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Review

# Keap It in the Family: How to Fish out New Paradigms in Keap1-Mediated Cell Signaling

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**Abstract:** Keap1 is associated with cytoprotective signaling. These roles of Keap1 typically focus on its role as a degrader of the antioxidant response (AR) transcription factor, Nrf2, and the inhibition of this process upon Keap1 labeling by electrophiles. However, work from several laboratories has reemphasized both the important role of Nrf2 binding in negatively regulating AR, and the fact that electrophile Keap1 modification dissociates Nrf2 from Keap1. This model appears to be applicable to other signaling modes regulated by Keap1. Here we use recent data we have derived using experiments in zebrafish and cultured cells to discuss different models of Keap1 regulation. Chemical reactivity is one of the key parameters to consider when one first encounters a molecule. Organic chemists typically segregate molecules based on reactivity. A compound can be defined as electrophilic (willing to accept electrons) or nucleophilic (willing to donate electrons), for instance. Other classifications, such as Brønsted acid (willing to donate a proton) or Brønsted base (willing to accept a proton), follow a similar logic. Such parameters need to be tempered by considering the propensity of the specific functional groups within the molecule to undergo their stereotypical reactions: a molecule could be very nucleophilic, or mildly Brønsted basic, for example. Such relative scales are important when considering a chemical reaction, as the context — namely, how electrophilic, or acidic, etc., one molecule is relative to another — is crucial to predicting how a reaction will proceed. Such considerations are equally relevant to biological systems. Consistent with the need to maintain homeostasis, most of biological compounds discussed in biochemical textbooks, for instance, most amino acids, DNA, and the vast majority of cofactors and vitamins (including ATP), are *kinetically* stable in standard biological conditions. Nature has learned to selectively activate many such small molecules allowing selective functionalization and transformation, giving rise to incredibly complicated molecular architectures. Indeed, the simple fact that a small molecule is kinetically inert opens avenues for enzymatic catalysis, and can enable controlled generation of reactive intermediates. Indeed, many reactive small-molecular entities are transient intermediates – i.e., highly reactive species – along enzymatic reaction landscapes. These include metallo-complexes, phosphate esters, o-quinone methides, and radicals whose stability and reactivity need to be carefully managed by virtue of being surrounded by a polypeptide (or other inert biopolymeric) scaffold, i.e., within an active site. Generation of such high-energy intermediates free in solution would constitute a waste of energy, and would, based on several precedents, result in them reacting with numerous cellular components indiscriminately. Notably however, nature has also learned to accommodate several subclasses of intrinsically reactive small molecules that are freely diffusive. Unlike their aforementioned sheltered cousins, such free, reactive compounds pose several problems to biological systems. This is because they can (semi)-indiscriminately react with key biomolecules, changing function or information storage, potentially causing deleterious outputs. Oftentimes production of these free reactive species is elevated under stress, and/or they can be generated through xenobiotic/exogenous processes. Perhaps the two most well-recognized classes of freely-diffusive reactive small-molecules are reactive oxygen species (ROS) and reactive electrophilic species (RES). Although many RES and ROS are only moderately kinetically reactive with proteins and other biomolecules (second order rate constants for reaction of many enals and peroxide with cysteine are 1–10 M<sup>-1</sup>s<sup>-1</sup>) and others have very short half-lives in cells, several lines of evidence show that these molecules are biologically relevant when produced in low dosages and in specific spatiotemporal state of affairs, regulating context-specific signaling pathways. Perhaps the best piece of evidence for this is that specific proteins have evolved to ‘sense and respond’ to reactive molecules. The most well-characterized sensing pathway is detoxification. Such reactivity-deactivating processes serve to eliminate the threat of reactive species. Despite well-characterized clearance processes, direct protein labeling can also modulate protein function, and such processes have also been

