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*Published in:*  
Current Opinion in Toxicology

*DOI:*  
[10.1016/j.cotox.2016.10.003](https://doi.org/10.1016/j.cotox.2016.10.003)

*Publication date:*  
2016

*Licence:*  
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*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

*Citation for published version (APA):*

Hayes, J. D., Ebisine, K., Sharma, R. S., Chowdhry, S., Dinkova-Kostova, A. T., & Sutherland, C. (2016). Regulation of the CNC-bZIP transcription factor Nrf2 by Keap1 and the axis between GSK-3 and  $\beta$ -TrCP. *Current Opinion in Toxicology*, 1, 92-103. <https://doi.org/10.1016/j.cotox.2016.10.003>

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# Regulation of the CNC-bZIP transcription factor Nrf2 by Keap1 and the axis between GSK-3 and $\beta$ -TrCP

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**Abbreviations:** ARE, antioxidant response element; tBHQ, *tert*-butyl hydroquinone; bZIP, basic-region leucine zipper; CDDO-Im, 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; CNC, cap'n'collar; Cul1, cullin-1; Cul3, cullin-3; DEM, diethyl maleate; ECH, erythroid cell-derived protein with CNC homology; FBS, fetal bovine serum; GSK-3, glycogen synthase kinase-3; HMOX1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; MEFs, mouse embryonic fibroblasts; Neh, Nrf2-ECH homology; NQO1, NAD(P)H:quinone oxidoreductase-1; Nrf2, NF-E2 p45-related factor 2; NSCLC, non-small cell lung cancer; p70<sup>S6K</sup>, p70 ribosomal S6 kinase; p90<sup>RSK</sup>, p90 ribosomal S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphoinositide 3-kinase; PIP<sub>3</sub>, phosphatidylinositol (3,4,5)-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; ROS, reactive oxygen species; SCF, Skp1–Cul1–F-box; SFN, sulforaphane;  $\beta$ -TrCP,  $\beta$ -transducin repeat-containing protein;

**Key words:** Nrf2, Keap1, GSK-3, PKB/Akt, PI3K, PTEN,  $\beta$ -TrCP

## Abstract

The transcription factor NF-E2 p45-related factor 2 (Nrf2) mediates adaptation to oxidative stress by inducing cytoprotective genes including *heme oxygenase-1* (*HMOX1*) and *NAD(P)H:quinone oxidoreductase-1* (*NQO1*). Nrf2 is principally controlled by Kelch-like ECH-associated protein 1 (Keap1), which allows constitutive ubiquitylation and rapid degradation of Nrf2 by the cullin-3 (Cul3)-RING ubiquitin ligase CRL<sup>Keap1</sup> under non-stressed conditions. Simultaneously, glycogen synthase kinase-3 (GSK-3) also negatively controls Nrf2 through phosphorylation of a DSGIS-containing destruction motif in Nrf2, which then allows binding by  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) and ubiquitylation of the transcription factor by the Skp1–Cul1–F-box (SCF) ubiquitin ligase designated SCF <sup>$\beta$ -TrCP</sup>. It is well documented that oxidative stressors activate Nrf2 by antagonizing Keap1. We now show that both *tert*-butyl hydroquinone (tBHQ) and diethyl maleate (DEM), but not sulforaphane, induce *Hmox1* and *Nqo1* in *Keap1*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs). Moreover, expression of *Hmox1* and *Nqo1* in *Keap1*<sup>-/-</sup> MEFs is substantially blunted by inhibition of either phosphoinositide 3-kinase (PI3K, using LY294002) or protein kinase B (PKB)/Akt, using MK-2206), whereas inhibition of GSK-3 (using CT99021) induces expression of *Hmox1* and *Nqo1*. Herein, we provide evidence that Nrf2 is subject to repression by both Keap1 and the axis between GSK-3 and  $\beta$ -TrCP. One likely scenario is that loss of the phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>) 3-phosphatase activity of PTEN caused by tBHQ and DEM results in an increase in the levels of PIP<sub>3</sub> produced by phosphoinositide 3-kinase (PI3K), and hence 3-phosphoinositide-dependent protein kinase-1 (PDK1) activity, which then stimulates protein kinase B (PKB)/Akt signaling.

## Introduction

Yuet Wai Kan and colleagues first described NF-E2 p45-related factor 2 (Nrf2, encoded by *NFE2L2*) when they identified it as the third human cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factor to be cloned [1]. Shortly thereafter, Masayuki Yamamoto and colleagues reported an orthologous cDNA from chicken encoding a transcription factor that they called erythroid cell-derived protein with CNC homology (ECH) [2]. The protein is now designated Nrf2, rather than ECH. In mammalian species, Nrf2 regulates the expression of approximately 250 genes, each of which contains an antioxidant response element (ARE, 5'-TGACNNNGC-3') sequence in its regulatory region(s) [3,4].

