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Succination of Keap1 and Activation of Nrf2-Dependent Antioxidant Pathways in FH-Deficient Papillary Renal Cell Carcinoma Type 2

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Fumarate hydratase (FH) is a tumor suppressor, but how it acts is unclear. Two reports in this issue of *Cancer Cell* reveal that FH deficiency leads to succination of Keap1, stabilization of Nrf2, and induction of stress-response genes including *HMOX1*, which is important for the survival of FH-deficient cells.

The fumarate hydratase gene (*FH*) encodes a TCA cycle enzyme and functions as a tumor suppressor gene. Heterozygous germline *FH* mutations result in hereditary leiomyomatosis and renal cell cancer (HLRCC), a syndrome characterized by smooth muscle tumors and papillary renal cell carcinoma type 2 (pRCC-2) (Tomlinson et al., 2002). In tumors, the wild-type *FH* allele is lost, and FH function is abrogated. pRCC-2 tumors in patients with HLRCC tend to metastasize early, and currently, there is no therapy.

How FH suppresses tumor formation is unknown. FH loss causes fumarate accumulation in tumor cells, and fumarate is a competitive inhibitor of 2-oxoglutarate-dependent prolyl hydroxylase domain-containing proteins (PHD) that hydroxylate HIFα. When hydroxylated, HIFα is recognized by the pVHL E3 ubiquitin ligase complex and is degraded. Because *VHL* is frequently mutated in renal cancer leading to HIF stabilization, a model whereby HIF is upregulated in pRCC-2 as a consequence of PHD inhibition by fumarate is attractive. However, the importance of PHD inhibition and HIF stabilization in the development of FH-deficient tumors remains unknown.

Keap1 is the substrate recognition subunit of a Cul3-based E3 ubiquitin ligase complex that regulates Nrf2, a pivotal transcription factor in the antioxidant response. Keap1 proteins dimerize through an N-terminal BTB domain, which through an intervening region (IVR) is linked to a C-terminal DC domain that contains a β-propeller made up largely of kelch repeats (Figure 1A). Two β-propellers in a Keap1 dimer interact with an Nrf2 monomer. Nrf2 contains two different Keap1-interacting motifs. Binding through both motifs is required for Nrf2 degradation, which led to a "hinge and latch" model (Hayes et al., 2010; Taguchi et al., 2011). Under normal conditions, Keap1 promotes Nrf2 ubiquitylation and proteosomal-mediated degradation. However, in the presence of electrophiles or reactive oxygen species, Keap1 is modified at several reactive Cys residues, resulting in Nrf2 stabilization and the activation of a protective gene expression program that includes *HMOX1*, an archetypal stress response gene (Hayes et al., 2010).

In this issue of *Cancer Cell*, Ooi et al. (2011) and Adam et al. (2011) show that FH loss results in Keap1 inactivation and Nrf2-dependent activation of antioxidant pathways. Through gene expression analyses, both groups discovered that FH deficiency was associated with increased expression of antioxidant genes, and this was accompanied by the accumulation of Nrf2. Reconstitution of FH-deficient

