Select Heterozygous Keap1 Mutations Have a Dominant-Negative Effect on Wild-Type Keap1 In Vivo

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Abstract

Under homeostatic conditions, Keap1 constitutively mediates the proteasomal degradation Nrf2. However, tertiary changes in Keap1 in response to the cellular environment allow for liberation of Nrf2 to transcriptionally regulate downstream cytoprotective genes that aid in cell survival. KEAP1/NRF2 somatic mutations causing constitutive NRF2 activation have been estimated to occur in approximately 25% of human lung tumors, with similar rates believed to exist in other tissue subtypes. As the stoichiometry between Keap1 and Nrf2 is 2:1, we hypothesized that heterozygous Keap1 mutations could suppress wild-type Keap1 (Keap1WT) activity by functioning as a dominant-negative protein through heterodimerization of mutant Keap1 (Keap1mutant) with Keap1WT. When Keap1G430C or Keap1I364C mutants were expressed in lieu of Keap1WT, premature juvenile mortality was observed. To test the hypothesis of a dominant-negative effect, Keap1-null mice were engineered to coexpress both Keap1WT and Keap1mutant transgenes, a phenotype analogous to that previously observed from Keap1C273&288A substitutions also diminished Keap1WT activity in vivo. To further delineate involvement of the dominant-negative heterodimer, transgenic mice with a deletion or strategic mutation in Broad-complex, Tramtrack and Bric-a-Brac (BTB) domain that disallowed Keap1 dimerization were generated in the presence of Keap1WT, and failed to induce the dominant-negative effect in vivo. These results thus demonstrate that sequestration of Keap1WT into a Keap1mutant–Keap1WT heterodimer leads to a dominant-negative effect in vivo and gives rise to Nrf2 activation.

Introduction

Keap1 is a cytoplasmic protein that aids in the constitutive degradation of Nrf2 (1). Oxidation of key cysteines on Keap1 in response to changes in the cellular environment allows for disruption of Nrf2 ubiquitination and Nrf2 accumulation due to decreased protein turnover (2). Nrf2 subsequently translocates to the nucleus, where it forms a heterodimer with a small Maf protein to transcriptionally regulate inducible expression of cytoprotective genes (3) via cis-acting antioxidant/electro-philic responsive elements (ARE/EpRE; ref. 4). As Nrf2 regulates a multitude of detoxification and antioxidant enzymes, Nrf2-deficient mice are susceptible to a variety of electrophilic or oxidative insults (5–8). When murine Keap1 is disrupted, constitutive Nrf2 activation manifests as in vivo juvenile lethality due to the Nrf2-mediated formation of hyperkeratotic obstructive lesions in the esophagus (9). Importantly, simultaneous deletion of the Nrf2 or small Maf in tandem with Keap1 (9, 10) can prevent premature lethality in double-null mice, indicating that Keap1 is essential in preventing hyperkeratosis caused by excessive activity of the Nrf2-small Maf heterodimer complex. These and other studies have revealed that Keap1 acts as a sensor for electrophilic and oxidative stresses to maintain proper Nrf2 activity. However, Keap1 function can be abolished or diminished to generate a cytoprotective response when conditions dictate.

Available lines of evidence suggest that integrity of the Keap1 tertiary structure is crucial in mediating the ubiquitination of Nrf2. Keap1 consists of 3 major domains, a Broad-complex, Tramtrack and Bric-a-Brac (BTB) domain, an intervening region (IVR), and a double glycine repeat plus C-terminal (DC) domain. Each of these domains has a defined function and plays an integral role in Keap1 activity. The BTB domain is critical for the homodimerization of Keap1 (11), whereas the IVR domain has an important role in binding to Cul3 and supporting the E3 ligase complex necessary for Nrf2 ubiquitination (1). This region also houses critical electrophile-responsive cysteines. Covalent modifications of these cysteines cause changes in Keap1 that interfere with Nrf2 ubiquitination/degradation and allow for increased Nrf2
levels, in essence, enabling the cytoprotective response (12). Two critical cysteines in the IVR are C273 and C288, both of which are known to be responsive to oxidative stress, and mutations cause constitutive Nrf2 activation in vivo (13). Finally, the DC domain is essential for interaction with Neh2 domain of Nrf2 (14). The Neh2 domain of Nrf2 contains the DLG and ETEL motifs that independently interact with the pocket on the bottom of Keap1 DC domain (15).