Nrf2 is a short-lived transcription factor, but upon exposure to oxidative stressors it is stabilized, rapidly accumulates, and increases transactivation of ARE-driven genes [5]. The instability of Nrf2 protein is largely controlled by Kelch-like ECH-associated protein 1 (Keap1), a redox-sensitive substrate adaptor for the cullin-3 (Cul3)-based ubiquitin ligase CRL<sup>Keap1</sup> [6•,7•], and a prevailing view is that alleviation of the repression exerted by Keap1 on Nrf2 is entirely responsible for induction of ARE-driven genes by oxidative stressors. It is however much less widely appreciated that Nrf2 is also negatively regulated by glycogen synthase kinase-3 (GSK-3)<sup>1</sup>, and it has been proposed that inhibition of GSK-3 contributes to induction of ARE-driven genes [8•]. Critically, in this case, GSK-3 is active under normal homeostatic/basal conditions and it phosphorylates a destruction motif in Nrf2 that is recognized by  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) [9••], a F-box-containing protein that acts as a substrate adaptor/receptor within the Skp1-Cul1-F-box (SCF) ubiquitin ligase SCF <sup>$\beta$ -TrCP</sup> [10•,11•]. Through this joint enterprise between GSK-3 and  $\beta$ -TrCP, it is envisaged that inhibition of GSK-3 activity, ostensibly upon cell stimulation by insulin, growth factors or amino acids [12], diminishes ubiquitylation of Nrf2 by SCF <sup>$\beta$ -TrCP</sup>, thereby allowing the transcription factor to accumulate and transactivate ARE-driven genes.

In this 'opinion' article, we describe the dual regulation of Nrf2 by Keap1 and by the combined actions of GSK-3 and  $\beta$ -TrCP. In particular, we highlight the putative role played by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in triggering Keap1-independent activation of Nrf2. One **distinct possibility** is that loss of the phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>) 3-phosphatase activity of PTEN, stimulated by **certain** inducing agents, results in an increase in the levels of PIP<sub>3</sub> that is produced by phosphoinositide 3-kinase (PI3K). **As a consequence of increased PIP<sub>3</sub> levels**, 3-phosphoinositide-dependent protein kinase-1 (PDK1) activity **is augmented**, which then stimulates protein kinase B (PKB)/Akt

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<sup>1</sup> GSK-3 is represented by two isoenzymes, designated GSK-3 $\alpha$  and GSK-3 $\beta$ .

signaling [13]. In turn, the increase in PBK/Akt activity (ultimately resulting from PTEN inhibition) leads to phosphorylation of an *N*-terminal Ser residue in GSK-3 that causes loss of its kinase activity, and thus an increase in the abundance of Nrf2 and induction of ARE-driven genes. Through its ability to increase PDK1 activity, via production of PIP<sub>3</sub>, PI3K may also repress GSK-3 activity by stimulating p70 ribosomal S6 kinase (p70<sup>S6K</sup>), p90 ribosomal S6 kinase (p90<sup>RSK</sup>) and certain protein kinase C (PKC) isoforms [14-16], and the possible regulation of Nrf2 by these pathways that lie downstream of PI3K is also discussed briefly.

### **Positive regulation of gene expression by Nrf2**

#### **Nrf2 targets antioxidant and detoxication genes in normal cells**

In normal non-transformed cells, Nrf2 positively regulates the basal and/or stress-inducible expression of many ARE-driven antioxidant genes (Table 1, part (i)) as well as those involved in the generation of NADPH (Table 1, part (ii)) [17,18]. As a consequence, Nrf2 plays a unique role in controlling cellular redox homeostasis [19]. Moreover, because Nrf2 regulates basal and/or inducible expression of phase I and phase II drug-metabolism genes (Table 1, parts (iii) and (iv), respectively), as well as the ‘phase III detoxification’ efflux pumps (Table 1, part (v)), it contributes to intrinsic drug resistance and mediates induction of compensatory defenses against xenobiotics [20].

