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Keap 1: the new Janus word on the block

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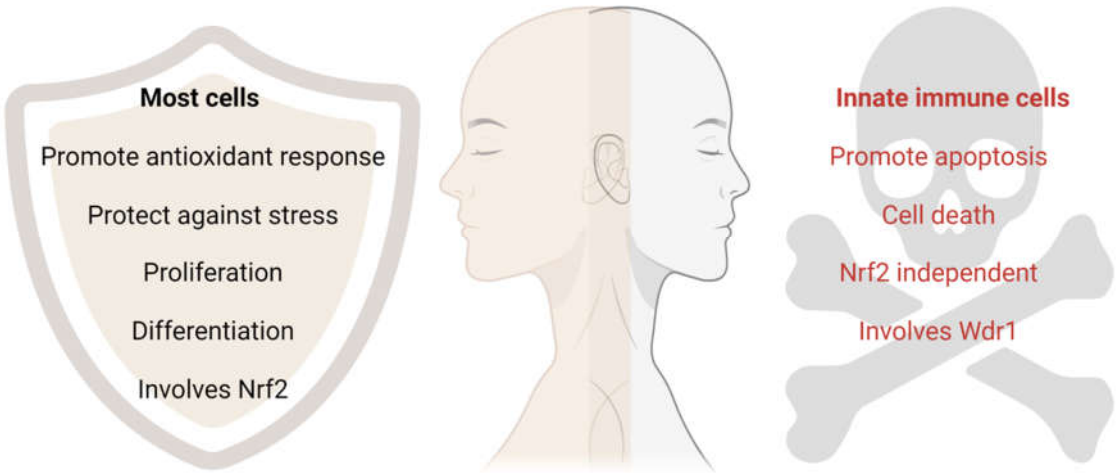
Abstract

Here we draw insights from the latest serendipitous findings made on the opposing roles of a validated drug-target protein Keap1. We weigh up how natural reactive electrophiles and electrophilic small-molecule drugs in clinical use directly impinge on seemingly conflicting, yet both Keap1-electrophile-modification-dependent, cell-survival- vs. cell-death-promoting behaviors. In the process, we convey how understanding reactive chemical-signal regulation at a single-protein-specific level is an enabling necessity in deconstructing otherwise intricate reactive-small-molecule-responsive cellular pathways. We hope this opinion piece further spurs the broader interests of basic and pharmaceutical research communities toward better understanding of molecular mechanisms underpinning reactive small-molecule-regulated signaling subsystems.

Key words: Electrophile signaling; drug mechanism; immunology; antioxidant response; apoptosis.

Table of Contents Graphic



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Keap on learning

History contains numerous allusions to the contradictions that arise in historical myths. One classic example is Iranian dualism, the concept that both good and bad are derived from eternity, concepts commonly debated in Zoroastrianism. Many contradictions are also manifest in cultural mythical figures, such as Tata Duende, a Mayan cultural spirit believed to have their feet pointing backwards, making them hard to track. Perhaps a more familiar exemplar is the Roman god of beginnings and endings, Janus, from whom the month January is derived. This figure was supposed to have two faces allowing them to see both forward and backward (in time). From this figure we also derive a phrase describing one of the key linguistic incarnations of contradictions: words which can convey the antonym of their conventional meaning. Good examples of such Janus words are peruse (which can mean both to browse and read thoroughly) and oversight (which can mean to watch over carefully, or to do the opposite). Along a similar theme, in this paper we will discuss our new findings on Keap1 and how it can function to promote viability, but also to promote apoptosis.

Keap fit?

Keap1's canonical role is to bind the transcription factor Nrf2 in the cytosol. This function anchors Nrf2 in the cytosol preventing activation of Nrf2's transcriptional program. Genes under the control of Nrf2 tend to be cytoprotective, particularly towards reactive small-molecule stress; they can also promote proliferation and differentiation[1]. Indeed, Nrf2 can upregulate a battery of detoxifying genes, falling into several different classes (detoxification, redox balance, protein quality control, metal homeostasis, metabolism, and inflammation among others); upregulation of such genes is called the antioxidant response (AR). Keap1 also functions as an adaptor of the E3 ligase, CUL3. This property of Keap1 aids to reduce the levels of Nrf2 in addition to preventing Nrf2 nuclear accumulation. Keap1's inhibition of Nrf2 is thus an essential function.[2]

Keap1 is also a particularly cysteine-rich protein. These cysteines perform an important stress-sensing function.[3] In the presence of oxidants and electrophiles, it is believed that Keap1 is electrophile- or oxidant-modified, and this modification leads to a loss of Keap1's Nrf2-binding ability, promoting Nrf2 accumulation and upregulation of Nrf2's transcriptional program. Thus, the cell is able to sense and respond to potentially-damaging small molecules.

