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## **The Keap1/Nrf2 pathway in health and disease**

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Dual regulation of transcription factor Nrf2 by Keap1 and by the combined actions of  $\beta$ -TrCP and GSK-3

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## Abstract

NF-E2 p45-related factor 2 (Nrf2) is a master regulator of redox homeostasis that allows cells to adapt to oxidative stress and also promotes cell proliferation. In this review we describe the molecular mechanisms by which oxidants / electrophilic agents and growth factors increase Nrf2 activity. In the former case, oxidants / electrophiles increase the stability of Nrf2 by antagonizing the ability of Kelch-like ECH-associated protein 1 (Keap1) to target the transcription factor for proteasomal degradation via the Cullin-3–based E3 ubiquitin ligase CRL<sup>Keap1</sup>. In the latter case, we speculate that growth factors increase the stability of Nrf2 by stimulating phosphoinositide 3-kinase (PI3K)–protein kinase B (PKB)/Akt signaling, which in turn results in inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3), and in so doing prevents the formation of a DSGIS motif-containing phosphodegron in Nrf2 that is recognised by the  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) Cullin-1–based E3 ubiquitin ligase complex SCF <sup>$\beta$ -TrCP</sup>. We present data showing that in the absence of Keap1 the electrophile *tert*-butyl hydroquinone (tBHQ) can stimulate Nrf2 activity and induce the Nrf2-target gene *NAD(P)H:quinone oxidoreductase-1* (i.e. *NQO1*), whilst simultaneously causing inhibitory phosphorylation of GSK-3 $\beta$  at Ser-9. Together, these observations suggest that tBHQ can suppress the ability of SCF <sup>$\beta$ -TrCP</sup> to target Nrf2 for proteasomal degradation by increasing PI3K–PKB/Akt signaling. We also propose a scheme that explains how other protein kinases that inhibit GSK-3 could stimulate induction of Nrf2-target genes by preventing formation of the DSGIS motif-containing phosphodegron in Nrf2.

## Background

Four cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factors have been described in mammalian species, designated nuclear factor-erythroid 2 p45 (NF-E2 p45), and NF-E2 p45-related factor 1 (Nrf1<sup>1</sup>), Nrf2 and Nrf3 [1]. They bind their cognate DNA sequences in the regulatory regions of target genes as heterodimers with the small musculoaponeurotic fibrosarcoma (Maf) bZIP proteins, MafF, MafG and MafK [2-4]. Expression of the NF-E2 p45 and Nrf3 transcription factors are restricted primarily to haematopoietic tissue and placenta, whereas Nrf1 and Nrf2 are widely expressed across mammalian tissues [5-7]. Each of the CNC-bZIP transcription factors is functionally distinct, as evidenced by the markedly different phenotypes observed upon their knockout in the mouse [8, 9].

Since its discovery in 1994 as a protein that binds a DNA sequence consisting of a tandem repeat of the activating protein 1 (AP1) site present in the  $\beta$ -globin gene locus control region [10], the work of Masayuki Yamamoto, Tom Kensler and many others has revealed that Nrf2 plays a central role in cancer chemoprevention and to be a master regulator of redox homeostasis [11-15]. More recently it has become apparent that Nrf2 can markedly influence intermediary metabolism by controlling carbon flux through the pentose phosphate pathway, inhibiting lipid synthesis, increasing  $\beta$ -oxidation of fatty acids, and supporting mitochondrial respiration [16-19].

