Biochemical Pharmacology 85 (2013) 705–717

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation

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ARTICLE INFO

Article history:
Received 1 October 2012
Accepted 27 November 2012
Available online 5 December 2012

Keywords:
Nrf2
Keap1
Oxidative stress
Cell defence
Regulation

ABSTRACT

The transcription factor Nrf2 (NF-E2-related factor 2) plays a vital role in maintaining cellular homeostasis, especially upon the exposure of cells to chemical or oxidative stress, through its ability to regulate the basal and inducible expression of a multitude of antioxidant proteins, detoxification enzymes and xenobiotic transporters. In addition, Nrf2 contributes to diverse cellular functions including differentiation, proliferation, inflammation and lipid synthesis and there is an increasing association of aberrant expression and/or function of Nrf2 with pathologies including cancer, neurodegeneration and cardiovascular disease. The activity of Nrf2 is primarily regulated via its interaction with Keap1 (Kelch-like ECH-associated protein 1), which directs the transcription factor for proteasomal degradation. Although it is generally accepted that modification (e.g. chemical adduction, oxidation, nitrosylation or glutathionylation) of one or more critical cysteine residues in Keap1 represents a likely chemico-biological trigger for the activation of Nrf2, unequivocal evidence for such a phenomenon remains elusive. An increasing body of literature has revealed alternative mechanisms of Nrf2 regulation, including phosphorylation of Nrf2 by various protein kinases (PKC, PI3K/Akt, GSK-3β, JNK), interaction with other protein partners (p21, caveolin-1) and epigenetic factors (micro-RNAs -144, -28 and -200a, and promoter methylation). These and other processes are potentially important determinants of Nrf2 activity, and therefore may contribute to the maintenance of cellular homeostasis. Here, we dissect evidence supporting these Keap1-dependent and -independent mechanisms of Nrf2 regulation. Furthermore, we highlight key knowledge gaps in this important field of biology, and suggest how these may be addressed experimentally.

1. Introduction

The exposure of cells to a range of environmental toxicants, mutagens and potential carcinogens has been linked to the pathogenesis of a broad range of diseases including cancer, neurodegenerative disease, cardiovascular disease and inflammation [1]. To protect against such insults, eukaryotic cells have developed complex signalling cascades to detoxify potentially harmful substances and maintain cellular redox homeostasis. One of these signalling cascades is responsible for the induction of cytoprotective and detoxifying enzymes consisting of phase I (cytochrome P450s) and phase II (detoxifying and antioxidant proteins) enzymes [2]. The co-ordinated expression of these genes removes the insult and attempts to restore the cell to a basal state by conferring a resistance to stress, thus preventing damage to cellular components sensitive to redox changes (i.e. proteins, lipids and DNA) [3]. The ubiquitously expressed cap'n'collar bZip transcription factor Nrf2 is largely responsible for the basal and inducible expression of proteins involved in drug metabolism, the oxidative stress response and cytoprotection. Supplementary to its primary role in cytoprotection, Nrf2 is also linked to differentiation, proliferation, growth, apoptosis and it is thought that Nrf2 has evolved from an original role in haematopoiesis and the regulation of cell differentiation from early lineages [4]. Whilst a study by Chan et al., showed that Nrf2 is not essential for growth,

Abbreviations: DEA-NO/AM, acetoxymethylated diethylamine-NONO-ate; LC-ESI MS/MS, liquid chromatography electronspray ionisation tandem mass spectrometry; MRM, multiple reaction monitoring; tBHQ, tert-butylhydroquinone; CDDO-Me, methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate; CDDO-Im, 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole.

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http://dx.doi.org/10.1016/j.bcp.2012.11.016