Recent genotyping of human cancers have revealed several somatic mutations that cause constitutive Nrf2 activation (16–19). Although the epidemiological data on tumor samples has been limited thus far, it was noted that the mutation rate for KEAP1 and NRF2 is estimated to be 25% in lung tumors when both NFE2 and KEAP1 mutations are tallied together (20). Empirical evidence from these studies has suggested that KEAP1 mutations primarily cluster in the IVR and DC domains, whereas NRF2 mutations concentrate in the DLG and ETEL motifs (21–22). The high frequency of mutagenesis in these critical regions required for KEAP1-mediated degradation of NRF2 strongly supports the contention that high levels of NRF2 activity facilitates a selective advantage for tumor growth, can cause chemotherapeutic resistance, and correlates with a poor prognosis (18, 22).

We previously identified two lung-derived KEAP1-DC mutations, one of which is a homozygous mutation at G364 (to cysteine; G364C), whereas the other is a heterozygous mutation at G430 (to cysteine; G430C; ref. 17). The heterozygous G430C mutation was a somatic mutation identified from a human patient, whereas the G364C mutation was identified from cancer-derived cell lines. To clarify how a heterozygous G430C mutation could enable a selective advantage for cancer cell growth in vivo, we have sought to establish a model system in mice that is capable of transgenically expressing Keap1 mutant (Keap1mutant) in lieu of wild-type Keap1 (Keap1WT). To this end, we exploited the mouse Keap1 gene regulatory region, KRD (Keap1 gene regulatory domain; ref. 13), to drive transgenic expression of the mutant constructs.

In this study, we demonstrate in vivo how relevant heterozygous mutations analogous to human lung cancers could cause dominant-negative effects on residual Keap1WT levels. Such heterozygous mutations offer a selective advantage to the tumor by increasing Nrf2 activity and inducing cytoprotective genes that are capable of eliciting a phenotype consistent with tumor survival and a multidrug-resistance phenotype.

Materials and Methods

Plasmid construction

Mutant mouse Keap1 cDNAs were generated by PCR-based mutagenesis using a site-directed mutagenesis kit (Stratagene). The HA-tagged cDNAs were subcloned into the BstHII site of pE-E BOS (23) to generate expression plasmids. These cDNA clones were subsequently ligated into pBluescript II SK (+) utilizing SalI and EcoRI sites. The KRD-Keap1G430C, KRD-Keap1G364C, and KRD-Keap1mutantKRD transgene constructs were generated using the vector harboring a 3.7-kb Keap1 KRD fragment (13). Mutations were confirmed by sequencing.

CMV-based expression vectors of FLAG-tagged Keap1WT and Keap1mutant were generated as described (13). Sequences of the primers used are available upon request.

Transfection and luciferase assays

The 293T cell line is a human renal epithelial cell line transformed with adenovirus EIA and SV40 large T antigen. The cell line was originally purchased from American Type Culture Collection. We have used frozen primary stocks of this cell line in this laboratory for years (13). Because 293T cells were used only for the overexpression experiments, in this study we routinely monitored the cell morphology, but did not test or authenticate the cell line further. Transfection experiments were performed as described (9) by using Lipofectamine-plus (Invitrogen). Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega). Keap1 and Nrf2 mutant expression plasmids were transfected into 293T cells in combination with a pNpo1-ARE reporter plasmid (24) and pRL-TK transfection control plasmid.

Generation of transgenic mice

Transgenic mice were generated as described (13). Transgenic mice expressing Keap1WT or Keap1mutant were mated into a Keap1-null background to obtain compound mutant mice (Keap1+/−/−Tg). All compound mutant mice examined in this study were from a mixed genetic background, with contributions from 129Sv/J, C57BL/6j and ICR strains. More than 3 independent animals from each line were examined to determine body weights.

Histology

For hematoxylin and eosin (H&E) staining, stomachs and esophagi of P10 pups were fixed in 3.7% formalin and embedded into paraffin. For immunohistochemical staining, the stomachs of P10 pups were processed as previously described (10).