## Stimulation of Nrf2 activity by both oxidants / electrophiles and growth factors

A cardinal feature of Nrf2 is that its activity, and hence the expression of its target genes, is maintained at low levels under normal homeostatic conditions but increases rapidly in response to redox and electrophilic stressors as well as by stimulation by growth factors. The diverse biological effects of Nrf2 are exerted through its ability to mediate induction of genes that contain in their promoter regions an antioxidant response element (ARE<sup>2</sup>, 5'-<sup>A</sup>/<sub>G</sub>TGA<sup>C</sup>/<sub>G</sub>NNNGC<sup>A</sup>/<sub>G</sub>-3') [20] upon exposure to a wide spectrum of oxidants and soft electrophiles (see **Figure 1** for some examples) [21-23], or stimulation by epidermal growth factor [24, 25], fibroblast growth factor [26], insulin [27, 28], insulin-like growth factor [29], keratinocyte growth factor [30, 31], nerve growth factor [32], platelet-derived growth factor [33] or supply of glucose [34]. To date, approximately 250 genes that contain ARE sequences have been reported in mice and humans [35-39]. Many of the ARE-containing Nrf2-target genes are involved in: 1) maintaining the glutathione and thioredoxin antioxidant defence systems within the cell; 2) the detoxication of soft electrophiles and oxidants that could compromise cellular redox status and cellular function by modifying protein thiols; 3) the repair of damaged tissue. Thus in the presence of electrophiles or growth factors, Nrf2 increases expression of genes encoding proteins and enzymes that contribute to glutathione-based antioxidant defence (i.e. the cystine/glutamate transporter, glutamate-cysteine ligase catalytic and modifier subunits, glutaredoxin, glutathione peroxidase and glutathione reductase), thioredoxin-based antioxidant defence (i.e. peroxiredoxin, sulfiredoxin, thioredoxin and thioredoxin reductase), drug-metabolism [i.e. aldo-keto reductases, glutathione S-transferases, and NAD(P)H:quinone oxidoreductase-1 (NQO1)], drug efflux pumps [i.e. multidrug resistance-associated proteins] and cytoprotective proteins associated with heme and iron metabolism [i.e. biliverdin reductase, ferrochelatase, ferritin heavy and light, and heme oxygenase-1 (HMOX1)]. Induction of the above members of the ARE-gene

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<sup>1</sup> Nrf1, Nrf2 and Nrf3 have also been called NF-E2 p45 like 1 (Nfe2l1), Nfe2l2 and Nfe2l3, respectively.

<sup>2</sup> The ARE has also been designated the electrophile response element (EpRE).

battery helps suppress the levels of reactive oxygen species and/or electrophiles in the cell and prevent oxidative stress [40, 41].

In addition to the well-documented role of Nrf2 in orchestrating adaptation to oxidative stress, increasing evidence indicates that Nrf2 also contributes to cell growth and the repair of damaged tissue [42-45]. In this case, activation of Nrf2 is presumed to facilitate cell growth by: 1) increasing nutrient availability; 2) augmenting the levels of NADPH, ATP and metabolic intermediates required for synthesis of macromolecules; 3) increasing production of signaling molecules; 4) limiting inflammation. Thus activation of Nrf2 results in the up-regulation of genes encoding transporters (i.e. the glucose transporter GLUT1, cystine/glutamate transporter SLC7A11, glycine transporter SLC6A9, and fatty acid translocase CD36) that are involved in the cellular uptake of glucose, amino acids and fatty acids. Also, activation of Nrf2 results in the up-regulation of enzymes involved in: carbohydrate metabolism (i.e. glucose-6-phosphate 1-dehydrogenase, isocitrate dehydrogenase 1, malic enzyme 1, 6-phosphogluconate dehydrogenase, transaldolase, and transketolase isoform 1) that regenerate NADPH; the  $\beta$ -oxidation of fatty acids (i.e. acetyl-CoA thioesterase, acetyl-CoA oxidase, carboxylesterase 1, and stearoyl-CoA desaturase) that produce ATP; purine nucleotide biosynthesis (i.e. phosphoribosyl pyrophosphate amidotransferase and methylenetetrahydrofolate dehydrogenase 2) that aid DNA synthesis. Moreover, Nrf2 also regulates directly Notch1 and augments liver regeneration (ALR), both of which contribute to tissue repair [43, 45]. Taken together, the findings outlined above provide an important insight into the biochemical pathways by which activation of Nrf2 stimulates cell growth.