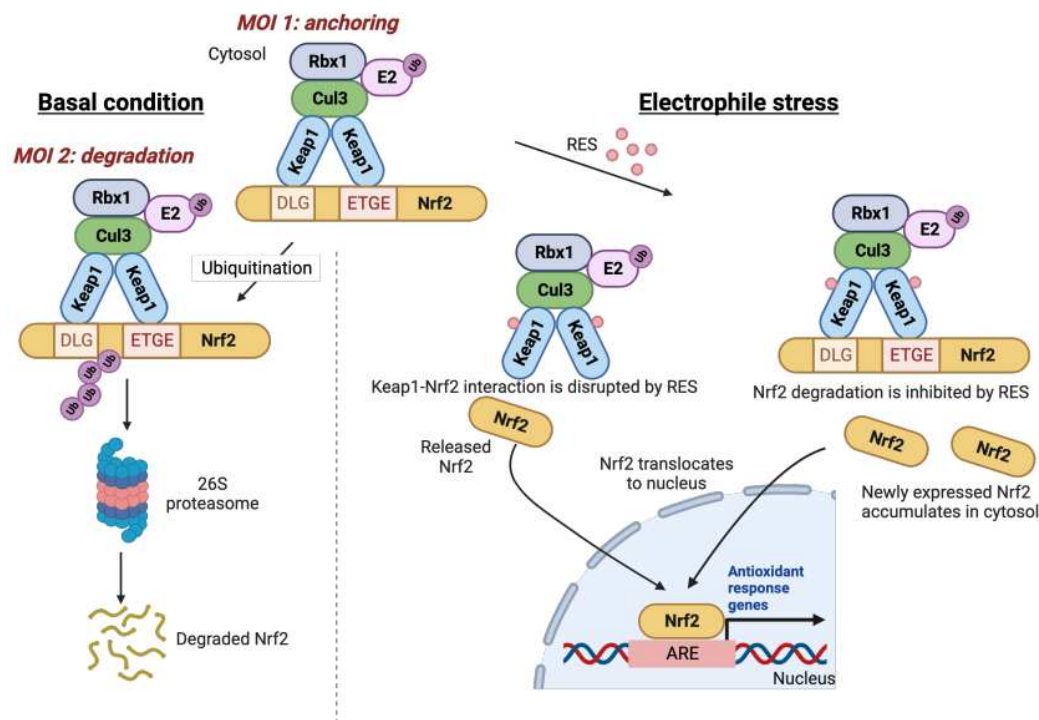
implicated in important evolutionary events essential for survival. Unfortunately, given the indiscriminate reactivity of many ROS and RES, it is highly challenging to provide precise and unequivocal proof that RES or ROS act directly on specific proteins under physiological conditions. In the context of ROS regulation, several antioxidant response systems appear to function through formation of disulfide bonds (often in processes mediated by detoxification enzymes, a process we have referred to as 'changing of hands'), or through formation of protein sulfenic acids. Of course, such conclusions heavily leverage mutagenesis and in vitro experimentation. Moreover, signaling occurs in the backdrop of an already complex system whose complexity is exacerbated due to build up of pleiotropic molecules that are interconvertible. Thus, there is arguably room for improved and expanded interpretation in all these data. Similar studies have been performed in RES research, with unavoidable limitations and challenges in data interpretations. Although, accumulating lines of evidence have demonstrated that under spatiotemporally-defined generation of RES in limited quantities, specific RES modifies specific proteins, leading to modulation of diverse cellular and organismal responses. Thus, our opinion remains that despite it being difficult to furnish unequivocal proof of direct ROS and RES signaling, the preponderance of evidence indicates that context-specific localized build up of reactive species modulates specific signaling pathways.

**Keywords:** Keap1; electrophile; RES; Nrf2; Wdr1

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### 1. Keap1/Nrf2, a (mostly) metazoan family affair crucial to protect against stress