development or erythropoiesis in mammalian cells, these authors suggest that Nrf2 could still have originally played this role in an avian system, due to observations made by Itoh et al. of high Nrf2 expression in chicken hematopoietic cells [5,6]. Furthermore, there is still evidence of Nrf2s role in haematopoiesis in a mammalian system as it regulates the expression of haemoxygenase-1 (HO-1) which is involved in the handling of iron [7]. In addition, recent findings show that Nrf2 is functionally involved in lipid deposition in the liver. Using proteomic analysis (iTRAQ) to compare wild type (WT) and Nrf2 knockout ($Nrf2^{-/-}$) mice, it was shown that basally, Nrf2 regulates a number of proteins involved in the synthesis and metabolism of fatty acids and other lipids [8]. The most potent known Nrf2 inducers; the triterpenoids CDDO-Me and CDDO-Im (synthesised from oleanolic acid) are lipid soluble molecules which have been shown to reduce the accumulation of lipids in the livers of mice on a high fat diet via the Keap1/Nrf2 pathway [9]. The importance of the basal control of lipid metabolism by Nrf2 is not understood. However it is possible that this process is under the control of Nrf2 when the cell is in a basal, energy-sufficient state yet, in times of stress and when Nrf2 is induced, lipid synthesis and other biosynthetic pathways are down-regulated to compensate for the energy requirements of cell defence mechanisms.

A vital factor in the functioning of many transcription factors is their spatio-temporal regulation and this is no different in the case of Nrf2; it is just as important that Nrf2 is switched on in response to a stimulus as it is that it is switched off when the stimulus has been removed. It is for this reason that this pathway is highly regulated with a number of different mechanisms responsible for preventing the aberrant activation of Nrf2. One of the most important mechanisms that regulate the cells response to inflammatory, hypoxic, oxidative and xenobiotic stimuli is proteasomal degradation; and the Nrf2 pathway is no exception to this. [10]. In unstressed conditions, the level of Nrf2 protein in the cell is maintained at very low levels by its inhibitor Keap1, which sequesters Nrf2 in the cytosol and facilitates its degradation via the proteasome. Under conditions of stress or in the presence of Nrf2 activating compounds, this degradation is hindered and Nrf2 translocates to the nucleus. Here, Nrf2 heterodimerises with small masculoaponeurotic fibrosarcoma (Maf) proteins which in turn facilitate the binding of Nrf2 to the Antioxidant Response Element (ARE), a *cis*-acting enhancer sequence (TCAG/CXXXGC) in the promoter region of Nrf2-regulated genes [11,12] (Fig. 1a). These Nrf2-regulated genes can be classified into phase II xenobiotic-metabolizing enzymes antioxidants, molecular chaperones, DNA repair enzymes, and anti-inflammatory response proteins [13] and they reduce reactive compounds such as electrophiles and free radicals to less toxic intermediates whilst increasing the ability of the cell to repair any damage ensued. Importantly, Nrf2 has been shown to possess an ARE sequence within its own promoter region providing a platform for Nrf2 to initiate its own transcription further enhancing the adaptive cell defence response [11]. Following its nuclear import, Nrf2 recruits transcriptional machinery to effectively transactivate the ARE-driven genes. This machinery includes co-activators such as receptor associated co-activator (RAC3) which initiates the transactivation domain of Nrf2 whilst the presence of other co-regulators such as CREB binding