Despite being long accepted, this particular model was experimentally difficult to validate due to the varied proteins that regulate Nrf2. Examples include the phospho-dependent E3 ligase

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β-TRCP, which can also degrade Nrf2;[4] and several kinases, such as protein kinase C, that can regulate Nrf2 nuclear import[5]. Furthermore, Nrf2 requires heterodimerization to function correctly in the nucleus.[6] All these processes could be regulated by reactive species and could contribute to reactive small-molecule-induced AR. Moreover, the precise reactive species that can cause AR were also unclear due to the fact that these molecules can interconvert, and promote production of other reactive species in vivo.[7]

Some years ago, we addressed this question directly using REX technologies. We specifically used T-REX, a technique that can deliver a specific electrophile to a specific, pre-elected protein, allowing the consequences of specific protein—specific electrophile engagement to be assayed in living systems.[8] Using a luciferase reporter to assess AR, western blotting for AR-upregulated proteins, or qPCR, we discovered that AR levels are similar between cells bulk-exposed to a quintessential native electrophilic stressor, 4-hydroxynonenal (HNE), and cells in which Keap1 was specifically labeled with HNE at a precise time, an event selectively enabled by T-REX. This result indicates that the vast proportion of AR induced upon HNE treatment is attributable to on-target HNEylation of Keap1.[9] These data put Keap1 right in the middle of the health promoting AR (**Figure 1**).

Keap off?

With several lines of evidence, including our own data above, indicating Keap1 performs a cytoprotective function, we were intrigued by the proposals that Keap1 is a principal target of the recently approved anti-multiple-sclerosis drug, Tecfidera. This is because Tecfidera is believed to function through immune suppression, and has been linked to neutropenia and lymphopenia.[10] These phenotypes are not linked to the traditional Nrf2 response, and indeed data indicate that the mechanism of Tecfidera is not dependent on Nrf2 as Nrf2 knockout mice still maintain drug-induced phenotypic responses following Tecfidera administration.[11] We set about addressing this mechanism using a variant of T-REX, called Z-REX, that can deliver HNE (in this case used as a proxy for Tecfidera) to Keap1 in the majority of cells in developing zebrafish embryos. Using this technique, we found that there was a significant loss of neutrophils and macrophages several hours after Keap1 is HNEylated in vivo. This loss was specific to innate immune cells. Intriguingly, the canonical Keap1-Nrf2-AR signaling cascade was also triggered in other tissues of the fish.[12]

We found that neutrophil and macrophage loss was inhibited by apoptosis inhibitors, and further involved upregulation of caspase-3 activity, which in fish localized to innate immune cells and

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was not present in other tissues post Z-REX. Experiments using T-REX, i.e., in cultured mammalian cells, showed that following on-target HNEylation of Keap1, there was a reduction in WDR1 binding to Keap1. Further experimentation implicated cofilin, a known interactor of WDR1 in this process, as well as players in the mitochondrial-targeted apoptosis pathway. When expression levels of many of these players were reduced by RNA interference or inactivated through mutation in zebrafish embryos, neither whole-animal Tecfidera nor HNE treatment was able to reduce the numbers of neutrophils or macrophages (compared to innate immune cell depletion otherwise observed in control embryos treated with Tecfidera or HNE). These data indicate that Tecfidera and HNE can trigger an apoptotic pathway by hitting Keap1 that proceeds through the release of WDR1 from Keap1 providing this occurs in specific cells (**Figure 1**). This mechanism appears to be important for the pharmaceutical program of Tecfidera.