### **Molecular basis for the dual regulation of Nrf2 by Cullin-based ubiquitin ligases**

A key feature of Nrf2 biology is that like other stress-responsive transcription factors it is a highly unstable protein because under normal homeostatic conditions it is continuously targeted for proteasomal degradation [46, 47]. The Nrf2 protein can be subdivided into seven regions, called Nrf2–ECH homology (Neh) domains 1-7. The intrinsic instability of Nrf2 can be attributed primarily to Neh2 and Neh6 as they contain several destruction motifs, whereas Neh1, Neh3, Neh4 and Neh5 are required for transactivation activity [41].

The first major repressor of Nrf2 to be discovered was Kelch-like ECH-associated protein-1 (Keap1), and it was identified using the Neh2 domain of Nrf2 as bait in a yeast two-hybrid screen [48]. It was initially thought that Keap1 sequesters Nrf2 in the cytoplasm, and that upon treatment with electrophiles the CNC-bZIP transcription factor is released by Keap1 to become free to translocate to the nucleus [48, 49]. Several models were advanced to explain the nuclear accumulation of Nrf2 following treatment with stressors: firstly, it was proposed that the release of Nrf2 from Keap1 required phosphorylation of Ser-40 in the Neh2 domain of Nrf2 by protein kinase C [50-52]; secondly, it was demonstrated that reactive Cys residues in a domain of Keap1 called the intervening region (IVR) serve as sensors for electrophiles and it was proposed that the chemical modification of these Cys residues triggered a conformational change in Keap1 that allowed release of Nrf2 [53]. Subsequently, several groups recognised that Keap1 targets Nrf2 for proteasomal degradation [54, 55], and several groups showed in close succession that Keap1 is a substrate adaptor for the Cullin-3–Rbx1 E3 ubiquitin ligase [56-59]. The mammalian Keap1 protein comprises 624 amino acids and contains at least 4 separate Cys-based stress sensors, in the IVR and elsewhere, that are triggered by reactive oxygen/nitrogen species and electrophiles [41]. It is a dimeric protein with two Kelch-repeat domains that each binds Nrf2 through either DLG or ETGE motifs in the Neh2 domain, and it seems improbable that Keap1 releases Nrf2 in a non-ubiquitylated state [60-62]. Indeed, the binding between Keap1 and Nrf2 is tighter when electrophilic agents modify the ubiquitin ligase substrate adaptor [63]. It seems likely that upon stress,

newly translated Nrf2 is able to bypass Keap1 [64], possibly because the latter protein is saturated with Nrf2 that is not ubiquitylated and degraded, and the modified Keap1 is ultimately degraded by autophagy [65].

The second major repressor of Nrf2 to be discovered was  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP). Prior to the discovery, it was recognized that mutant Nrf2 lacking the high-affinity ETGE Keap1-interaction motif (i.e. Nrf2 <sup>$\Delta$ ETGE-V5</sup>) was not particularly stable even though CRL<sup>Keap1</sup> could not target the mutant protein for degradation. Systematic deletion mapping across the Nrf2 <sup>$\Delta$ ETGE-V5</sup> transcription factor identified amino acids bordering the N- and C-terminal boundaries of the Neh6 domain (i.e. residues 329-339 and 363-379 of mouse Nrf2) that conferred instability on the protein [66]. The N-terminal and C-terminal Neh6 regions contain the sequences DSGIS and DSAPGS, respectively, that resemble the consensus binding sequence for  $\beta$ -TrCP, a substrate adaptor for the S-phase kinase-associated protein-1 (Skp1)–Cullin-1–F-box ubiquitin ligase that is responsible for turnover of I $\kappa$ B $\alpha$  and  $\beta$ -catenin; the  $\beta$ -TrCP consensus site has been reported to be DSG $\phi$ XS [67, 68]. Whilst neither the DSGIS motif nor the DSAPGS motif conforms exactly to the  $\beta$ -TrCP consensus site, both have been found to recruit  $\beta$ -TrCP and support ubiquitylation of Nrf2 by SCF <sup>$\beta$ -TrCP</sup> [69, 70]. Unlike the DLG and ETGE motifs in the Neh2 domain that are both required for CRL<sup>Keap1</sup> ubiquitylation of Nrf2, the DSGIS and DSAPGS motifs in the Neh6 domain function independently of each other [70]. The reason for this is uncertain, as both Keap1 and  $\beta$ -TrCP are dimeric substrate adaptors, but one possibility is that whereas the Lys ubiquitin acceptor residues for CRL<sup>Keap1</sup> lie between the DLG and ETGE motifs in Neh2, the Lys ubiquitin acceptor residues for SCF <sup>$\beta$ -TrCP</sup> may lie outwith the region encompassed by the DSGIS and DSAPGS motifs.