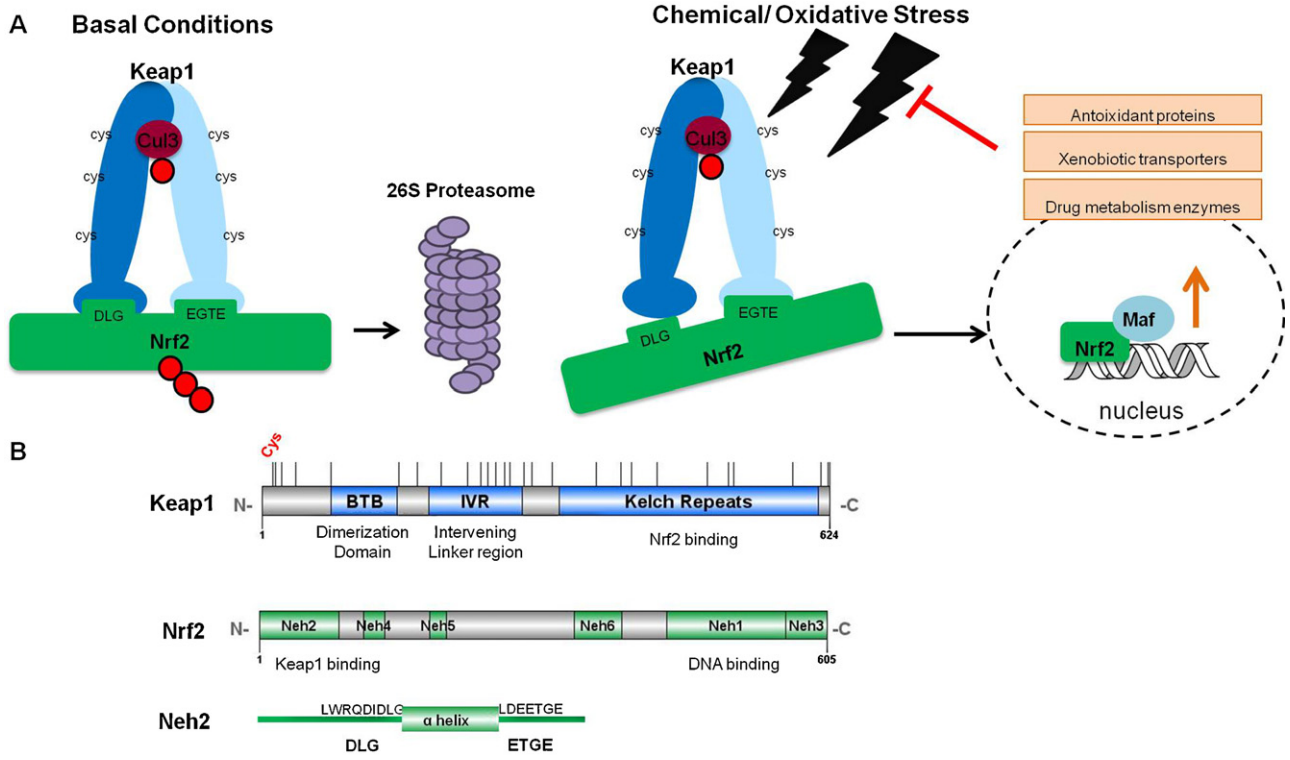
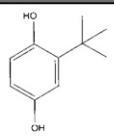
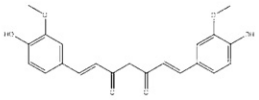
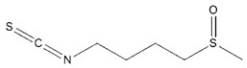
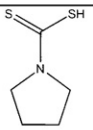
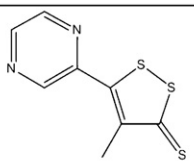
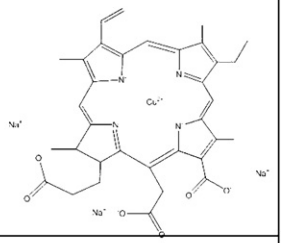
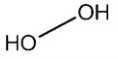
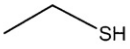


Fig. 1. (A) Schematic overview of the Nrf2 pathway. Under basal conditions, Nrf2 is sequestered in the cytosol by a Keap1 homodimer which facilitates the ubiquitination and proteasomal degradation of Nrf2. When the cell is faced with an insult such as chemical or oxidative stress, a conformational change in Keap1 mediated via its reactive cysteine residues results in the release of Nrf2 from one Keap1 molecule. Nrf2 can no longer be ubiquitinated and degraded therefore Keap1 becomes fully saturated with Nrf2, allowing newly synthesised Nrf2 to accumulate and translocate to the nucleus. Here Nrf2 heterodimerises with small Maf proteins and binds to the antioxidant response element (ARE). This activates the expression of a battery of genes responsible for removing the insult, conferring increased resistance to stress and returning the cell to a basal state. (B) Keap1 and Nrf2 Protein Domains. Keap1 contains a number of functional domains including the Broad complex, Tramtrack and Bric-a-brac (BTB), the intervening linker domain (IVR), the double glycine/Kelch repeats and the C-terminal region. The BTB is responsible for the dimerisation of two Keap1 molecules whilst the Kelch repeats contain the region responsible for binding Nrf2, facilitated in particular by a number of arginine residues (Arg-380, -415, -483). Keap1 contains a number of reactive cysteine residues also highlighted. Nrf2 contains Neh1-6 domains of which Neh1 binds to the ARE within DNA whilst Neh4/5 are transactivation domains. Neh2 is responsible for binding to Keap1 via the ^{29}DLG^{31} and ^{79}ETGE^{82} motifs which flank an α helix region containing the lysine residues for Keap1-mediated ubiquitination.