#### **Constitutive activation of Nrf2 in cancer cells increases anabolic metabolism**

In non-small cell lung cancer (NSCLC) cell lines in which repression of Nrf2 by Keap1 is lost as a consequence of somatic mutations in *Keap1* or *NFE2L2*, the resulting high levels of Nrf2 activity can increase anabolic metabolism if its up-regulation coincides with a growth stimulus, such as sustained activation of PI3K [21••]; PI3K pathway alterations in cancer are associated with somatic mutations in a number of oncogenes including *EGFR*, *HER2*, *MET* and *RAS* [22,23]. Under these circumstances, Nrf2 orchestrates overexpression of *G6PD*, *PGD*, *TKT* and *TALDO1* (involved in the pentose phosphate pathway), along with *PPAT* and *MTHFD2* (involved in purine synthesis), and results in glucose being redirected away from glycolysis towards ribose-5-phosphate and nucleotide production [21••]. In addition, such circumstances result in overexpression of *ME1* and *IDH1*, which along with *G6PD* and *PGD* are involved in generation of NADPH that is required for synthesis of macromolecules; these genes have all been reported to contain ARE sequences [21••]. High Nrf2 activity in NSCLC cells also results in the glycolytic intermediate 3-phosphoglycerate being channeled towards serine biosynthesis by increasing the activity of activating transcription factor 4 (ATF4), which in turn induces expression of *PHGDH*, *PSAT1*, *PSPH*, *SHMT1* and *SHMT2* [24•]. These

findings suggest that in cancer cells, constitutive up-regulation of Nrf2 in conjunction with activation of the PI3K pathway, increases macromolecule synthesis and promotes tumorigenesis.

### Repression of Nrf2 by Keap1

Because many xenobiotic inducers of NQO1 and GST enzyme activities contain a thiol-reactive electrophilic moiety (i.e. are soft electrophiles), Paul Talalay and colleagues predicted in 1988 that they would be recognized within the cell through reaction with sulfhydryl groups in a ‘sensor’ protein [25]. Thus following the discovery that Nrf2 mediates basal and/or inducible expression of *Nqo1* and *Gsta1* in mice [26•,27], it seemed possible that Nrf2 activity would be regulated through a ‘sensor’ protein that contains unique thiol groups.

Yeast two-hybrid screening enabled identification of Keap1 as a repressor protein that binds to the Nrf2-ECH homology (Neh)2 domain of Nrf2 [28•], and confers instability on Nrf2 [29]. In mammalian species, Keap1 contains about ten reactive Cys residues (i.e. with thiolate anion side-chain) because they are located adjacent to basic amino acids. Consistent with the hypothesis that Keap1 represents an electrophile ‘sensor’ protein, many agents that induce Nrf2-target genes form adducts with Cys-151, Cys-273, Cys-288, Cys-434 and Cys-613 in Keap1 [30•,31,32,33•]. Most importantly, the ability of inducing agents to modify these Cys residues is inversely associated with the ability of CRL<sup>Keap1</sup> to ubiquitylate Nrf2 [34•], and manipulation of the basic environment around Cys-151 in Keap1 diminishes the ability of inducing agents such as *tert*-butyl hydroquinone (tBHQ) and sulforaphane (SFN) to stabilize Nrf2 protein [33•]; see Figure 1 for structures of tBHQ and SFN. Using knock-in of mutant forms of Keap1 that retain substrate adaptor activity but lack individual electrophile sensors, it has been demonstrated that Cys-151, Cys-273 and Cys-288 recognize different inducers [35]. Besides forming adducts with xenobiotics, Cys-216 and Cys-613 in Keap1 can form an intramolecular disulfide bridge following exposure to H<sub>2</sub>O<sub>2</sub>, and this is thought to contribute to activation of Nrf2 by reactive oxygen species (ROS) [36,37]. Importantly, inducing agents stimulate conformational changes in the complex formed between Keap1 and Nrf2 that block ubiquitylation of the transcription factor by CRL<sup>Keap1</sup> [38].

### Control of Nrf2 via the combined actions of the PTEN–GSK-3 pathway and β-TrCP

#### Evidence that the PI3K–PKB/Akt–GSK-3 pathway regulates Nrf2

Jeffrey Johnson and colleagues first reported that the PI3K inhibitor LY294002 suppressed basal and tBHQ-stimulated Nrf2 activity in human IMR-32 neuroblastoma cells

[39•,40] but the observation was largely ignored because it could not be accommodated within the canonical 'Keap1-Nrf2 pathway'. The notion that PI3K contributes to Nrf2-mediated gene induction was confirmed by Antonio Cuadrado and colleagues, who showed that pre-treatment of rat PC-12 pheochromocytoma cells with LY294002 substantially attenuates the ability of carnosol to increase endogenous Nrf2 protein levels and induce *HMOX1* [41•]. Subsequently, Salazar et al [8•] demonstrated that PI3K increases Nrf2 activity by stimulating PKB/Akt, which in turn inhibits GSK-3. They also provided evidence, using ectopic expression of constitutively active and dominant negative mutants, that GSK-3 $\beta$  activity prevents nuclear accumulation of Nrf2. In this context, it should be noted that GSK-3 is active in cells under normal conditions, and PKB/Akt is one amongst a number of protein kinases that inhibit GSK-3 by catalyzing phosphorylation of Ser-9 and Ser-21 in GSK-3 $\beta$  and GSK-3 $\alpha$ , respectively [42•,43]; note, GSK-3 can also be inhibited by p70<sup>S6K</sup>, p90<sup>RSK</sup> and PKC [12,44], and all of these are regulated by PI3K through PDK1 [14-16,45,46].