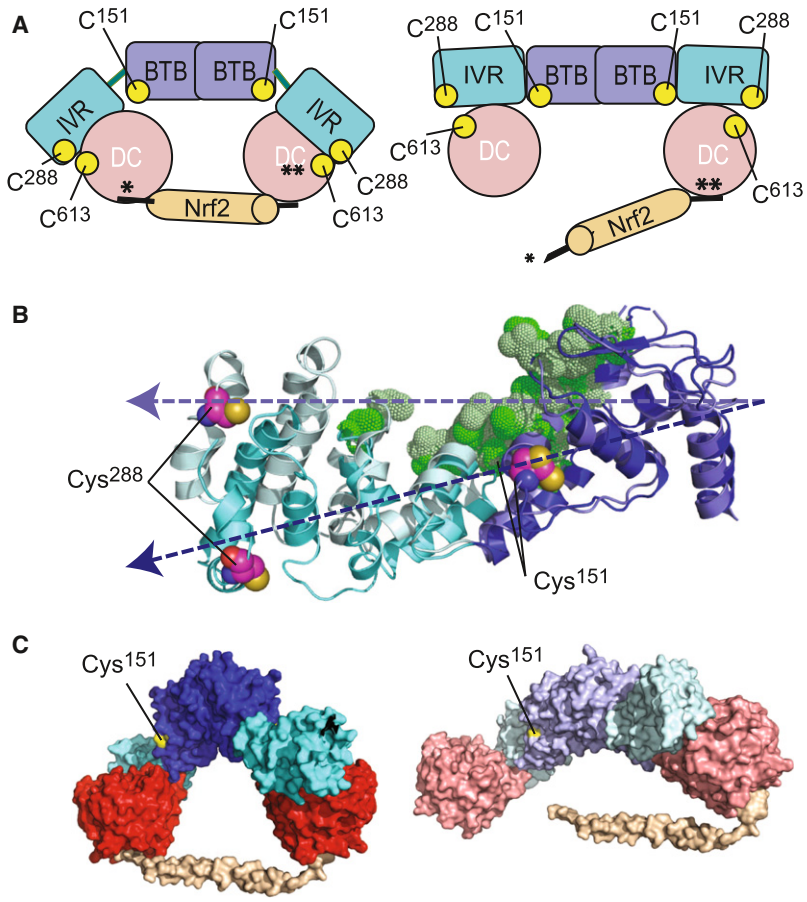

Figure 1. A Proposed Update of the Keap1 "Hinge and Latch" Model of Nrf2 Degradation

(A) Cartoon model of Keap1 dimer bound to Nrf2. In the active state (left), two DC domains are oriented to simultaneously bind Nrf2 high-affinity (**) and low-affinity (*) sites. Right panel illustrates the inactive state induced by Cys^{151} and Cys^{288} modification. According to our model, Cys^{151} modification causes rotation to a linear conformation and the release of Cul3. We propose that Cys^{288} modification misorients the DC domain with respect to the IVR.

(B) Potential structural context of proposed "hinge." Two Keap1-related structures with BTB and IVR domains have been reported: KLHL11 (PDB accession code: 3i3n) and Gigaxonin (PDB accession code: 3hve). These two proteins exhibit the same domain organization as Keap1: BTB and IVR followed by a domain made up of kelch repeats. KLHL11 (faded tones) differs from Gigaxonin (deeper colors) by a rotation of the IVR domain (cyan) with respect to the BTB domain (blue) (the two states are marked by arrows). We propose that Keap1 exists in two conformations corresponding to these two structures and that rotation from one to the other ("hinge") is brought about by Cys^{151} modification. The "hinge" motion causes a change in the putative Cul3-binding site (green dots), which we determined by superimposing a Cul1-bound BTB domain (PDB accession code: 1ldk). Thus, the hinge motion may be linked to Cul3 release.

(C) Potential structural context of proposed "latch." Left view is a surface representation of a hypothetical degradation competent, V-shaped Keap1 dimer interacting with both Nrf2-binding motifs. Right view is a surface representation of a hypothetical degradation incompetent, linear Keap1 dimer that cannot bind both Nrf2 motifs simultaneously. Keap1 DC domains (from PDB accession code: 2flu, red) were placed relative to the IVR domains (cyan) using the following considerations: (1) EM density pictures suggesting a general positioning of the DC domain with respect to the IVR; (2) identification of a conserved DC surface patch, which included Cys^{288}, that was placed near the IVR; and (3) maintaining the "latch" distance of Nrf2 DC-binding motifs located on either side of a helix (wheat). The orientation of the DC domain toward a position unfavorable for binding the Nrf2 low-affinity site (right) may be triggered by either modification of Cys^{151} resulting in a rotation from a V-shape (left) to a linear dimer (right), as described in (B), or modification of Cys^{288} resulting in a misorientation of the DC domain with respect to the IVR.

cells with wild-type FH or an extramitochondrial FH decreased fumarate levels and restored Nrf2 regulation (Adam et al., 2011; Ooi et al., 2011). Complementarily, a membrane-permeable fumarate ester was sufficient to induce Nrf2 (Ooi et al., 2011). Thus, Nrf2 regulation appeared to be linked to fumarate levels.

Pollard and colleagues (Bardella et al., 2011) had previously shown that in FH-deficient tumor cells, fumarate spontaneously reacts with Cys thiol groups in proteins to form *S*-(2-succinyl) Cys (2SC) adducts, and both groups asked whether Keap1 was succinated. Using an anti-2SC antibody, ectopically expressed Keap1 was found to be succinated in FH-deficient, but not wild-type cells (Adam et al., 2011). MS/MS analyses revealed succination at several Cys previously shown to be electrophile targets, including Cys^{151} and Cys^{288} (Adam et al., 2011; Ooi et al., 2011).

How Cys^{151} and Cys^{288} modification affects Keap1 remains an unresolved question (Hayes et al., 2010). Notably, whereas Ser substitution of Cys^{288} abolishes Keap1 function and stabilizes Nrf2, Ser substitution of Cys^{151} does not affect Nrf2 degradation. However, substitution of Cys^{151} by amino acids with progressively bulkier side chains causes progressive loss of Keap1 function, suggesting that the effect at this site is steric (Hayes et al., 2010).