Besides Keap1 and  $\beta$ -TrCP, Donna Zhang and colleagues have shown that Nrf2 is repressed by the E3 ubiquitin ligase Hrd1 (also called synoviolin) [71]. Hrd1 is activated in response to endoplasmic reticulum stress and it is therefore questionable whether it contributes to the repression of Nrf2 under normal homeostatic conditions. Importantly, however, activation of Hrd1 leads to loss of Nrf2 activity in cirrhotic liver and therapeutic targeting of Hrd1 in a murine model suppresses liver cirrhosis [71].

### **Glycogen synthase kinase-3 allows regulation of Nrf2 by growth factors**

Many proteins that are ubiquitylated by SCF <sup>$\beta$ -TrCP</sup> are themselves phosphorylated by glycogen synthase kinase-3 (GSK-3), including I $\kappa$ B $\alpha$ ,  $\beta$ -catenin, Gli3 and securin [67, 68, 72, 73]. Salazar et al [74] first reported that GSK-3 inhibits the expression of Nrf2-target genes, and is able to phosphorylate the transcription factor. Evidence has subsequently been provided that GSK-3 phosphorylates Ser residues within the DSGIS motif [75, 76], and that this promotes ubiquitylation of Nrf2 by SCF <sup>$\beta$ -TrCP</sup> [69, 70]. By contrast, the DSAPGS degron does not appear to be influenced by GSK-3 [70].

Unlike most kinases, GSK-3 is active in the cell under resting conditions, but is inhibited by phosphorylation of an N-terminal Ser residue (i.e. Ser-9 and Ser-21 in GSK-3 $\beta$  and GSK-3 $\alpha$ , respectively) by protein kinase B (PKB)/Akt, and in turn PKB/Akt lies downstream of phosphoinositide 3-kinase (PI3K) [77]. As PI3K is closely associated with growth factor receptors and is intimately involved in cell growth and differentiation [78-80], it seems distinctly possible that the ability of epidermal growth factor, fibroblast growth factor, insulin, insulin-like growth factor, keratinocyte growth factor, nerve growth factor and platelet-derived growth factor to activate Nrf2 occurs through stimulation of the PI3K–PKB/Akt pathway and entails inhibition of GSK-3 and loss of repression by  $\beta$ -TrCP.

### **Regulation of Nrf2 by mitogen-activated protein kinases**

Various cancer chemopreventive agents that induce Nrf2-target genes stimulate mitogen-activated protein kinase (MAPK) signaling, including extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 kinases. Specifically, it has been reported that *tert*-butyl hydroquinone (tBHQ) activates ERK and p38<sup>MAPK</sup> [81-84], sulforaphane (SFN) activates ERK and can suppress activation of p38<sup>MAPK</sup> by aninomycin [82, 85], phenethyl isothiocyanate (PEITC) activates ERK and JNK [86, 87], dithiole-3-thione (D3T) activates ERK [88], and pyrrolidine dithiocarbamate (PDTC) activates ERK [89, 90]; see Figure 1 for structures. In the above studies, the use of kinase inhibitors and dominant-negative mutants to blunt gene induction suggest that ERK and JNK positively regulate Nrf2 activity. In marked contrast, p38<sup>MAPK</sup> has been reported to both positively and negatively regulate Nrf2 [84, 91-94] and this may reflect cell-specific differences in p38<sup>MAPK</sup> signaling pathways or response to chemicals.