Besides tBHQ and carnosol, other chemoprotective agents induce *HMOX1* and *NQO1* through an Nrf2-mediated mechanism that is sensitive to inhibition by LY294002. These include curcumin, ferulic acid, 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im)<sup>2</sup>, 4,17(20)-(cis)-pregnadiene-3,16-dione (*E*-guggulsterone) and nordihydroguaiaretic acid (see Figure 1 for structures of inducers) [47,48,49•,50,51]. By analogy with carnosol [8•], it seems plausible that these inducers also stimulate PKB/Akt and inactivate GSK-3. This prediction is not however supported by Lee et al [39•], who reported that treatment of IMR-32 cells with tBHQ did not activate PKB/Akt or increase inhibitory phosphorylation of GSK-3 whereas treatment with insulin did. By contrast, treatment of mouse embryonic fibroblasts (MEFs) with tBHQ has been reported to stimulate PKB/Akt and cause phosphorylation of GSK-3 $\alpha/\beta$  at Ser-21/9 [52]. It is not known whether this discrepancy is due to experimental differences or to intrinsic properties of IMR-32 cells and MEFs.

#### Evidence that PTEN and DJ-1 are 'sensor' proteins for agents that induce Nrf2-target genes

It is uncertain why xenobiotics that increase ARE-driven gene expression in a LY294002-sensitive manner are dependent on PI3K for their inducer activity. However, as a biotinylated analogue of CDDO-Im has been shown to activate the PI3K–PKB/Akt pathway by modifying covalently Cys-124 in the active center of PTEN [49•], it is possible that other LY294002-sensitive inducers of Nrf2-target genes similarly inhibit the PIP<sub>3</sub> 3-phosphatase activity of PTEN and in so doing stimulate Nrf2 activity via the PI3K–PKB/Akt–GSK-3 pathway. Consistent with this idea, the ability of *E*-guggulsterone to increase Nrf2 protein

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<sup>2</sup> CDDO-Im has also been called Bardoxolone.

abundance and induce *HMOX1* in MCF-10A cells is substantially attenuated by forced overexpression of a PTEN<sup>C124S</sup> mutant [50]. Moreover, 4-hydroxynonenal, which has been reported to induce Nrf2-target genes, is also capable of modifying Cys-71 in PTEN [53]. Within cells, the Cys-71 and Cys-124 residues in PTEN form a disulfide bridge upon treatment with H<sub>2</sub>O<sub>2</sub> and peptide growth factors, causing inactivation of the PIP<sub>3</sub> 3-phosphatase and an increase in PKB/Akt activity [13]. It is therefore reasonable to suppose that modification of Cys-71 and/or Cys-124 in PTEN will inhibit its phosphatase activity. The hypothesis that PTEN acts indirectly to repress Nrf2 is supported by the findings that the activity of Nrf2 is suppressed in MEFs that overexpress PTEN and also in PC-12 cells that ectopically express a PTEN-estrogen receptor fusion protein that can be activated by 4-hydroxytamoxifen [54]. Thus like Keap1, PTEN may serve as a ‘sensor’ of electrophiles and oxidative stressors within cells.

The proposed ‘sensor’ role for PTEN that controls Nrf2 via the PI3K–PKB/Akt–GSK-3 pathway raises the possibility that the Parkinsonism-associated protein DJ-1 attenuates this mode of regulation because it is a multifunctional protein that negatively controls PTEN [55,56]; the functions of DJ-1 include atypical peroxiredoxin-like peroxidase activity [57] and metal binding [58]. Most importantly, knockdown of DJ-1 has been reported to suppress Nrf2 activity, but as this was discovered before the concept that the PI3K–PKB/Akt pathway regulates Nrf2 gained traction, it was thought that DJ-1 increases Nrf2 activity by antagonizing Keap1 [59]. It now seems more likely that DJ-1 dampens the ability of PTEN to prevent the PI3K–PKB/Akt pathway from stimulating Nrf2 activity, rather than it antagonizing Keap1. An interesting point is that DJ-1 contains some reactive thiol groups that can be modified by ROS, including Cys-46, Cys-53 and Cys-106 [60], with Cys-46 and Cys-53 being susceptible to S-nitrosylation [61], and so it too might act as a ‘sensor’ protein. Intriguingly, DJ-1 may act as an adjunct ‘sensor’ by transferring nitric oxide from Cys-106 in DJ-1 onto Cys residues in PTEN, in a manner that results in loss of the PIP<sub>3</sub> 3-phosphatase activity of the latter protein [62].