Of the now plentiful examples of signaling pathways susceptible to target-specific RES regulation, including immune response, DNA damage, apoptosis, and transcription, one of the oldest known, the Keap1/Nrf2 pathway remains the best studied and most well-understood. In its simplest form, Nrf2 is a transcription factor that upregulates cytoprotective genes essential for safe-guarding against numerous reactive molecules. Keap1 is an inhibitor of Nrf2. In resting cells, Keap1 and Nrf2 form a cytosolic complex. This complex physically prevents Nrf2 from entering the nucleus [mode of inhibition (MOI) 1]. Moreover, when complexed to Nrf2, Keap1 can also promote Nrf2 degradation (MOI 2). Nonetheless, under conditions where cellular stresses are elevated with associated increased RES production, Keap1 can be modified ultimately augmenting Nrf2 expression, increasing Nrf2 transcriptional activity. The step-wise molecular events of Nrf2 signaling upregulation induced upon reactive small-molecule stress however remains unsettled. Several authors have proposed that Nrf2 is released from Keap1, boosting Nrf2 signaling, although a recent in vitro study indicates RES modification does not cause Nrf2 dissociation from Keap1.<sup>[1]</sup> Conversely, inhibition of Nrf2 degradation, and new synthesis of Nrf2 have also been proposed.<sup>[2]</sup> (Figure 1) Regardless of the intricacies, this pathway is almost entirely conserved between numerous non-vertebrate marine species, such as octopuses, as well as frog, fish, and higher vertebrates. Indeed, it is also apparent in relatively simple metazoans such as *Drosophila*, although *C. elegans* and hydra appear to lack a true analog of Keap1, and rely on other Nrf2 regulation methods.<sup>[3]</sup> Evidence for the necessity of the Keap1/Nrf2 pathway can be found from the fact that Keap1 homozygous knockout (KO) mice show severe hyperkeratosis and die in infancy; Nrf2/Keap1 double KO mice are viable.<sup>[4]</sup> Nrf2 KO mice alone are viable, but show severe issues in response to reactive small-molecule stresses.



**Figure 1. The Keap1-Nrf2-AR axis is regulated by RES.** Nrf2 anchors Keap1 through ETGE and DLG motifs, forming a complex in the cytosol [mode of inhibition (MOI) 1]. (*Left panel*) Under the *basal condition* (i.e., no electrophilic stress), Keap1 recruits Cul3, Rbx1, and E2, resulting in Nrf2 ubiquitination. The ubiquitinated Nrf2 is degraded by 26S proteasome (MOI 2). Nonetheless, under electrophilic stress with associated increase in the production of reactive electrophilic species (RES), RES covalently modifies Keap1 and disrupts Keap1's binding to Nrf2 (*middle panel*), leading to accumulation of free Nrf2 that can bind the antioxidant response element (ARE) within nuclear DNA, promoting transcriptional activation of numerous antioxidant response (AR) genes that defend the cell against electrophilic stress. Similar output of AR upregulation is elicited by RES modification of Keap1 inhibiting Nrf2 degradation (*Right panel*).

## 2. Duplication aids investigation of function

In our recent work,<sup>[5]</sup> we investigated the Keap1/Nrf2 system in zebrafish *D. rerio*. Zebrafish is a particularly interesting model system in which to study protein function due to a genome duplication that occurred in teleost fish, meaning that zebrafish contain two copies of many of their genes. This is indeed the case for Keap1 and Nrf2, which express isoforms labeled a and b. In the case of Keap1, on which we will focus here, Keap1b is often considered the paralog closer to human Keap1 than Keap1a, although there is overall good homology between both paralogs and the human protein (~50% identity for Keap1a and ~80% for Keap1b) (Figure 2a,b). In numerous instances, different paralogs have adopted tissue-specific functions, likely tailored to specific requirements of the particular tissues in which they are expressed.<sup>[6, 7]</sup> This is indeed often assumed in zebrafish biology. However, different zebrafish paralogs can also be expressed in the same tissues, although expression patterns over development may be different.<sup>[8]</sup> In our case, we found that both Keap1 paralogs are expressed in the head and the tail of the fish, albeit at different levels. Thus, both paralogs are likely present at least some of the time in the same tissues. Intriguingly, we found that knockdown of Keap1a and Keap1b caused disparate outcomes in terms of Nrf2 signaling in zebrafish embryos: when embryos were exposed to electrophilic stress, Keap1a depletion led to upregulation of Nrf2 signaling in both the head and the tail of zebrafish. The opposite was observed for Keap1b. These data are consistent with findings reported by others.<sup>[9]</sup> These data indicating a dampening effect of



RES-induced Nrf2 signaling by Keap1a and a stimulatory action by Keap1b under electrophilic stress, implied that the two paralogs of Keap1 in zebrafish have very different roles. We initially hypothesized that Keap1a and Keap1b sensed electrophiles differently, namely that Keap1a could not sense electrophiles whereas Keap1b could. However, further experiments leveraging precision electrophile-sensitivity interrogation tools revealed that this was not the case for these paralogs against several known Nrf2-inducing native RES. These outcomes indicated that another effect was at play.