Placing Cys^{151} and Cys^{288} residues in a structural context should help unravel their role. Our analysis of two Keap1-related structures (Zhuang et al. [2009]; see also Figure 1 legend) suggests that the IVR rotates with respect to the BTB domain, giving rise to either a linear or a V-shape dimer (Figure 1A). The rotation may affect the interaction with Cul3 because it causes a change in the conformation of a Cul3-binding "3-box" within the IVR (Zhuang et al., 2009) (see also Figure 1B). Interestingly, Cys^{151} is at the bending point, and we propose that succination induces the linear state and releases Cul3, resulting in Nrf2 stabilization (Figure 1C). Although the structural context of Cys^{288} is less clear, the IVR, where Cys^{288} is located, closely associated with the DC domain in a low-resolution electron microscopy model of Keap1 (Ogura et al., 2010). Interestingly, Cys^{288} was found to form part of a conserved surface patch that we postulate interacts with the DC domain and that placed Cys^{288} in close proximity with Cys^{613}. We propose that these two Cys stabilize the interaction between the IVR and DC domains either through a disulfide bond or metal coordination (together with Glu^{289} and His^{246}) and that modification of Cys^{288} (and Cys^{613}) abrogates this interaction (Figure 1A).

Ooi et al. (2011) report that Nrf2 target genes are induced also in sporadic pRCC-2. However, in contrast to familial

pRCC-2, mutations in *FH* have not been detected in sporadic pRCC-2, and sporadic pRCC-2 tumors do not accumulate succinated proteins (Bardella et al., 2011). Thus, how antioxidant genes are upregulated in sporadic pRCC-2 remains unclear.

The contribution of antioxidant genes to renal tumorigenesis in patients with HLRCC requires further exploration. However, there is precedent implicating Keap1 and Nrf2 in tumor development. Somatically acquired loss-of-function *KEAP1* mutations have been found in tumors (Taguchi et al., 2011). Nrf2 is also stabilized in tumors by mutation in either of the two motifs involved in Keap1 binding that are necessary for its degradation (Taguchi et al., 2011). Consistent with the notion that these two proteins function in concert, mutations in *KEAP1* and *NRF2* tend not to be observed together in the same tumor (Taguchi et al., 2011).

Understanding the biology of HLRCC-associated pRCC-2 will hopefully lead to new therapies. Because *FH* is lost in tumor cells, and this truncates the TCA cycle, these tumors may be exquisitely dependent on glycolysis for energy generation. As determined by 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) positron emission tomography (PET), FH-deficient tumors take up large amounts of glucose (Yamasaki et al., 2011). Although exploiting this dependency may be challenging (Yamasaki et al., 2011), this may offer an opportunity for therapeutic intervention.

Recently, HMOX1, which is required for heme synthesis and is upregulated in FH-deficient tumors, was proposed as a therapeutic target (Frezza et al., 2011). FH-deficient cells maintain segmental TCA cycle function and NADH generation by using glutamine and disposing of excess carbon through the synthesis of heme and its excretion from the cell as bilirubin (Frezza et al., 2011). Interestingly, inhibition of HMOX1 is synthetically lethal with FH deficiency. However, the selectivity of this approach in patients remains to be determined, particularly because HLRCC patients are heterozygous for *FH*, and mutant FH may be incorporated into FH homotetramers, markedly reducing FH activity in non-neoplastic cells. Nonetheless, this concept deserves to be studied further in primary xenografts and, subsequently, in patients.

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The Spliceosome as an Indicted Conspirator in Myeloid Malignancies

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Reports of whole-exome sequencing in myelodysplastic syndrome (MDS) patients by Yoshida et al. and Papaemmanuil et al. suggest spliceosome mutations have clinical relevance. Identifying the impact of these mutations on MDS pathogenesis holds promise for therapeutic modulation of mRNA splicing.

The myelodysplastic syndromes (MDSs) are a heterogeneous group of myeloid malignancies characterized by clonal hematopoiesis, impaired differentiation, peripheral blood cytopenias, and increased risk of progression to acute myeloid leukemia. Although recent studies have identified recurrent somatic mutations in most patients with MDS, approximately 20% of patients with MDS had no known somatic genetic or cytogenetic abnormalities in the largest studies to date. Two recent studies report the results of whole-exome sequencing in patients with MDS (Papaemmanuil et al., 2011; Yoshida et al.,