#### Repression of Nrf2 by GSK-3 through the actions of β-TrCP

It was initially proposed that repression of Nrf2 by GSK-3 involves nuclear exclusion of the transcription factor [8•], possibly following its phosphorylation by Fyn (downstream of GSK-3) and/or increased turnover of the transcription factor by Keap1 [63,64]. However, in a separate line of investigation, deletion analyses indicated that two regions within the Neh6 domain of Nrf2 conferred Keap1-independent instability on the transcription factor [65•]. Further investigation revealed that a DSGIS motif in the Neh6 domain (amino acids 334-338 in mouse Nrf2, and amino acids 343-347 in human Nrf2) is phosphorylated by GSK-3, and thereupon is bound more avidly by β-TrCP [9••,66,67•]. Thus rather than evoke nuclear



exclusion as the mechanism by which GSK-3 suppresses Nrf2 activity, the current model posits that under non-stressed conditions GSK-3 constitutively phosphorylates the DSGIS-containing degron in Nrf2, and in so doing allows continuous targeting of the transcription factor for proteasomal degradation by  $\beta$ -TrCP.

#### Selective and synergistic effects of PTEN and Keap1 on Nrf2-target gene expression

Hepatocyte-specific disruption of the *Pten* gene has little effect on expression of Nrf2-target genes in mouse liver. By contrast, hepatocyte-specific disruption of *Pten* in conjunction with hepatocyte-specific disruption of *Keap1* increases synergistically the overexpression of Nrf2-target genes in mouse liver when compared with hepatocyte-specific disruption of *Keap1* alone. Most obviously, the increase in expression of metabolic genes regulated via Nrf2 such as *G6pdx*, *Mthfd2*, *Pgd* and *Tkt* is pronounced in livers of *Pten*<sup>F/F</sup>::*Keap1*<sup>F/F</sup>::*Alb-Cre* double knockout mice, but the expression of these genes is only modestly increased in livers of *Pten* or *Keap1* single knockout mice [21••]. Also, overexpression of the prototypic antioxidant genes *Gclc*, *Gpx2* and *Nqo1*, which was obvious in livers of *Keap1*<sup>F/F</sup>::*Alb-Cre* single knockout mice, was increased further in hepatocyte-specific *Pten*<sup>F/F</sup>::*Keap1*<sup>F/F</sup>::*Alb-Cre* double knockout mice but the relative increase in expression was nowhere near as profound as the increase in expression of the metabolic genes [21••,68].

The above findings indicate that both Keap1 and Pten repress Nrf2 in mouse liver. Based on the fact that loss of Pten alone in hepatocytes does not substantially increase expression of prototypic members of the ARE-gene battery, a case could be made that repression of Nrf2 by Keap1 in mouse liver is dominant over its repression by Pten. This interpretation is however difficult to reconcile with the finding of many investigators that inhibition of PI3K by LY294002 suppresses basal and/or inducible ARE-driven gene expression in many cells including IMR-32, PC12, VSCM, ARPE-19, HUVEC and MCF-10A [39•,41•,47,48,49•,50]. Moreover, LY294002 and the PKB/Akt inhibitor MK-2206 diminish substantially Nrf2 activity in cells that lack functional Keap1, such as *Keap1*<sup>-/-</sup> MEFs and A549 cells [67•]. Clearly the experiments showing that inhibition of PI3K or PKB/Akt suppresses Nrf2 activity are based on *ex vivo* cell culture experiments. Further work is therefore required to establish the relative importance of Keap1 and PTEN in suppressing basal and inducible activity of Nrf2 *in vivo*, and an obvious place to start would be to compare their roles in mouse liver with that in extrahepatic tissues.

#### **Certain, but not all, inducers that activate Nrf2 antagonize both Keap1 and GSK-3**

Evidence suggests that tBHQ may simultaneously inhibit the Keap1-directed ubiquitylation of Nrf2 and the phosphorylation of Nrf2 by GSK-3 [52]. It is not however clear