**Figure 2.** Zebrafish Keap1a/b paralogs and human Keap1 exhibit highly-conserved amino-acid sequence, but their functions are dissimilar. (a) Keap1(C273) is conserved between human (h)Keap1 and zebrafish (z)Keap1b. In zKeap1b, the C273 (human ortholog numbering) residue is replaced by isoleucine. (b) Keap1 sequence is overall conserved among human and zebrafish orthologs, with ~50% sequence identity for zKeap1a and ~80% for zKeap1b (against human ortholog). The sequence alignment was performed with the Clustal Omega multiple sequence alignment program. (c) (left) Keap1 is an inhibitor of Nrf2 under basal condition, and introducing C273I to hKeap1 diminishes hKeap1's inhibitory function of Nrf2. However, under electrophilic stress, RES covalently modifies

Keap1, compromising wild-type (wt) hKeap1's regulation of Nrf2, but not hKeap1(C273I). (Right) zKeap1a functions with a similar mechanism as hKeap1(C273I), whose regulation of Nrf2 is not affected by RES. Nonetheless, zKeap1b's function is regulated by RES, similarly as in wt hKeap1. The detailed mechanism of action (MoA) of the two zKeap1 paralogs and their similarities in electrophile sensing versus differences in electrophile signaling have been reported recently.<sup>[5]</sup>

Additional experiments examining the two paralogs separately in cultured cells showed that despite being sensitive to electrophiles, electrophile modification of Keap1a does not allow RES-induced upregulation of Nrf2 signaling. However, Keap1b is permissive for this process. Such combination of permissivity and non-permissivity of RES-driven Nrf2 signaling could explain a large amount of our observations from fish, but not all. This is because if this model were true, knockdown of Keap1a should *not* increase RES-induced Nrf2 signaling. We thus postulated that Keap1a could function in a dominant-negative manner for RES-induced Nrf2 signaling, through the formation of Keap1a/b heterodimers. Indeed, when Keap1a was co-expressed with Keap1b in cultured cells, suppression of RES-promoted Nrf2 signaling was observed relative to Keap1b expression alone. Thus, Keap1a can prevent RES-driven Nrf2 signaling, even when the permissive paralog, Keap1b, is present. We next considered the mechanism by which such a process could function. In our hands, Nrf2 co-precipitated with both Keap1a and Keap1b paralogs, as well as a 1:1 mixture of the twain. However, only in the case of Keap1b, was there found to be a release of Nrf2 from Keap1(a/b) post electrophilic stress. This model is consistent with RES-induced Nrf2 signaling occurring through Nrf2 release that is possible in Keap1b, but not in Keap1a. Since Keap1b responds similarly to electrophiles as human Keap1, and this outcome is concordant with phylogenetic analyses, we searched for a residue that differs between Keap1b and Keap1a that may be responsible for the latter's non-canonical behavior. We narrowed down the search to cysteine residues in Keap1b that are mutated to hydrophobic residues in Keap1a. Through this method, we identified C273I as a potential candidate residue. Human Keap1(C273I) indeed showed similar dominant-negative properties for electrophile-triggered Nrf2 signaling (Figure 2).