Real-time PCR analysis

Real-time PCR was performed using an ABI7300 system (Applied BioScience). Total RNA was extracted from mouse forestomachs using ISOGEN (Nippon Gene). One microgram total RNA was utilized to synthesize cDNA using a Superscript II kit (Life Technologies). The mouse Nqo1 primers were described previously (25).

Immunoprecipitation and immunoblot analyses

To examine Keap1 dimerization, HA-tagged and FLAG-tagged Keap1 expression vectors were cotransfected into 293T cells. Whole-cell extracts were prepared in a lysis buffer (13) and subjected to immunoprecipitation using an anti-Flag M2-Agarose Affinity Gel (A-2220; Sigma). After washing in the lysis buffer, immune-complexes were visualized by immunoblot analysis using anti-HA (3F10; Roche) and anti-Flag (A-8592; Sigma) antibodies. Keap1 protein was detected by immunoblot analyses using a rat anti-mouse monoclonal Keap1 antibody (clone #144; ref. 26). Anti-α-tubulin antibodies (Sigma) were utilized as a loading control.

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Results

Cancer-analogous mutants Keap1<sup>G430C</sup> and Keap1<sup>G364C</sup> cannot repress Nrf2 in vivo

We previously identified somatic mutations of KEAP1 gene in human cancers or cancer-derived cells lines (16–19). Characterizations of representative mutants, such as KEAP1<sup>G430C</sup> from lung cancer or KEAP1<sup>G364C</sup> from lung cancer-derived cells lines, revealed that these KEAP1<sup>mutant</sup> failed to repress NRF2 activity (17). As these residues are highly conserved amongst vertebrates, we introduced identical mutations into the murine Keap1 cDNA and examined Nrf2-repressing activity of Keap1<sup>G430C</sup> and Keap1<sup>G364C</sup> by conducting similar reporter transfection assays. Expression plasmids of Keap1<sup>mutants</sup> were transfected into 293T cells in combination with a p<sup>Nqo1</sup>-ARE reporter plasmid (24). Keap1<sup>G430C</sup> and Keap1<sup>G364C</sup> failed to repress Nrf2 activity (Supplementary Fig. S1), indicating that both G430 and G364 residues are functionally important. Thus, mouse harboring either Keap1<sup>G430C</sup> or Keap1<sup>G364C</sup> mutant can serve to model the human conditions.

To determine how Keap1<sup>mutants</sup> repress Nrf2 activity in vivo, we utilized transgenic complementation rescue. Because this system critically relies on adequate physiological expression of Keap1, we expressed the Keap1 cDNAs under the regulatory influence of a 5.7-kb KRD portion of the Keap1 gene (Fig. 1A). The use of this KRD system has previously been validated (13). Transgenic mice expressing Keap1<sup>G430C</sup> and Keap1<sup>G364C</sup> were generated, and these transgene-derived Keap1 proteins were deemed Tg-Keap1 (Fig. 1A). Tg-Keap1 mice were mated with Keap1<sup>+/−</sup> mice to obtain Keap1<sup>+/−</sup>:Tg-Keap1<sup>G430C</sup> or Keap1<sup>+/−</sup>:Tg-Keap1<sup>G364C</sup> mice, which were further crossed with Keap1<sup>+/−</sup> mice to generate compound mutant mice (Keap1<sup>+/−</sup>:Tg-Keap1<sup>G430C</sup> or Keap1<sup>+/−</sup>:Tg-Keap1<sup>G364C</sup>). Four lines of transgenic mice expressing Tg-Keap1<sup>G430C</sup> (Fig. 1B) and 5 lines of transgenic mice expressing Tg-Keap1<sup>G364C</sup> (Fig. 1C) were found to be unable to rescue Keap1<sup>+/−</sup> mice from growth retardation and juvenile lethality. Transgenically expressed Keap1<sup>G430C</sup> failed to improve esophageal hyperkeratosis of Keap1-null mice (Fig. 1D).

