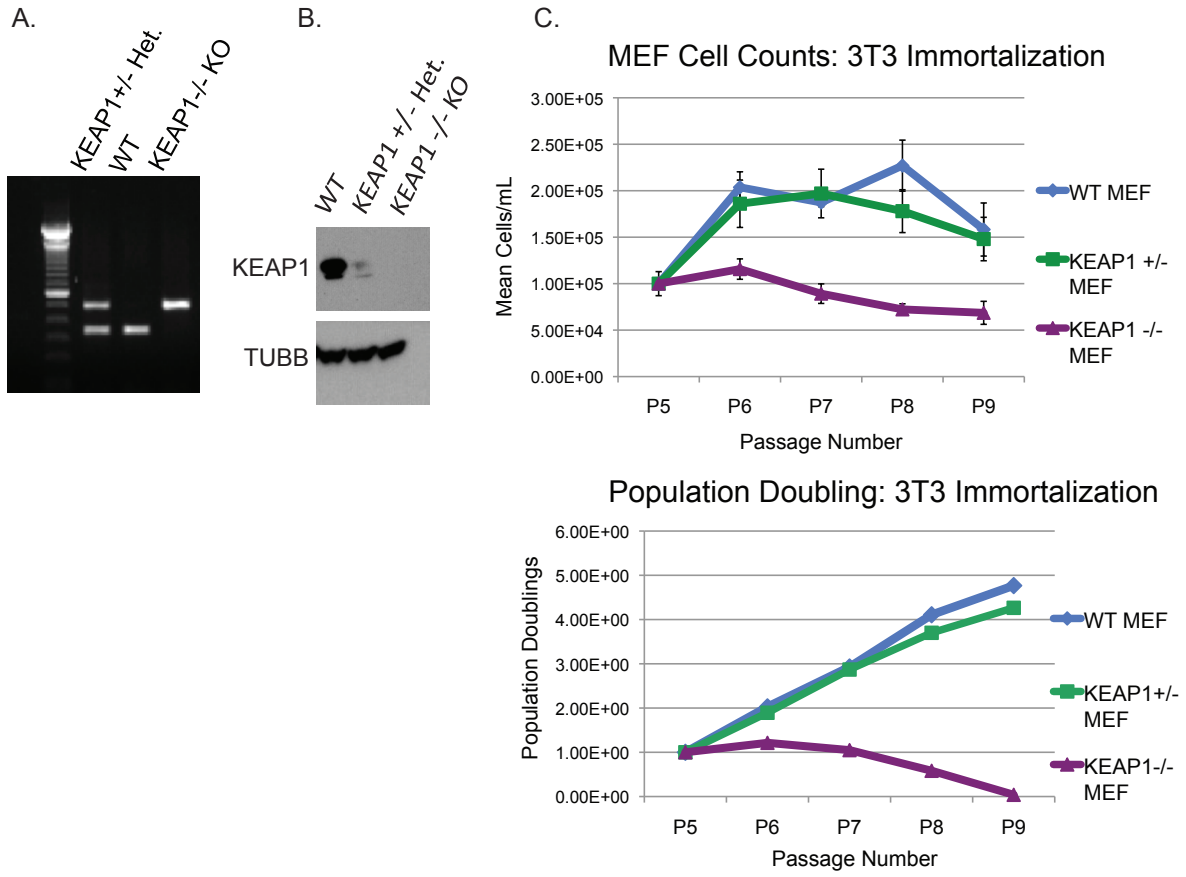

Figure 3.2 KEAP1 Knockout MEFs show decreased proliferation. A) WT, KEAP+/-, and KEAP1-/- MEFs were derived from littermate embryos. Genotyping by RT-PCR is shown. B) MEF genotypes were validated by western blot to detect levels of KEAP1. TUBB serves as a loading control. C) Top panel: 3T3 immortalization assay: 3E5 cells total or 1E5 cells/mL are counted and reseeded every 3 days to immortalize cells by serial passage. Bottom panel: The population doublings of each cell line are calculated from the 3T3 cell counts over time (plotted in the top panel).