Many of the investigators who examined the effects of MAPKs on Nrf2 activity considered that their activation resulted in phosphorylation of Nrf2 and that this event controlled translocation of the transcription factor from the cytoplasm to the nucleus. There are however at least three problems with this conclusion: firstly, it assumes the only mechanism of Nrf2 regulation is subcellular compartmentalization, and does not consider the impact of MAPK on transcriptional activation of the gene [95]; secondly, it does not consider the impact of MAPK on protein stability nor does it explain how knockdown or knockout of Keap1 is sufficient to induce Nrf2-target genes [96, 97]; thirdly, mutation of putative MAPK phosphorylation sites in Nrf2 have little impact on the activity of the transcription factor [90, 98]. The accumulated evidence suggests that both ERK and JNK positively regulate Nrf2 activity, but that their effects are probably indirect. Most, but not all studies, suggest that p38<sup>MAPK</sup> inhibits Nrf2, and again this appears to be indirect.

### **Regulation of Nrf2 by phosphatidylinositol-3-kinase**

Over the years it has been found consistently that inhibition of PI3K by Wortmannin or LY294002 blunts Nrf2-mediated induction of ARE-driven genes. Jeff Johnson and colleagues first used LY294002 to diminish induction of NQO1 by tBHQ in IMR-32 human neuroblastoma cells [99], and they subsequently used microarray analysis of gene induction by tBHQ in IMR-32 cells and primary astrocytes from *Nrf2*<sup>+/+</sup> and *Nrf2*<sup>-/-</sup> mice to link PI3K to the induction of Nrf2-target genes [100, 101]. Around the same time, Antonio Cuadrado and colleagues showed that stimulation of PI3K affected Nrf2 indirectly by demonstrating first that activation of PI3K by nerve growth factor required activation of PKB/Akt in order for HMOX1 to be induced [32], and secondly that Nrf2 was required for HMOX1 induction [102]. Thereafter, Salazar et al [74] proposed that GSK-3 provides the link between activation of the PI3K–PKB/Akt pathway and stimulation of Nrf2-mediated gene induction in that they reported activation of PKB/Akt resulted in inhibitory phosphorylation of GSK-3, and that this resulted in failure by GSK-3 to phosphorylate Nrf2.

### **Regulation of Nrf2 by glycogen synthase kinase-3**

As activation of PI3K and PKB/Akt cause inhibition of GSK-3 $\alpha$  and GSK-3 $\beta$  by phosphorylation of their Ser-21 and Ser-9 residues, we hypothesized that chemopreventive agents might up-regulate Nrf2 by activating PI3K and PKB/Akt, thereby preventing formation of the DSGIS-containing phosphodegron in the Neh6 domain of Nrf2. To test this idea, we treated *Keap1*<sup>-/-</sup> mouse embryonic fibroblast (MEF) cells with tBHQ or SFN. As expected, we found that under normal conditions the Nrf2-target genes *Nqo1* and *Hmox1* were overexpressed in the *Keap1*<sup>-/-</sup> MEFs, relative to *Keap1*<sup>+/+</sup> MEFs. However, both *Nqo1* and *Hmox1* could be further induced by tBHQ, but not by SFN, in the mutant MEFs (**Figure 2**). Most importantly, induction of *Nqo1* and *Hmox1* by tBHQ in the *Keap1*<sup>-/-</sup> MEFs was

accompanied by inhibitory phosphorylation of GSK-3 $\beta$  at Ser-9 and by activating phosphorylation of PKB/Akt at Ser-473. Also of note, the PI3K inhibitor LY294002 markedly reduced Nrf2 protein levels as well as the basal expression of Nqo1 and Hmox1 in *Keap1*<sup>-/-</sup> MEFs, and blocked induction of Nqo1 and Hmox1 by tBHQ. These data suggest that PI3K positively regulates Nrf2, and support the hypothesis that tBHQ, but not SFN, activates PI3K and PKB/Akt.