Table 1
The major classes of Nrf2 inducers and their structures. Where more than one example is given, the structure shown is that of the example in bold type.

Class	Example	Structure
Oxidisable diphenols, phenylenediamines, Quinones	**BHA:(2(3)-tert-butyl-4-hydroxyanisole)** tBHQ: tert-butylhydroquinone	
Michael reaction acceptors	**Curcumin (turmeric)** CDDO-Me, Zerumbone (ginger) Citral (plant oils)	
Isothiocyanates and sulfoxythiocarbamates	**Sulforaphane ,** Benzyl isothiocyanate, (cruciferous vegetables)	
Thiocarbamates	Pyrrolidine dithiocarbamate (PDTC)	
Dithiolethiones	**Oltipraz: 4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione** (R)-Lipoic acid	
Polyenes	**Chlorophyll**, Porphyrins, chlorophyllins	
Hydroperoxides	Hydrogen peroxide	
Trivalent arsenicals	Arsenic Trioxide	AS^{3+}
Heavy metals	Methyl mercury, Cadmium, Zinc	Hg^{2+}, Cd^{2+}, Zn^{2+}
Dimercaptans	Mercaptan	

protein (CBP), coactivator-associated arginine methyltransferase (CARM1) and protein arginine methyl-transferase (PRMT1), further enhance the ability of RAC3 to initiate the transactivation domain [14].

The discovery of the ARE lead to the identification of Nrf2 as the transcription factor capable of both binding to this DNA sequence and inducing the expression of cell defence genes and resulted in a burst of research into this pathway as a potential therapeutic target. The vast number of both natural and synthetic compounds able to induce Nrf2 can be divided into at least 10 groups outlined in Table 1. The pharmacological activation of Nrf2 by various compounds such as allyl sulfides, dithiolethiones, flavonoids, isothiocynates, polyphenols and triterpenoids has been proposed for use in the prevention of a number of diseases associated with oxidative stress [4]. A number of Nrf2 inducers, mostly plant-derived compounds with chemopreventive properties, such as sulforaphane (broccoli) [15], curcumin (turmeric) [16,17] and resveratrol (grapes) [18,19] are currently in clinical trials for a variety of cancers. Interestingly there is some dispute over the exact role of Nrf2 in cancer as it seems to play a dual role potentially acting as both a tumour suppressor and an oncogenic factor. Recent observations include mutations in the Nrf2 or Keap1 genes which result in aberrant expression or ineffective regulation of Nrf2. Raised Nrf2 levels have been detected across an array of cancer tissues including lung [20,21] and pancreas [22,23] and it is proposed that this provides cells with enhanced chemo-resistance as well as supporting increased proliferation thus promoting cancer growth and development [22]. Nrf2-deficient mice are more susceptible to toxicity by a number of compounds such as paracetamol and tobacco smoke and to many diseases (neurodegenerative, cancer, inflammation) but interestingly, $Nrf2^{-/-}$ mice do survive and are able to procreate which suggests that, although some constitutively active processes are under Nrf2 control (e.g. lipid homeostasis), Nrf2 is not necessarily vital for survival in

unstressed cells and is only called upon in the presence of stress or insult. [24]. What is evident, however, is that Nrf2 plays a major role in health and disease and it is not surprising that Nrf2 is considered to be a potential therapeutic target. For a detailed review of the potential of Nrf2 as a therapeutic target, see the review by Copple [25]. The development of a number of Nrf2 inducers as possible pharmacological agents without a complete knowledge of the workings of this pathway and its regulation heightens the need to further our understanding and to determine whether activation of Nrf2 would be beneficial in both the short- and long-term.

The protein primarily responsible for the regulation of Nrf2 is Kelch-like ECH-associated protein 1 (Keap1), which forms a homodimer responsible for sequestering Nrf2 in the cytosol, thereby rendering it inactive. It is an association between Keap1 and the actin cytoskeleton which prevents this complex entering the nucleus, limiting basal activity of the transcription factor [26].Additionally, Keap1 facilitates the Cul3-mediated poly-ubiquitination of Nrf2 leading to its proteasomal degradation. Whilst Keap1 seems to be the major mechanism by which Nrf2 levels are tightly controlled in the cell, recent research would suggest that the pathway is highly complex and supports a multi-faceted defence system. In this commentary we focus on the mechanisms by which Keap1 regulates the Nrf2 pathway under basal and stressed conditions and also how it is that Keap1 has become known as a redox-sensor with the role of sensing environmental conditions and facilitating the up-regulation of the cell defence pathway via Nrf2. Furthermore we explore a number of mechanisms by which Nrf2 can be regulated independently of Keap1 at the level of protein transcription, translation and by post translational modifications.