Keap at it

Similar to how the examples we described in the introduction show our longstanding interest with opposite, these data together form a clear and quite striking example of the importance of mechanistic nuance in biology: on the one hand, Keap1 electrophilic modification alone is sufficient to trigger AR-pathway-dependent cytoprotective responses; on the other hand, Keap1 electrophile engagement may trigger death of cells essential for innate immune defense. It is interesting to speculate why a simple modification of Keap1 can lead to such different outputs in innate immune cells versus other cells. A simple hypothesis is that expression levels of WDR1 may be higher in innate immune cells and/or the susceptibility to WDR1 is higher in innate immune cells. The second proposal indicates that there could well be other cell-type-specific variations that impact responses to changes in Keap1-interactomes that marshal response to stress. This could readily be due to changes in expression or localization in downstream players, such as cofilin, or the presence of negative regulators hitherto not identified. Given the growing appreciation of mechanistic nuances in electrophile signaling, the manifest and potentially non-degenerate cysteines present on Keap1, and Keap1's growing roles in biology beyond AR, it is critically important that further investigations into Keap1 *on-target* signaling be performed using methods that can precisely perturb this important protein. It is further important that these be executed in cell-type divergent, and context-specific manners. In such a way, we are sure that this system will keep on producing surprises that could well be medicinally relevant.

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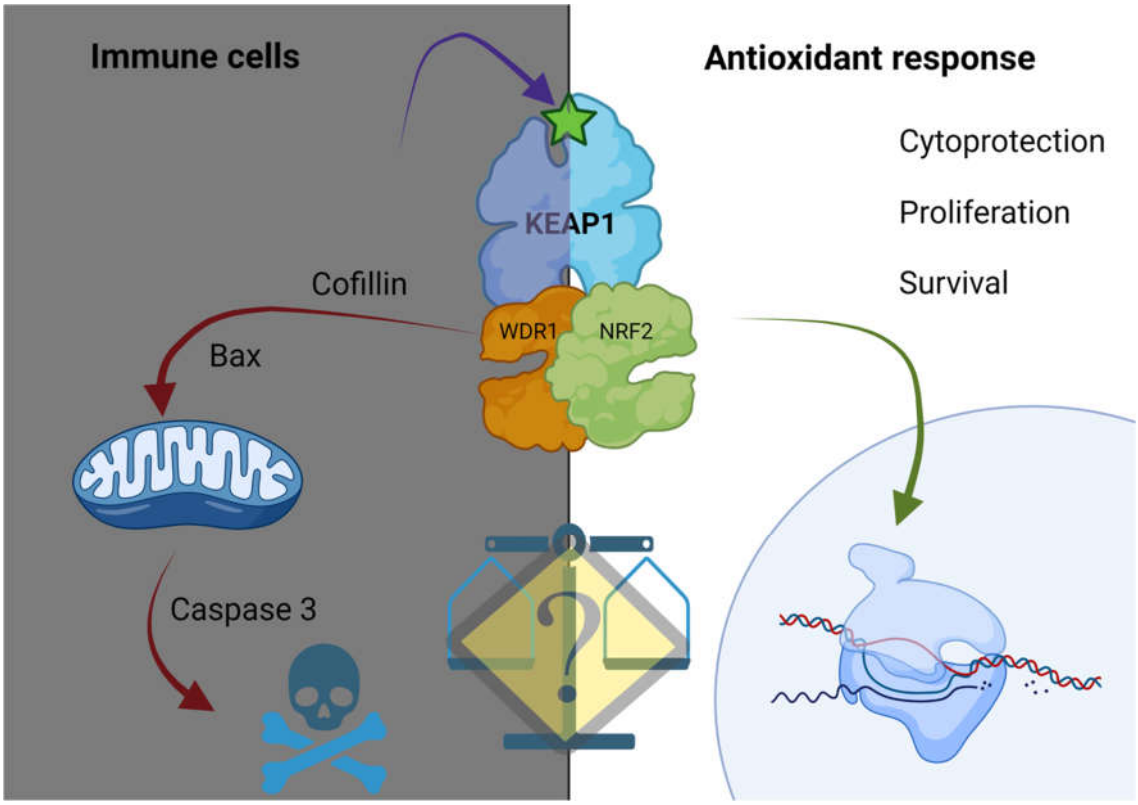

Figure 1. *The Janus role of Keap1 electrophilic regulation in cell survival vs. apoptosis.* ***Right panel:*** *the canonical Keap1-Nrf2 antioxidant response (AR) pathway activated by on-target electrophilic modifications of Keap1.* ***Left panel:*** *the newly-discovered Keap1-Wdr1 innate immune cell-specific mitochondrial-targeted apoptosis pathway promoted by on-target Keap1 electrophilic modifications. See text for the detailed discussions.*

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Conflict of Interest Small-molecule inhibitors derived from application of REX-technologies have been filed for patent applications by the authors' former institution, Cornell University (USA).

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