### 3. Cul3 comes into the fold

Intriguingly, recently it has been reported by Yumimoto et al. in a very thought-provoking paper, that Keap1a (and orthologs) interacts better than Keap1b (and orthologs) with Cul3, the E3 ligase responsible for Keap1-mediated Nrf2 degradation.<sup>[10]</sup> This has also been proposed as an explanation for why Keap1a is less responsive to electrophile and oxidant stressors. Indeed, we observed less Nrf2 co-immunoprecipitating with Keap1a than Keap1b, which could readily be explained by such a mechanism. A simple amalgamation of both models leads to the hypothesis that Keap1a is so efficient at degrading bound Nrf2 that it cannot release enough Nrf2 to allow a boost in Nrf2 signaling upon electrophile exposure; Keap1b does build up Nrf2 so it can release Nrf2 upon electrophile labeling. However, in the absence of electrophilic stress, we showed that in a system where Keap1a and Keap1b were equally expressed, Nrf2 co-precipitating with the Keap1 ensemble was similar, albeit lower than Nrf2 co-precipitating with Keap1b alone. Even in that ensemble system, there was no release of Nrf2 upon whole-cell electrophile stress and Nrf2 signaling was compromised. These data indicate that Nrf2 release from electrophile-modified Keap1, is crucial for Nrf2 signaling, at least in the expressing system used in this study. In the case of Yumimoto et al.'s paper, even though an overexpression system was used, similar comparisons were unfortunately not investigated. However, interesting data for heterozygotic Keap1a-like/WT mice are reported, in addition to WT/WT and Keap1a-like/Keap1a-like mice. Although statistical comparisons between Keap1a-like/WT heterozygote and the two homozygotes were not explicitly made, in some instances, including most assays for response to oxidative stress, only modest differences between the heterozygote and the WT homozygote were observed. Such a situation might be explained by the fact that sufficient reduction of Nrf2 occupancy on Keap1 to suppress Nrf2 signaling upon stress can only be obtained in the Keap1a-like/Keap1a-like mutant homozygote. It should also be noted that Keap1a contains the C273I mutation that is known to affect Keap1 activity, and hence it is unlikely

that solely increasing affinity to Cul3 would be responsible for all the differences between Keap1a and Keap1b. Nonetheless, Keap1a’s increased affinity to Cul3 could explain, for instance, why human KEAP1(C273I) elicits reduced Nrf2-activity suppression, whereas Keap1a does not in the absence of electrophile treatment (Figure 2c).

Obviously, future studies of Keap1-mutants should involve careful evaluations of phenotypes associated with heterozygotic states (or co-expression systems) to enable a deeper understanding of specific proteins’ behaviors. Such insights are particularly important to assess the release versus degradation inhibition model of Keap1/Nrf2 signaling. Nonetheless, all data pointing to various subtleties and likely overlapping signaling mechanisms, much of the emerging data, including ours and that of Yumimoto et al., are at least in our opinion, more consistent with a model where RES-induced Keap1/Nrf2 signaling event is dominated by Nrf2 release. Indeed, if Nrf2 signaling upon electrophile stress were solely caused by inhibition of Nrf2 degradation, Keap1a should be more able to upregulate Nrf2 signaling than Keap1b that is less proficient at Nrf2 degradation. In fact, Keap1a is dominant negative for Nrf2 signaling relative to Keap1b.

4. Keap1 plays away from home

That being said, as underscored by studies from several independent laboratories, Keap1 does not only interact with Nrf2. Indeed, a large number of proteins (often those containing an ETGE motif, Table 1), associated with numerous different cellular functions, are Keap1 interactors. Several of these Keap1 interactions are increased upon electrophilic or reactive oxygen stress.<sup>[11]</sup> This has led various propositions that several proteins compete with Nrf2 for a similar Keap1 binding site,<sup>[12]</sup> although the response to stressors indicates that regulation of these interactions must be different from the canonical Nrf2/Keap1 interaction. How this could factor into endogenous electrophile signaling systems in a specific biological context is poorly understood. Of course such concerns are particularly relevant to tissue-/cell-type-specific signaling differences. To some extent such concerns are mitigated in many of the systems used to study Keap1 and Nrf2, as they use overexpression, winnowing the contribution of these competitors. However, such contributions may be severely misregulated in cancer cells, that are the most common system studied. Thus, Keap1-Nrf2-signaling / regulation is best assayed in close-to-physiological systems such as zebrafish and mice. This point further underlines the need to incorporate a broad range of model systems for better understanding of Keap1/Nrf2 signaling modalities.