2. Keap1 dependent regulation

2.1. Identification of Keap1 as an inhibitor of Nrf2

The identification of an inhibitor of Nrf2 arose from observations by Itoh et al. that the deletion of the Neh2 region of the Nrf2 protein resulted in a marked increase in Nrf2 activity in erythroblasts and led to the proposal that this region was responsible for the negative regulation of Nrf2 via an interaction with a repressor protein [27]. Itoh et al. then used yeast-two-hybrid – with the Neh2 domain of Nrf2 as the bait – to identify a mouse protein homologous to the *Drosophila* protein Kelch and termed the protein Keap1. Later observations by Dhakshinamoorthy et al. further confirmed this idea by cloning the rat homologue of Keap1 by purifying Nrf2-interacting proteins [28]. Following these discoveries, the domains of Keap1 were characterised and it was noted that two Keap1 molecules are able to bind to one Nrf2 molecule [10] and that the BTB domain is responsible for the homodimerisation of Keap1 and the subsequent inhibition of Nrf2 [29] (see Fig. 1b for domain structures of Nrf2 and Keap1). When transfected into cells it was observed that Nrf2 would accumulate in the nucleus, however when co-transfected with Keap1 the two would co-localise in the cytoplasm. Moreover, in the presence of both Keap1 and a panel of electrophiles (e.g. diethylmaleate (DEM)), this co-localisation is lost and Nrf2 again localises in the nucleus [30]. This promoted the idea that Keap1 sequesters Nrf2 in an inactive form in the cytoplasm until faced with an oxidative or electrophilic insult when Nrf2 is freed and translocates to the nucleus. The importance of Keap1 is highlighted by the observation that Keap1-deficient mice ($Keap1^{-/-}$) do not survive longer than 3 weeks postnatally due to hyperkeratosis of the digestive system resulting in ulceration of the stomach [31]. Embryonic fibroblasts isolated from $Keap1^{-/-}$ mice showed constitutive activation of Nrf2 and the inducibility of Nrf2 regulated cell defence genes was hugely reduced [31], demonstrating that Keap1 is vital in the regulation of the Nrf2 pathway in vivo.

Having initially established that Keap1 plays an role in the regulation of the Nrf2 pathway, is was questioned whether under basal conditions, Keap1 is simply binding Nrf2 and sequestering it in the cytosol thus preventing its translocation to the nucleus or whether Keap1 also provided a functional role in this pathway. It was quickly determined that the latter was the more likely.

2.2. Ubiquitination and proteasomal degradation of Nrf2

Under basal conditions, low levels of Nrf2 are maintained to prevent the constitutive activation of the oxidative stress response. It is the interaction with Keap1 that facilitates the proteasomal degradation and high turnover of Nrf2 protein resulting in a half life of approximately 10–20 min [32,33]. In the absence of oxidative stress, Nrf2 is sequestered in the cytosol by the Keap1 homodimer which acts as a substrate adaptor for the ubiquitination of Nrf2 in a cullin-3 (Cul3) dependent manner [34]. Nrf2 binds the DGR site of each Keap1 subunit via 2 distinct binding motifs in its Neh2 domain, one high affinity, ^{79}ETGE^{82}, and one low affinity, ^{29}DLG^{31} [10] (Fig. 1). The hinge and latch hypothesis proposes that the high affinity binding site (hinge) allows Nrf2 to bind Keap1 whilst still able to move freely, whilst the low affinity binding site acts as the latch that impedes the movement of Nrf2 and positions the lysine residues within the Neh2 region for ubiquitination [10]. When bound at both sites, Nrf2 is perfectly positioned to undergo poly-ubiquitination via the Cul3 E3 ligase and is consequently degraded by the 26S proteasome ensuring extremely low basal levels of Nrf2 in the cell (Fig. 1a).