whether other inducing agents antagonize both Keap1 and GSK-3. To examine this possibility, and test whether antagonism of GSK-3 entails activation of PI3K and PKB/Akt, we treated *Keap1*<sup>-/-</sup> and *Keap1*<sup>+/+</sup> MEFs with tBHQ or diethyl maleate (DEM), with the latter chosen because it is a potent inducing agent that has been employed in gene expression profiling and chromatin immunoprecipitation combined with high-throughput DNA sequencing [4,28<sup>•</sup>]. As anticipated, immunoblotting showed that the basal level of mRNAs for both *Hmox1* and *Nqo1* is higher in *Keap1*<sup>-/-</sup> than *Keap1*<sup>+/+</sup> fibroblasts. Treatment of *Keap1*<sup>+/+</sup> fibroblasts with tBHQ (Figure 2A) or DEM (Figure 2B) induced both *Hmox1* and *Nqo1*. Whilst induction was less obvious in *Keap1*<sup>-/-</sup> than *Keap1*<sup>+/+</sup> fibroblasts, both tBHQ and DEM increased *Hmox1* and *Nqo1* mRNA in *Keap1*<sup>-/-</sup> MEFs. Interestingly, the PI3K and PKB/Akt inhibitors, LY294002 and MK-2206, diminished both basal and inducible *Hmox1* and *Nqo1* mRNA levels in both wild-type and mutant MEFs. By contrast, the GSK-3 inhibitor CT99021 increased basal levels of *Hmox1* and *Nqo1* mRNA in both *Keap1*<sup>+/+</sup> and *Keap1*<sup>-/-</sup> MEFs, which is consistent with previous results using the GSK-3 inhibitors LiCl and PDZD-8 [8<sup>•</sup>]. This is an interesting finding because, unlike typical inducing agents, CT99021 is not a soft electrophile. It is also notable that CT99021 did not significantly increase the expression of *Hmox1* or *Nqo1* in *Keap1*<sup>-/-</sup> MEFs treated with tBHQ above the elevated level stimulated by the inducing agent, but this was not the case for DEM.

Like tBHQ and DEM, treatment of *Keap1*<sup>+/+</sup> MEFs with SFN increased the abundance of *Hmox1* and *Nqo1* mRNA, and this was blunted by LY294002 and MK-2206 (Figure 2C). However, unlike the situation with tBHQ and DEM, treatment of *Keap1*<sup>-/-</sup> MEFs with SFN did not increase further the elevated basal levels of *Hmox1* or *Nqo1* mRNA in the mutant fibroblasts; similar results have been observed by workers in the laboratory of Antonio Cuadrado<sup>3</sup>. This implies that certain inducing agents inhibit Keap1 but not GSK-3.

### Concluding comments and future directions

This article provides an overview of the mechanisms by which Nrf2 is repressed by CRL<sup>Keap1</sup> and by GSK-3. We have also presented evidence that tBHQ and DEM can induce *Hmox1* and *Nqo1* through a Keap1-independent mechanism, and that the GSK-3 inhibitor CT99021 can also stimulate Keap1-independent induction of *Hmox1* and *Nqo1*. We speculate that inducing agents may modify Cys-46, Cys-53 and Cys-106 in DJ-1, and/or Cys-71 and Cys-124 in PTEN, and in so doing cause inhibition of GSK-3 by increasing PIP<sub>3</sub>-based PDK1 signaling that results in activation of PKB/Akt, thereby preventing formation of the DSGIS-

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<sup>3</sup> Personal communication from Professor Antonio Cuadrado and Dr Ana I Rojo.

containing phosphodegron in Nrf2 (Figure 3). The possibility that the positive regulation of Nrf2 by PI3K via GSK-3 might be mediated, at least in part, by kinases other than PKB/Akt, such as p70<sup>S6K</sup>, p90<sup>RSK</sup> and certain PKC isoforms [14,16], warrants investigation.

As both soft electrophiles and growth stimuli increase Nrf2 activity, it might be anticipated that the two classes of activator result in co-induction of antioxidant/detoxication and anabolic metabolism genes. However, genetic experiments suggest that antagonism of repression of Nrf2 by Keap1 results in induction of a distinct battery of genes from that resulting from antagonism of the PIP<sub>3</sub> 3-phosphatase activity of PTEN. Specifically, disruption of both *Keap1* and *Pten* in mouse hepatocytes results in the marked transactivation of metabolic genes such as *G6pdx*, *Mthfd2* and *Pgd*, whereas disruption of either *Keap1* or *Pten* alone does not induce these metabolic genes: knockout of *Keap1* alone increased modestly the expression of *Me1* and *Tkt* whilst increasing significantly the expression of *Gpx2* and *Nqo1*, whereas knockout of *Pten* alone increased very modestly the expression of *Me1* and *Tkt* but did not increase the expression of *Gpx2* or *Nqo1* [21••,68]. The molecular basis for this selectivity in induction of Nrf2-target genes upon inhibition of Keap1 and/or PTEN requires further study.

An important area that needs to be addressed in future concerns the priming of Nrf2 for phosphorylation by GSK-3. In particular, GSK-3 substrates usually have to be phosphorylated (i.e. primed) by another protein kinase before GSK-3 is able to phosphorylate them [69•]. Further work is required to identify protein kinases that prime Nrf2 for phosphorylation by GSK-3.