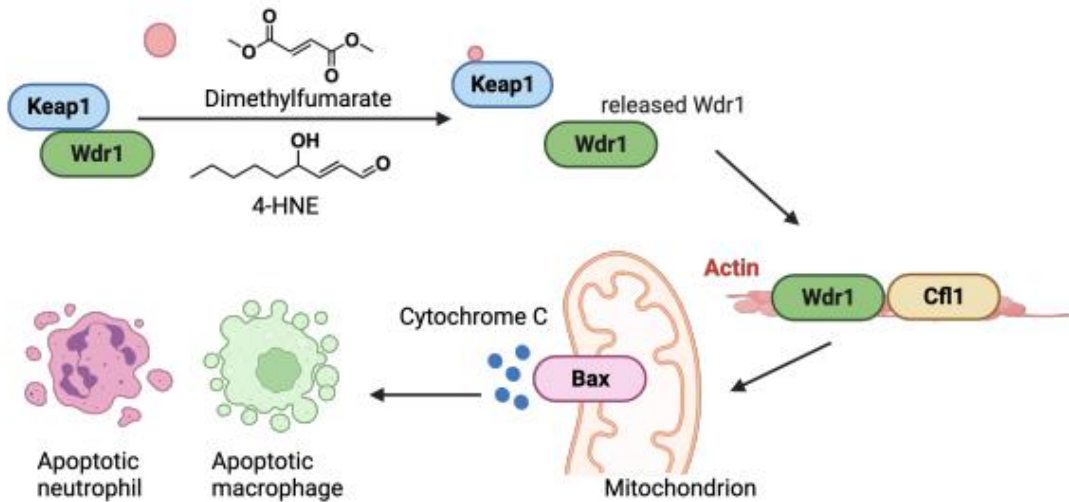
**Table 1. Selective Keap1 interactors and their binding motifs.** The E(T/S)GE motif is present in several proteins reported to interact with Keap1. All binders listed reportedly manifest functional responses as a result of binding.

Keap1 interactor	Binding motif	Functionally-relevant interaction
Nrf2 <sup>[15]</sup>	ETGE and DLG	Keap1 promotes Nrf2 degradation.
Nrf1 <sup>[16]</sup>		Keap1 stabilizes Nrf1.
MCM3 <sup>[17]</sup>	ETGE	Competes with Nrf2 for binding Keap1 and promotes antioxidant response.
PALB2 <sup>[18]</sup>		
DPP3 <sup>[19]</sup>		
CDK20 <sup>[20]</sup>		
FAM117B <sup>[21]</sup>		
WTX <sup>[22]</sup>	ENGE	
FAM129B <sup>[23]</sup>		
PTMA <sup>[24]</sup>		
SQSTM1 <sup>[25]</sup>		
HBXIP <sup>[26]</sup>		
BPTF <sup>[27]</sup>	unknown	
PPP1R13L <sup>[12]</sup>		

IKBKB <sup>[28]</sup>	ETGE	Keap1 promotes IKBKB degradation and subsequent NF-κB pathway activation.
PGAM5 <sup>[29]</sup>	ESGE	PGAM5-KEAP1-Nrf2 complex regulates stress-induced mitochondrial retrograde trafficking.
Wdr1 <sup>[13, 14]</sup>	ETGE	Keap1-Wdr1 interaction regulates apoptosis pathway in neutrophils/macrophages.
RELA <sup>[30]</sup>	N-terminal region	RELA associates with Keap1 and suppresses Nrf2.
Cul3 <sup>[31]</sup>	N-terminal region	Cul3 binds IVR domain of Keap1 and promotes Nrf2 ubiquitination.
EGFR <sup>[32]</sup>	unknown	EGFR regulates tyrosine phosphorylation of Keap1.
STK3 <sup>[33]</sup>	unknown	STK3 promotes phosphorylation of Keap1 and suppresses Nrf2 activity.