Double transgenic Keap1 complementation rescue approach for assessment of Keap1 function

To examine Keap1<sup>mutant</sup> function in vivo, we aimed to reconstitute comparable expression levels of Keap1<sup>WT</sup> and Keap1<sup>mutant</sup> proteins on a Keap1<sup>-null</sup> background. This method was referred to as a double transgenic Keap1 complementation
rescue approach. To this end, 3 transgenic mouse lines were selected among several lines generated per each construct. We selected line 10 of Keap1G430C, line 6 of Keap1G364C, and line 38 of Keap1WT, as expression levels of the Keap1 mRNAs were almost comparable in the forestomachs of these mouse lines (data not shown). Indeed, in subsequent immunoblot analyses, line 38 and line 10 expressed comparable amounts of Tg-Keap1WT and Tg-Keap1G430C proteins, respectively (Fig. 2A), and line 6 expressed almost similar levels of Tg-Keap1G364C protein to that of Tg-Keap1WT in line 38 (Fig. 2B). These results thus verified that line 10, line 6, and line 38 express Tg-Keap1G430C, Tg-Keap1G364C, and Tg-Keap1WT proteins at comparable levels, which justified the use of a double transgenic Keap1 complementation rescue approach.

Forestomach expression of Nqo1, a prototypical Nrf2-target gene, was determined in Keap1G430C and Keap1G364C transgenic mice (lines 10 and 6, respectively) and compared with those of Tg-Keap1WT mice (line 38). Tg-Keap1WT had lower Nqo1 mRNA levels than Tg-Keap1G430C (Fig. 2C) and Tg-Keap1G364C (Fig. 2D), demonstrating that Keap1mutants are unable to repress Nrf2 activity in vivo.

Cancer-derived Keap1G430C mutant functions in a dominant-negative manner in vivo

Several unique KEAP1 heterozygous mutants have been detected in cancer samples derived from human patients (16, 19, 22, 27). A subset of these mutations cluster in the Nrf2-KEAP1 interface and lead to constitutive Nrf2 activation. We surmised that, as the Keap1G430C mutant was identified as a somatic, heterozygous mutation in vivo, the Keap1G430C mutant might function in a dominant-negative manner (16, 27). Indeed, when tested in luciferase reporter assays, the Keap1G430C was able to impair Keap1WT activity (Supplementary Fig. S2).

Therefore, to examine the dominant-negative effects of Keap1mutants in vivo, transgenic mice expressing Keap1WT (line 38) and Keap1G430C (line 10) were bred into a Keap1-null background. We conducted genotyping by directly sequencing genomic PCR products (Fig. 3A). Nqo1 gene expression in forestomachs of Keap1−/−:Tg-Keap1WT::Tg-Keap1G430C mice was substantially higher than that of Keap1−/−:Tg-Keap1WT mice (Fig. 3B), suggesting that downstream Nrf2 activity is increased due to dominant-negative effects caused by the coexpressed Keap1G430C. Importantly, although the double transgenic compound mice are viable, their body weight is reduced when compared with Keap1WT transgenic mice (Fig. 3C). Consistent with the body-weight reduction, esophageal hyperkeratosis was observed in Keap1−/−:Tg-Keap1WT::Tg-Keap1G430C mice but to a lesser degree than in the Keap1−/− mice (Fig. 3D). These results support our contention that the cancer-analogous Keap1G430C mutant functions in a dominant-negative manner in vivo.
Keap-1 loss-of-dimerization mutants cannot inhibit Nrf2 activity in vivo

Ubiquitination of Nrf2 requires Keap1 homodimerization, a process that requires the Keap1 BTB domain for Keap1–Keap1 interactions. (1, 11). Thus, for a dominant-negative effect to occur it would likely require the sequestration of one Keap1WT protein by one Keap1mutant protein to create an inactive dimer that could not inhibit the Nrf2 activity. To model this phenomenon in vivo, a Keap1mutHKVVL mutant was experimentally generated that substituted the HKVVL sequence (96–100 residues) in the BTB domain with 5 alanine residues. The HKVVL sequence in Keap1 corresponds to a critical region required for the dimerization of BTB-containing proteins (28). When HA-tagged Keap1mutHKVVL and FLAG-tagged Keap1WT constructs were cotransfected into 293T cells, HA-tag immunoprecipitation failed to produce viable Keap1mutHKVVL–Keap1WT dimers, a result consistent with deletion of the entire BTB domain (ΔBTB; Fig. 4A).