Although it has not been clearly established how tBHQ stimulates PI3K, Mike Sporn and colleagues have shown that the semi-synthetic triterpenoid CDDO-Im, which is a potent inducer of Nrf2-target genes, can activate signaling downstream of PI3K by adducting to the catalytic Cys-124 residue of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [104], the enzyme that cleaves the product of the PI3K reaction, phosphatidylinositol (3,4,5)-trisphosphate (PI3,4,5P<sub>3</sub> or PIP<sub>3</sub>). Importantly, all Cys-dependent protein tyrosine phosphatases are redox sensitive [105]. We therefore propose that tBHQ either modifies Cys-124 in PTEN or another protein tyrosine phosphatase that impinges on PI3K signaling. We envisage that tBHQ and CDDO-Im increase PI3,4,5P<sub>3</sub> levels by inhibiting PTEN, and this causes activation of PDK1, which in turn activates PKB/Akt resulting in inhibition of GSK-3 and failure to produce the DSGIS-containing phosphodegron.

Besides influencing the stability of Nrf2 by catalyzing formation of the DSGIS-containing phosphodegron, it has been reported that GSK-3 indirectly controls the subcellular localization of Nrf2 because it lies upstream of Src non-receptor tyrosine kinases that phosphorylate mouse Nrf2 at Tyr-568 and human Nrf2 at Tyr-576. In this case, treatment of cells with H<sub>2</sub>O<sub>2</sub> has been reported to result in phosphorylation of Nrf2 at Tyr-568/Tyr-576 by Fyn, Src, Yes and Fgr, which triggered nuclear export and degradation of the transcription factor [106, 107]. Further work is required to establish the precise pathway by which H<sub>2</sub>O<sub>2</sub> activates the Src kinases and the putative involvement of GSK-3 in the process.

### Concluding comments

Nrf2 plays an indispensable role in maintaining redox homeostasis, and it has become clear in recent years that it also controls cell growth. It is well known that Nrf2 activity is increased upon treatment of cells with electrophilic agents, because such chemicals antagonize the ability of Keap1 to direct the transcription factor to ubiquitylation by Cul3–Rbx1. It is however much less well appreciated that Nrf2 activity is controlled by growth factors. The body of literature we have reviewed suggests that GSK-3 plays a pivotal role in the regulation of Nrf2 by growth factors because it catalyzes formation of the DSGIS-containing phosphodegron that is recognised by SCF <sup>$\beta$ -TrCP</sup>. We have emphasized the ability of the PI3K–PKB/Akt pathway to inhibit GSK-3. However, as shown in **Figure 3**, other kinases besides PKB/Akt can inhibit GSK-3 $\alpha/\beta$  by catalyzing phosphorylation of their Ser-21/9 residues, such as mTOR–p70<sup>S6K</sup>, ERK–p90RSK, p38<sup>MAPK</sup> and PKC signaling pathways [77], suggesting other mechanisms by which Nrf2 can be regulated. It is intriguing that the kinases that potentially inhibit GSK-3 include ERK and PKC, which have been considered to be positive regulators of Nrf2 [50, 83, 84, 90]. It is also notable that GSK-3 has a strong preference for substrates that have already been phosphorylated by a ‘priming’ kinase at a Ser or Thr residue that is situated four or five residues to the C-terminal side of the amino acid that it phosphorylates [108]. It is not known whether Nrf2 has to be ‘primed’ before it can be modified by GSK-3, nor is it known whether induction of such a ‘priming’ kinase might serve as an alternative means of regulating Nrf2. Our preliminary investigations with peptide-based mini-protein assays suggest priming greatly enhances phosphorylation of Nrf2 by GSK-3. It is becoming clear that the regulation of Nrf2 stability and function is far more complex than just control by Keap1, and the input of multiple growth factor signaling pathways opens up an exciting new chapter in Nrf2 research and its association with human disease.