### **Acknowledgements**

We thank Professor Masayuki Yamamoto for providing the MEFs used in this study, and for his advice. We also thank Professor Antonio Cuadrado for invaluable discussions and for sharing with us data about the inability of sulforaphane to stimulate Keap1-independent gene expression. We gratefully acknowledge the Medical Research Council for their financial support (MR/N009851/1).

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- of outstanding interest

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## Graphical Abstract

The cartoon depicts the approximate increases in Nrf2 activity, and the resulting induction of antioxidant and detoxication cytoprotective genes (such as *Gclc*, *Gclm*, *Gpx2*, *Hmox1*, *Nqo1*, *Srxn1* and *Txnrd1*) compared with metabolic genes (such as *G6pdx*, *Me1*, *Mthfd2*, *Pgd* and *Tkl1*), that are observed in the mouse upon pharmacological inhibition of Keap1 and Pten by *tert*-butyl hydroquinone (tBHQ), or disruption of the *Pten* gene alone, or disruption of both *Keap1* and *Pten* genes. The relative size of the arrows has been chosen to provide a rough estimate of the amount of Nrf2 protein targeted for proteasomal degradation, as opposed to the amount of Nrf2 translocated to the nucleus to support ARE-driven gene expression (or stimulate other indirect gene transactivation), and also the strength of repression through Keap1 and the PTEN–PI3K–PKB/Akt–GSK-3 pathway.

## Table 1. Genes positively regulated by Nrf2 in mouse and the human

(see attachment)

This table has been compiled from information contained in references 3, 4, 17 and 18. Please note that in the column designated ‘Name of gene’, the abbreviation ‘p’ (against genes *CYP1B1*, *CYP2B9*, *GSTA1*, *GSTM1*, *GSTP1*, *UGT1A1* and *UGT2B7*) signifies ‘polypeptide’. Under the column designated ‘Function of gene product’ the abbreviations are as follows: 4-HNE, 4-hydroxy-2-nonenal; PG, prostaglandin; PAH, polycyclic aromatic hydrocarbon. Under the column designated ‘Species’, m signifies mouse, and h signifies human.

## FIGURES

### Figure 1. Structures of xenobiotics that activate Nrf2.

Agents that have been reported to induce Nrf2-target genes in a LY294002-sensitive manner include: i) *tert*-butyl hydroquinone (tBHQ); ii) carnosol; iii) curcumin; iv) ferulic acid; v) 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im); vi) 4,17(20)-(*cis*)-pregnadiene-3,16-dione (*E*-Guggulsterone) and vii) nordihydroguaiaretic acid. Other agents that have been widely used to induce Nrf2-target genes include: viii) sulforaphane and viii) diethyl maleate (DEM).

**Figure 2. Inhibition of GSK-3 suppresses basal and inducible expression of Nrf2-target genes by xenobiotics.**

Petri-dishes (60 mm) were seeded with *Keap1*<sup>+/+</sup> and *Keap1*<sup>-/-</sup> MEFs, and grown for about 36 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS until they reached 70% confluence. Thereafter, they were maintained in DMEM containing low serum (0.5% FBS) for 16 h, before being transferred to fresh medium containing 0.5% FBS and treated with 50  $\mu$ M tBHQ, 100  $\mu$ M DEM, or DMSO vehicle control for either 6 hr or 12 hr, to measure Hmox1 or Nqo1 mRNA, respectively. In some instances, the fibroblasts were pre-treated for 60 min with 10  $\mu$ M LY294002, 5  $\mu$ M MK-2206 or 5  $\mu$ M CT99021 immediately prior to transfer to fresh medium to which was added the relevant kinase inhibitor along with tBHQ, DEM or DMSO for either 6 hr or 12 hr as required. Whole cell lysates were prepared from *Keap1*<sup>+/+</sup> and *Keap1*<sup>-/-</sup> MEFs that had been treated with tBHQ, DEM or DMSO vehicle control. Hmox1 and Nqo1 mRNA levels were measured by TaqMan qRT-PCR using standard methods and using Actin mRNA as an internal reference control. (A) Results for Hmox1 and Nqo1 mRNA from MEFs that had been treated with DMSO alone are shown in open bars with white background; those from MEFs pre-treated with LY294002 (abbreviated to LY) are shown in diagonally cross-hatched bars on white background; those from MEFs pre-treated with MK-2206 (abbreviated to MK) are shown in spot-containing bars on white background; those from MEFs pre-treated with CT99021 (abbreviated CT) are shown in chequered bars on white background. Data from MEFs pre-treated with DMSO, LY, MK or CT that had then been treated with 50  $\mu$ M tBHQ are depicted in similarly shaded bars but on a light grey background. (B) Results for MEFs treated with 100  $\mu$ M DEM are set out as for panel A.