5. Nrf2 – the black sheep of the family?

Using a co-immunoprecipitation strategy comparing Keap1 associators before and after electrophile treatment, we recently uncovered that Keap1 can interact with Wdr1 in a manner that, similar to the interactions outlined above, appeared to be competitive with Nrf2. However, contrary to Nrf2-inhibitory proteins, the Keap1/Wdr1 interaction was decreased upon electrophile treatment. Wdr1 release from electrophile-modified Keap1 triggered apoptosis in immune cells in live zebrafish embryos (Figure 3). No apoptosis was detected elsewhere in other cells in the embryos. These data, that were used to explain how the approved multiple sclerosis drug Tecfidera, and presumably its emerging congeners such as Vumerity and Bafiertam, lower neutrophil counts, potentially explaining the mode of action of such drugs.<sup>[13]</sup> These findings also indicate that Keap1 electrophile engagement can radically rewire cellular signaling pathways in a cell-type-specific manner. Indeed, we identified several proteins in addition to Wdr1 whose Keap1 interaction was impeded by electrophile treatment. These proteins could play similarly important contextual roles in marshaling electrophile stress, through a unified electrophile-induced release mechanism. The role of Cul3 (affinity to /expression of) and other competing protein factors could readily help tune the context dependence of such signaling modes.



**Figure 3.** Dimethyl fumarate and 4-HNE induce neutrophil- and macrophage-specific apoptosis by disrupting Keap1-Wdr1 interaction. Upon covalent engagement with dimethyl fumarate or 4-HNE, Keap1 releases Wdr1, which later forms a complex with cofilin (Cfl1) and actin.<sup>[13]</sup> The Wdr1-Cfl1-actin complex upregulates cytochrome C released from mitochondria through Bax, resulting in



downstream apoptotic event in neutrophils and macrophages. Wdr1-Keap1 interaction reportedly occurs through the ETGE motif of Wdr1.<sup>[14]</sup>

## 6. Letting go to relieve stress

In sum, by (re)appreciating that at least to some extent Nrf2 signaling functions through Nrf2 release, we believe that new insights into Keap1-mediated regulation are possible. We do not believe that this is the sole way Keap1 can regulate its targets, but application of this postulate provides a simple screening regimen to identify candidates involved in specific responses to specific small-molecule stress stimuli. Regardless of how various models are reached, performing experiments in live model organisms as much as possible is often proven helpful, with strong back up from results in cell culture models. Furthermore, mounting evidence has shown that using approaches that trigger controlled protein-specific RES modification, in addition to conventional bulk-treatment procedures or approaches that broadly upregulate endogenous stress signaling, proffers new avenues to uncover such nuanced regulatory mechanisms. After all, in light of the (semi)-indiscriminate reactivity of RES as outlined in the beginning of our opinion piece, the unique advantage of precision tools – when deployed alongside appropriate suite of technical and biological controls – is unsurprising.

**Author Biographies:** Marcus J. C. Long was born in Yorkshire, UK. He was a chemistry scholar at Oxford University, and performed his PhD thesis research at Brandeis University, USA. He subsequently performed postdoctoral research in the Aye research group. He is now a senior postdoctoral researcher at the University of Lausanne. Kuan-Ting Huang was born and raised in Taiwan. He developed methods for chemical labeling of intact antibodies in his BSc and MSc study under the supervision of Prof. Chun-Cheng Lin, and received degrees in Chemistry from National Tsing Hua University. In 2019, he joined the Aye laboratory at EPFL, Switzerland, to study electrophile-regulated signaling pathways and ligand-protein interactions, and to pursue his PhD degree in chemical biology. Yimon Aye was born and raised in Burma. She studied Chemistry at Oxford University (2000-2004), and achieved her doctoral degree in Organic Chemistry at Harvard University in 2009. Her postdoctoral research at MIT focused on the mechanism and regulation of the enzyme ribonucleotide reductase. The Aye laboratory, established in mid-2012, pioneered the use of photocaged electrophiles (REX technologies) as a unique means to map and mine the precision ramifications of protein-electrophile modifications and ensuing signaling in multiple living systems. Contributions made by her laboratory (<https://leago.epfl.ch>) have been recognized by international accolades, both from the US and European research communities.

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