To test physiological significance of the Keap1 loss-of-dimerization mutant (dimer-mutant), we generated 3 lines of KRD-Keap1mutHKVVL transgenic mice and bred these lines into a Keap1-null background (Keap1−/−), as is the case for Keap1−/− mice (Fig. 4B). Protein expression from the transgenes (line 36 of Keap1mutHKVVL and line 48 of Keap1ΔBTB) was confirmed to be approximately comparable with that of Tg-Keap1WT (line 38) by immunoblotting (Fig. 4C). Nqo1 expression was not repressed in Keap1−/−::Tg-Keap1WT, Keap1−/−::Tg-Keap1mutHKVVL and Keap1−/− mice at P10. Black double-ended arrows indicate the thickened cornified layer.

Keap1 loss-of-sensor-activity mutants cause dominant-negative effects in vivo

To further examine characteristics of the Keap1 mutants, we prepared Keap1C273&288A, a loss-of-sensor-activity mutant (sensor-mutant), as C273 and C288 residues have been shown to be critical in maintaining repressor activity of Keap1 in vivo (29). We examined whether this mutant functions as a dominant-negative molecule in vivo by using the double transgenic Keap1 complementation rescue approach. It has been suggested that cysteine-to-alanine substitution of these residues (C273&288A) leads to key tertiary changes that mimic the derepression of Nrf2 caused by electrophiles (13, 30). Thus, if the dominant-negative hypothesis is true, loss of these
cysteines in a heterozygous manner would lead to a phenotype similar to the Keap1WT::Keap1G430C mouse model, caused by sequestration of Keap1WT into a dimer with sensor-mutant Keap1. Indeed, concomitant expression of Keap1C273&288A and Keap1WT demonstrated that the Keap1C273&288A functions as a dominant-negative molecule in luciferase reporter assays (Fig. 5A). To exclude negative effects of C273&288A substitution on Keap1 dimerization, we also performed an immunoprecipitation assay. No significant difference in dimer formation was observed between Keap1 WT and Keap1C273&288A (WT and CA, respectively, in Fig. 5B), indicating that the C273&288A substitution did not affect Keap1 dimerization.

We then crossed transgenic mice harboring Keap1WT with Keap1mutHKVVL, Keap1DBTB, or Keap1C273&288A on a Keap1-null background. Genotyping of these double transgenes was carried out by restriction digestion of PCR products (Supplementary Fig. S3A). Immunoblotting analyses demonstrated that the Tg-Keap1C273&288A expression was comparable to the Tg-Keap1WT expression, and in double transgenic mice overall expression of Keap1 was increased (Supplementary Fig. S3B).

In forestomachs of wild-type and dimer-mutant Keap1 double transgenic mice (Keap1WT::Tg-Keap1mutHKVVL; Keap1WT::Tg-Keap1DBTB) Nqo1 expression was similar to that of wild-type Keap1 transgenic mice (Keap1WT::Tg-Keap1WT; Fig. 5C). In contrast, in wild-type and sensor-mutant double transgenic mice (Keap1WT::Tg-Keap1WT::Tg-Keap1mutHKVVL) Nqo1 expression was significantly higher than that of Keap1WT mice at adulthood (Fig. 5C). In forestomachs of Keap1WT::Tg-Keap1mutHKVVL mice Nqo1 expression was lower than that of Keap1WT::Tg-Keap1DBTB mice (Supplementary Fig. S4A). We surmise 2 plausible interpretations of the latter result. One is that the Tg-Keap1WT homodimer still resides in the double transgenic mice, the other is that the Tg-Keap1WT and Tg-Keap1mutHKVVL dimer still retains some residual activity despite the dominant-negative nature of Keap1mutHKVVL molecule. Either or both mechanisms should give rise to the lower Nrf2 activity.
(Supplementary Fig. S4B). Hyperkeratosis of the esophagus was observed in these sensor-mutant and wild-type Keap1 double transgenic mice at P10, but not in dimer-mutant and wild-type Keap1 double transgenic mice (Fig. 5D). Taken together, these results demonstrate that, similar to the Keap1G430C mutant, the Keap1C273&288A mutant functions in vivo in a dominant-negative manner. These results support our contention that for the dominant-negative activity to occur, formation of the mutant Keap1 heterodimer for sequestration of Keap1WT is a requirement.