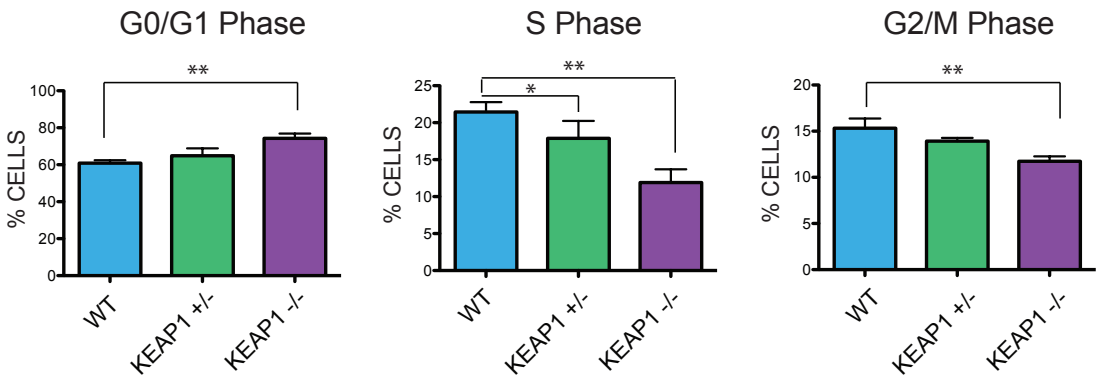

Figure 3.3 KEAP1 Knockout MEFs show altered cell cycle profiles. The DNA content flow cytometry by propidium iodide is shown. The percent of each cell line (WT, KEAP1+/- and KEAP1-/-) in each phase of the cell cycle was determined from primary, untransformed MEF lines between passage 2 and passage 4 in FlowJo Software. Bars represent 3 biological triplicate experiments each with 3 technical replicates per experiment. Statistical significance is noted as follows: *=p<0.05; **=p<0.005.

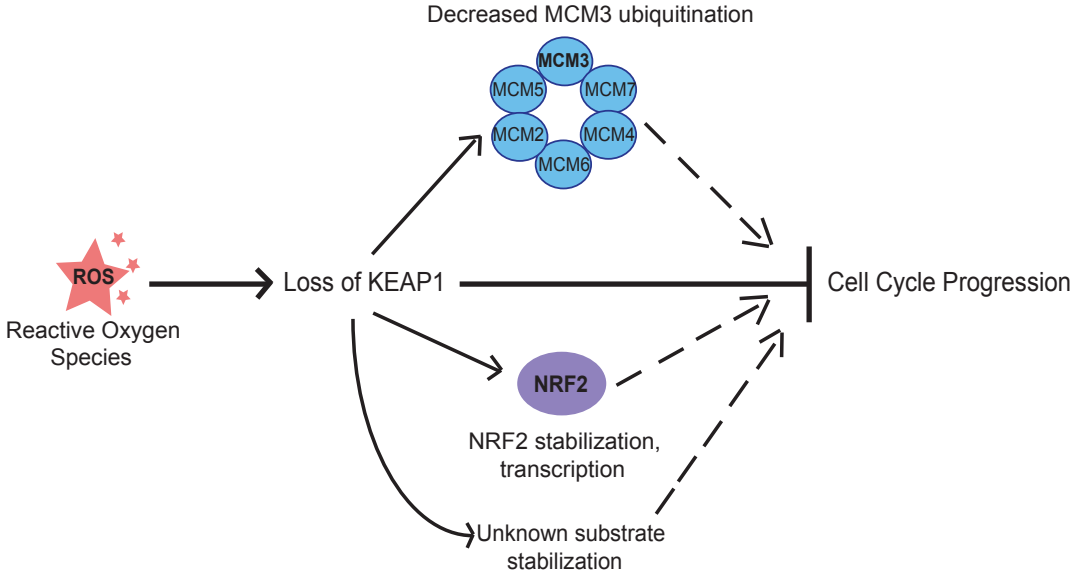

Figure 3.4 Model of KEAP1 Regulation of Normal Cell Cycle Progression. Loss of KEAP1 function, which can happen in response to elevated ROS, leads to decreased proliferation in normal cells. Dotted lines represent the potential contributions of known KEAP1 substrates towards the observed KEAP1 loss-of-function phenotype.

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