When the cell experiences conditions of (oxidative) stress or in the presence of electrophiles, it is proposed that subsets of the cysteine residues in Keap1 are modified. These modifications potentially result in a conformational change in the protein which results in the release of Nrf2 from the low affinity binding site (latch), disturbing the transfer of ubiquitin. Keap1 molecules become saturated with Nrf2 that is no longer targeted for degradation and newly synthesised, free Nrf2 accumulates in the cytosol. Consequently, Nrf2 translocates to the nucleus where it binds to the ARE, activating the transcription of a host of cell defence genes (Fig. 1b). The importance of Keap1 in this process is clear, as the half life of Nrf2 increased from approximately 7.5–15 min. in unstressed COS1 cells co-expressing mNrf2-V5 and mKeap1 to ~30 min in cells under the same conditions co-expressing the mutant $mNrf2^{\Delta ETGE}$-V5 with wild-type mKeap1 [35]. The changes in Nrf2 half-life associated with oxidative stress and specific electrophiles varies immensely between cell lines due to the dramatic differences in detectable basal levels of the protein [36–38]. There is also conflicting evidence on the mechanism by which Nrf2 becomes free in the cell, some stating that electrophiles cause the dissociation of the Keap1–Nrf2 complex [39,40] whilst others suggest that the electrophiles cause the dissociation of Cul3 from Keap1 thus preventing the proteasomal degradation of Nrf2 [41–43]. There is also the possibility that whilst the ubiquitination of Nrf2 ceases, ubiquitination of Keap1 increases under conditions of chemical/oxidative stress however this is only seen with certain Nrf2 inducers [44]. Furthermore, there are those who believe that Nrf2 is primarily a nuclear protein and this nuclear localisation is responsible for the basal expression of cell defence genes and that the degradation of Nrf2 via Keap1 is downstream of Nrf2 transcriptional activity [45,46]. Whilst this would explain how Nrf2 is capable of regulating the basal expression of genes despite being constantly degraded via Keap1, this idea requires the nucleo-cytoplasmic shuttling of Keap1 which others claim not to observe [47]. What is certainly clear is that Keap1 is responsible for

the proteasomal degradation of Nrf2 and that the half-life of Nrf2 is somehow dependent on the redox state of the cell and that this is at least partially via the interaction with the redox-sensitive Keap1.

2.3. Autophagic degradation of Keap1

The proteasome has long been considered the machinery utilised to carry out the degradation of specific proteins targeted for destruction for reasons such as misfolding. On the other hand, autophagy was generally regarded to be a bulk-degradation pathway for the recycling of a multitude of non-specific cellular organelles and proteins. However, there is growing evidence supporting the notion that the latter pathway is capable of degrading specific, targeted proteins [48–51].

The substrate adaptor Sequestosome1 (p62) acts as a scaffold protein in various signalling pathways via its multiple protein–protein interactions and binds to both poly-ubiquitinated proteins as well as the autophagic machinery, specifically LC3, targeting specific proteins for degradation via the autophagic pathway [52,53]. Until recently Nrf2 was thought to be one of very few substrates for Keap1 however a physical and functional relationship between Keap1 and p62 has been elucidated [54–58]. Evidence suggests that p62 has a role in regulating Keap1 degradation via autophagy, altering the ability of the cell to respond to various stresses via this pathway [58]. The ectopic expression of p62 in a panel of cell lines resulted in reduced levels of Keap1 protein whilst siRNA-mediated knockdown of p62 resulted in increased levels of Keap1 protein, decreased Nrf2 protein (without changes in mRNA) concurrent with reduced mRNA and protein levels of a number of Nrf2-regulated genes [58]. This change was associated with a 2-fold increase in the half-life of Keap1 in the absence of p62 [58]. Further observations involving this complex indicate a role for this interaction in hepatocellular carcinoma whereby liver-specific autophagy deficient mice ($Atg^{-/-}$) show p62 accumulation and the formation of p62/Keap1 aggregates alongside the development of the aforementioned carcinoma; moreover, this p62 accumulation leads to the consistent activation of Nrf2 which promotes the development of human hepatocellular carcinoma [59]. The proposed model is that under conditions of stress, a conformational change in Keap1 releases Nrf2 from the low affinity binding site and p62 takes advantage of this empty site and binds to Keap1 via an STGE motif, a sequence similar to Nrf2s ETGE motif [54] and also to LC3 which is associated with the autophagosome membrane, therefore providing a link between the Keap1–Nrf2 complex and autophagic degradation adding another level to the complexity of the regulation of this pathway.