Discussion

Recently, several somatic KEAP1 mutations have been identified from genotypic screens of cancers and cancer-derived cell lines (reviewed in ref. 20). Cancers that have shown increased propensity for KEAP1 mutations include lung, gall bladder, head and neck, and breast cancers (17, 18, 22, 31, 32). Intriguingly, it has become clearer that selective pressures in the microenvironment of some cancers enables mutations to occur that allow for constitutive activation of NRF2. Subsequent heightened expression of antioxidant- and drug metabolism–related enzymes and drug-resistant transporters provides a major advantage for cancer cells in evading the effects of chemotherapeutic drugs. NRF2-mediated activation of these enzymes allows for increased levels of glutathione, increased detoxification, and increased efflux transport (3, 33, 34). Along similar lines, constitutive activation of NRF2 aids in cellular proliferation, although these mechanisms are currently poorly understood. Translational research has also demonstrated that these mechanisms are critical to progression-free survival, with a poor prognosis...
Demonstration of Dominant-Negative Effect of Keap1 Mutants

Figure 6. Double transgenic complementation models demonstrating the multiple requirements need to mechanistically produce a dominant-negative effect. The model examines how Keap1 theoretically functions in normal conditions, in a cancer environment after a Keap1 mutation, in an experimental condition where a mutant heterodimer cannot form, and experimentally when critical sensor cysteines are mutated. A, in the presence of wild-type Keap1 alone, Keap1 properly homodimerizes and promote polyubiquitination and proteasomal degradation of Nrf2. B, coexpression of Keap1WT and Keap1G430C leads to a nonfunctional heterodimer and Keap1 activity decreases, resulting in increased Nrf2 activity. C, when mutations in Keap1 occur in regions critical to homodimerization, there is an inability for the mutant homodimer to inhibit Keap1WT activity and these mutations cause no phenotypic changes. D, heterozygous Keap1mutHKVVL protein failed to associate with Keap1WT, thus in essence creating a Keap1 protein with an inability to form a mutant heterodimer and Keap1 activity decreases, resulting in increased Nrf2 activity.

of patients that have mutations in regions essential for functional KEAP1 activity (22, 31, 35). NRF2 mutations in the DLG and ETGE motifs also significantly interfere with KEAP1-mediated degradation of NRF2 in cancers (22). Thus, the identification of a significant number of KEAP1 and NRF2 mutations and epidemiological links of KEAP1 mutations to a poor prognosis highlight the importance of KEAP1 in preventing the cancer progression.

In this study, we have demonstrated that mutated forms of KEAP1 analogous to documented cancer genotypes, Keap1G430C and Keap1G364C, lose the ability to repress Nrf2 activity in vitro. Furthermore, when one of these mutants Keap1G430C is coexpressed with similar levels of Keap1WT, it leads to a dominant-negative effect and phenotypic observations typical of Nrf2 hyperactivity. Previous demonstrations using luciferase reporter assays have identified that G430C and G364C mutations lead to loss of Keap1 function and constitutive Nrf2 activation (17). However, it is difficult to extrapolate the in vitro system results to a heterozygous cancer environment, as luciferase assays rely on a complex mixture of reagents. Furthermore, the results do not necessarily translate to the in vivo situation. Thus, as summarized in Figure 6, we utilized the double transgenic complementation model system in which both Tg-Keap1WT and Tg-Keap1mutant are coexpressed to approximately similar levels. We attempted to better represent the type of heterozygous mutation likely to be found in the human condition in which homozygous mutations are less prevalent (20). Our present analysis enabled an in vivo assessment under physiologic conditions in terms of expression levels and cellular environment. This result strongly supports the idea that a "single-hit" heterozygous KEAPI mutation is sufficient to generate a selective advantage in the cancer microenvironment.