(C) Results for MEFs treated with 5  $\mu$ M SFN are set out as for panel A. In panels A, B and C, results that are significantly higher than the DMSO vehicle control with *p*-values < 0.01 or <0.001 are indicated with double (\*\*) or triple (\*\*\*) asterisk signs, respectively, and results that are significantly lower than the DMSO vehicle control with *p*-values <0.01 are indicated with double (\$\$) signs; ns = not significant. Also, results from MEFs pre-treated with LY, MK or CT before treatment with the tBHQ, DEM or SFN inducers that are significantly lower or higher than those for MEFs treated with the inducer alone (i.e. without pre-treatment with kinase inhibitor) are indicated similarly. Statistical analysis was performed using a two-way Anova.

**Figure 3. Stimulation of Nrf2-mediated induction of ARE-driven gene expression by oxidative stressors as well as growth stimuli.**

The transactivation activity of Nrf2 is increased by: i) redox perturbation and ii) growth and/or metabolic stimuli. In the first case (see upper left-hand side of cartoon) soft electrophiles can adduct to Cys-151, Cys-273, Cys-288 or Cys-434 [30-35], or excess levels of ROS can cause formation of an intra-molecular disulfide bridge within Keap1 between Cys-226 and Cys-613 (indicated by dotted line) [36,37], and antagonize its ability to act as a substrate adaptor for Cul3. Modification of these Cys residues in Keap1 diminishes ubiquitylation of Nrf2, between the DLG and ETGE motifs within the Neh2 domain by CRL<sup>Keap1</sup>, and in so doing induces the ARE-gene battery. In the second mode of regulation (see upper right-hand half of cartoon) growth factors and somatic mutations causing activation of certain oncogenes, such as *EGFR*, *HER2* and *RAS*, stimulate the PI3K pathway [22,23], which through activation of PKB/Akt results in inhibition of the constitutively active GSK-3 through phosphorylation of an N-terminal serine in the kinase [12]. By analogy with CDDO-Im [49•], we speculate that besides inhibiting Keap1, soft electrophiles such as tBHQ and DEM may positively regulate Nrf2 by forming adducts with Cys-71 and/or Cys-124 in PTEN, causing loss of its lipid PIP<sub>3</sub> 3-phosphatase activity, and in so doing cause activation of PDK1 and PKB/Akt that results in inhibition of GSK-3; PTEN inhibition may also be triggered by ROS causing formation of a disulfide bridge between Cys-71 and Cys-124 (indicated by a dotted line) [13]. In addition, electrophiles or ROS may positively regulate Nrf2 by modifying Cys-46, Cys-53 and Cys-106 in DJ-1 and in this case heighten inhibition of the PIP<sub>3</sub> 3-phosphatase activity of PTEN by DJ-1 [60,61]. Alternatively, amino acids can stimulate the mTOR-p70<sup>S6K</sup> pathway and inhibit GSK-3 [12,14,15], and this represents another mechanism by which repression of Nrf2 by GSK-3 might be alleviated (see top right-hand side of cartoon). In all of these instances, the resulting loss of GSK-3 activity causes a decrease in phosphorylation of the DSGIS motif in the Neh6 domain of Nrf2, which is recognized by β-TrCP, and so diminishes ubiquitylation of Nrf2 by SCF<sup>β-TrCP</sup>, allowing accumulation of the transcription factor and induction of its target genes [9••,66,67•]. It should be noted that by comparison with other substrates [69•], it is probable that GSK-3 requires Nrf2 to be phosphorylated by another ‘priming’ kinase before it is able to phosphorylate the transcription factor within the DSGIS motif, which introduces a further tier of regulation. The genes that are induced following inhibition of Keap1 differ from those activated upon inhibition of PTEN [21••,68]. As shown in the bottom left-hand part of the cartoon, induction of ARE-driven genes upon inhibition of Keap1 augments antioxidant and detoxication capacity, which allows adaptation to exposure to oxidative stressors.

However, as shown in the bottom right-hand part of the cartoon, the genes that are transactivated by Nrf2 following loss of Keap1 and PTEN increases both antioxidant/detoxication genes and those involved in metabolism; some of the metabolic genes that are induced may not contain ARE sequences, and their induction is indirect [24•]. Loss of PTEN activity in conjunction with loss of Keap1 appears to augment expression of those antioxidant/detoxication genes that are induced upon loss of Keap1, and to induce metabolism genes involved in generation of NADPH and purines that are either not overexpressed or only modestly overexpressed in cells/tissues lacking Keap1. In particular, hepatocyte-specific ablation of both *Keap1* and *Pten* in the mouse results in large increases in expression of *G6pdx*, *Mthfd2* and *Pgd* that are not observed in the single knockout animals, and to increase significantly expression of *Tk1*, *Me1* and *Taldo1* [17••].