2.4. Keap1 as a sensor of stress

Having uncovered some of the mechanisms by which the Nrf2 pathway is regulated via the degradation of various components under both basal and stressed conditions, the next question to be answered is how the system senses the wide variety of stresses that result in the de-repression of Nrf2.

The Keap1 protein is relatively cysteine rich with 4.3% of all residues being cysteines; approximately double the number in the average protein [60]. Cysteine residues are usually found within

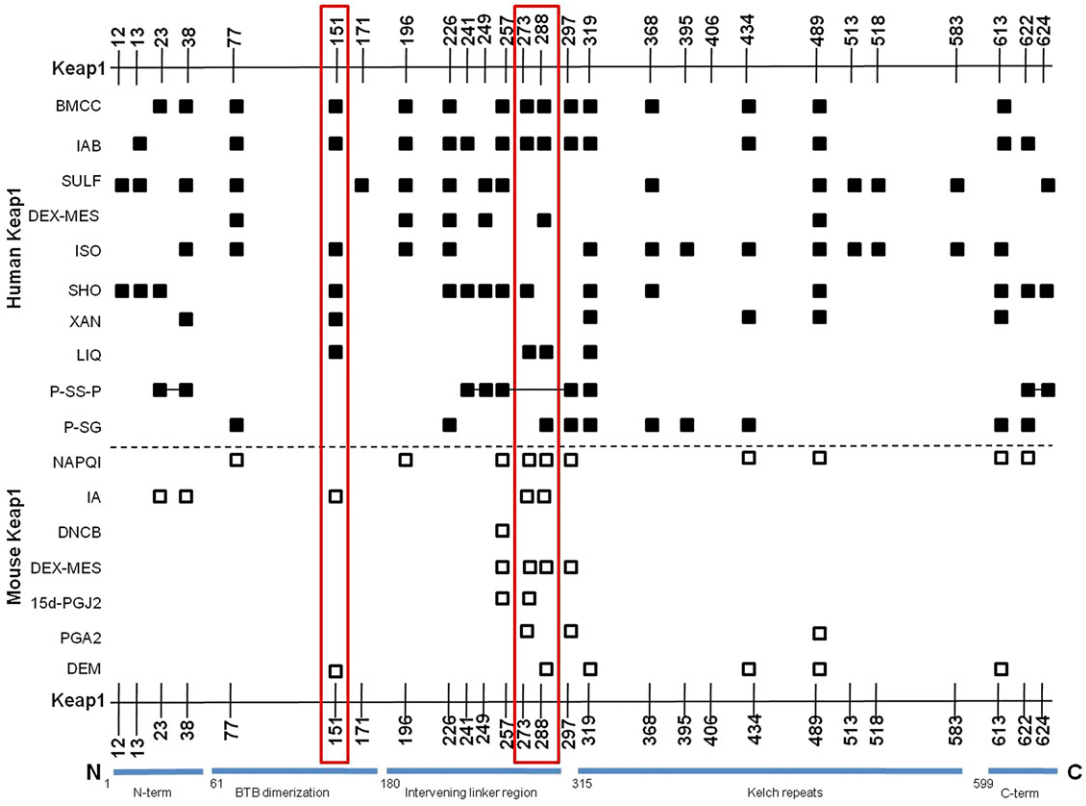


Fig. 2. Schematic overview of human and mouse Keap1 cysteine residues and their modification by electrophiles. Electrophile modification of human Keap1 cysteine residues (black box) and mouse Keap1 cysteine residues (white box). The boxes outline the three critical reactive cysteines of Keap1 (C151, C273 and C288). The domains of Keap1 are outlined at the bottom of the figure and the cysteine numbers are annotated at the top and bottom of the figure. The abbreviations of the electrophiles are as follows: BMCC, 1-biotinamido-4-4(4′-[maleimidiethylcyclohexane]-carboxamido)butane [121,122]; IAB, N-iodoacetyl-N-biotinylhexylenediamine [121,123]; SULF, sulforaphane [124]; DEX-MES, dexamethasone-21-mesylate [125]; ISO, isoliquiritigenin; SHO, 10-Shogaol; XAN, xanthohumol [122]; LIQ, liqustilide [126]; P-SS-P, protein disulfide; P-SG, s-glutathionlyation [66]; NAPQI, N-acetyl-p-benzoquinoneimine; IA, iodoacetamide; DNCB, dinitrochlorobenzene [74]; DEX-MES [65,74]; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2 [67,74]; PGA2, prostaglandin A2; DEM, diethylmaleate [67] (adapted from Holland et al. [62]).

the functional domains of proteins and are redox-active and responsive to the local environment [61]. It is Keap1's 27 cysteine residues that are proposed to be the main mechanism whereby Keap1 is able to sense electrophilic or oxidative stresses [62]. The majority of Keap1 cysteine residues are flanked by basic amino acids which increase the reactivity of the cysteine residue by lowering the predicted pK_a value [63]. Moreover, the majority of Nrf2 inducers are electrophilic and capable of reacting with cysteine sulfydryl groups and forming direct covalent adducts with them. It is postulated that these residue modifications disrupt the Keap1–Nrf2 association allowing Nrf2 to translocate to the nucleus [64]. The hypothesis that the modification of cysteines in Keap1 may be essential to the regulation of the Nrf2 pathway was first put forward by Dinkova-Kostova et al., who determined that a number of Nrf2 inducers, whilst being structurally dissimilar, were all reactive with thiol groups at rates that correlated with their potency [65]. They showed that electrophiles are capable