The stoichiometry of Keap1 is another critical aspect in understanding mechanistically how the dominant-negative effect can occur. As Keap1 heterozygous mice exhibit a benign phenotype (36), it was difficult to comprehend how a single KEAP1 mutation in a human cancer could lead to efficacious increases in chemoresistance. A key finding was the discovery that the ETGE and DLG motifs in the Nrf2-Neh2 region bind to Keap1 at two sites (37, 38), with a binding stoichiometry between Keap1 and Nrf2 of 2:1 (Fig. 6A). This two-site substrate-binding model led to the hypothesis that a Keap1mutant protein could sequester Keap1WT into an inactive heterodimer (Fig. 6B), leading to a functional loss of Keap1WT that could be 50% or more.

To demonstrate that a dominant-negative heterodimer was mechanistically possible, deletion of the entire BTB domain (ΔBTB) and a point mutation in a critical amino acid sequence (mutHKVVL) were conducted, and the resultant transgenes were placed in the double transgenic complementation system. The Keap1mutHKVVL protein failed to associate with Keap1WT, thus in essence creating a Keap1 protein with an inability to form a mutant homodimer (Fig. 6C). Although the BTB domain is essential for mediating Nrf2 degradation, a BTB domain mutant would theoretically not sequester existing Keap1WT. In this study, the Keap1mutHKVVL mutant had no effects on Keap1WT when coexpressed, and caused no observed phenotypic changes or increases in Nrf2 activity when compared with the Keap1G430C mutant. Interestingly, epidemiological evidence also supports the hypothesis that a BTB mutant has no real selective advantage, and few mutations in the BTB domain have been reported in tumors to date. These data strongly support the notion that the BTB domain is essential for dimerization and that sequestration of Keap1WT into a Keap1mutant–Keap1WT heterodimer leads to a dominant-negative effect in vivo.

Similar to the Keap1G430C mutant, the Keap1C273&288A also diminishes Keap1WT activity in vivo. C273 and C288 are critical reactive residues that respond to electrophiles, and sulfhydryl modification of these amino acids can cause changes in the tertiary structure of Keap1 that lead to loss of Nrf2 degradation (Fig. 6D), allowing Nrf2 levels to increase and drive
transcription of Nrf2-mediated target genes (37, 38). Keap1<sup>C273&288A</sup> mutants, in effect, mimic the electrophile-activated conformation of Keap1. Structural analysis revealed that in the Neh2 domain, which contains the Keap1-interacting DLG and ETGE motifs, flanks the central α-helix of Nrf2 containing 7 lysines. Keap1 targets these lysines to enable Cul3-mediated ubiquitination of Nrf2 (37) by locking the central α-helix of the Neh2 domain in a position suitable for ubiquitin ligation (37, 39, 40).

Thus, the current study demonstrates that the Keap1<sup>C273&288A</sup> mutants have a dominant-negative effect on Keap1<sup>WT</sup> activity, leading to hyperkeratosis and increased Nrf2 activity in a manner consistent with the Keap1<sup>G430C</sup> mutant. The data also add insight into the role of the Keap1<sup>WT</sup> homodimer, as a single cysteine modification at either 273 or 288 on either Keap1 protein may be enough to cause increased Nrf2 activity. This would mean that unmodified thiols on both Keap1 proteins are a requirement for repression of Nrf2 activity. Thus, the dominant-negative Keap1<sup>C273&288A</sup> mutants confirm the validity of the Keap1<sup>G430C</sup> model and add mechanistic insight into the signaling that occurs in response to electrophilic modification.

In conclusion, our results using the double transgenic complementation mouse models provide evidence for the presence of a dominant-negative effect in vivo that is analogous to heterozygous mutations that have been identified in human patients. Furthermore, heterozygous mutations in critical signaling cysteines (i.e., Keap1<sup>C273&288A</sup>), which are representative of the tertiary changes in Keap1 that occur by electrophilic cysteine oxidation also aid in the understanding of the mechanistic inactivation of Keap1 that leads to increases in Nrf2 activity. Finally, Keap1 mutants that disallowed Keap1–Keap1 interactions demonstrated that homodimerization was a requirement to elicit a dominant-negative effect. Taken together, these in vivo models provide a clear explanation on how a heterozygous, dominant-negative mutation in KEAP1 in cancer cells could lead to a selective advantage in the cancer microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Demonstration of Dominant-Negative Effect of Keap1 Mutants


Select Heterozygous \textit{Keap1} Mutations Have a Dominant-Negative Effect on Wild-Type Keap1 \textit{In Vivo}

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