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**Figure 1.** Chemical structures of electrophilic agents that induce Nrf2-target genes. The structures are (i) *tert*-butyl hydroquinone; (ii) sulforaphane; (iii) phenethyl isothiocyanate; (iv) dithiole-3-thione; (v) pyrrolidine dithiocarbamate; (vi) 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; (vii) bardoxolone methyl.

**Figure 2.** Keap1-independent induction of Nrf2-target genes by *tert*-butyl hydroquinone but not by sulforaphane. Duplicate sets of 60-mm petri-dishes of wild-type and *Keap1*<sup>-/-</sup> MEFs, plated in DMEM medium containing 10% FBS, were grown in DMEM containing low serum (0.1% FBS) for 16 h. Thereafter, they were transferred to fresh medium containing 0.2% FBS and treated with tBHQ, SFN or vehicle control for various time intervals. In some instances, the fibroblasts were pre-treated for 30 min with 10 μM LY294002 immediately prior to transfer to fresh medium and treatment with tBHQ or SFN. **(A)** Whole cell lysates were prepared from *Keap1*<sup>-/-</sup> MEFs that had been treated for 18 h with tBHQ, SFN or DMSO vehicle control. Thereafter, Nqo1 enzyme activity was measured in the 10,000 x g supernatant by the ‘Prochaska’ bioassay method [103]. Data obtained from the MEFs that had been pretreated with LY294002 are shown in solid bars. Results that were significantly higher than the DMSO vehicle control with p-values of 0.01-0.001 or <0.001 are indicated with double (\*\*) or triple (\*\*\*) asterisk signs, respectively; ns = not significant. **(B)** Nqo1 mRNA levels were measured in *Keap1*<sup>-/-</sup> MEFs that had been treated for 12 h with tBHQ, SFN or DMSO. Data from MEFs that had been pretreated with 10 μM LY294002 prior to treatment with tBHQ or SFN are shown in solid bars. **(C)** MEFs from *Keap1*<sup>-/-</sup> and *Keap1*<sup>+/+</sup> mice were treated with tBHQ, SFN or DMSO for 2 h, either with or without pretreatment with LY294002. Whole cell lysates were probed with antibodies specific for Nrf2, Nqo1, Hmox1, phospho-Ser473 Akt (i.e. activated PKB/Akt), total Akt, phospho-Ser9 GSK-3β (i.e. inactive GSK-3β), total GSK-3, and Gapdh.

**Figure 3.** Regulation of Nrf2 by phosphorylation of its Neh6 domain. Evidence indicates that GSK-3 negatively controls Nrf2 by phosphorylating the DSGIS motif in its Neh6 domain and promoting degradation via the actions of SCF<sup>β-TrCP</sup>. As indicated in **(1)**, shown on mid right-hand side, phosphorylation of most validated substrates by GSK-3 requires ‘priming’ (i.e. pre-phosphorylation) by an alternative kinase. Therefore Nrf2 degradation could be regulated by control of ‘priming’. As indicated in **(2)**, shown on mid left-hand side, GSK-3α/β is itself inhibited by phosphorylation of an N-terminal Ser-21/9 residue. The main kinases reported to date to catalyze inhibitory phosphorylation of GSK-3 are members of the AGC class of Kinases (including PKB/Akt, PKC and p70<sup>S6K</sup>). Moreover, p90<sup>RSK</sup> and p38<sup>MAPK</sup> have also been reported to inhibit GSK-3. Therefore, as indicated in **(3)**, shown in upper half, activation of PKB/Akt, p70<sup>S6K</sup>, p90<sup>RSK</sup>, p38<sup>MAPK</sup> and PKC could inhibit GSK-3 and reduce Neh-6 phosphorylation and Nrf2 degradation.