of dissociating Keap1 from the Neh2 region of Nrf2, from which they deduced that the sensor of the inducers must be Keap1, as the Neh2 region contains no cysteine residues. Using mass spectrometry analysis, the group went on to show that a number of cysteine residues that lie in the linker region between the BTB domain and Kelch-repeats of the Keap1 protein; C257, C273, C288 and C297 are especially reactive [65]. The idea that Keap1 may be under the control of oxidation/reduction and alkylation opened up a number of opportunities for the exploration of the importance of the role these residues play in the regulation of the Nrf2 pathway.

This led to a vast quantity of literature regarding which cysteine residue(s) are required for an Nrf2 response and a general consensus on a selection of cysteines reactive to particular electrophiles developed. Published data demonstrates that there is not a single cysteine or selection of cysteines that are reactive to all electrophiles [66] rather that there are some residues which are more reactive than others (see Fig. 2 for an overview). As seen in Fig. 2, it is clear that there are reactive cysteines across the whole Keap1 sequence. However the residues that are reactive to most electrophiles are those in the intervening linker region (IVR) of Keap1 thus is it possible that modification of any one/combination of these cysteines is sufficient to disrupt the association with Nrf2. The modification of a subset of cysteine residues in Keap1 by Nrf2 inducers with similar structures supports the hypothesis of a "cysteine code" and may underlie the ability of Nrf2 to respond to such a diverse array of compounds [67]. Point mutation of Cys151 results in reduced activation of Nrf2 in response to some inducers (tBHQ, DEM, sulforaphane) but not to others (CDDO-Im, mitro-oleic acid, cadmium chloride) compared to wild type cells [68]. It is proposed that Cys151 is important in the de-repression of Nrf2 whilst Cys273 and Cys288 are more important in the basal repression of Nrf2. Transgenic expression of mutant Keap1 ($Keap1^{C273A}$/$Keap1^{C288A}$) into $Keap1^{-/-}$ mice prevents the ability of Keap1 to inhibit the constitutive activation of Nrf2, whilst mutant Cys^{C151S} retained the ability to suppress Nrf2 but had decreased expression levels of Nrf2-regulated genes both before and after electrophilic insult [69]. McMahon et al. propose that Keap1 quantifies stress by monitoring endogenous levels of messengers (NO, Zn^+ and alkenals) which imply the presence of stress within the cell, using at least 3 unique cysteine-based sensors to confer the appropriate Nrf2 response [70]. They go on to propose that whilst the NO-donor DEA-NO/AM does not form direct adducts with Cys151, this cysteine may undergo *S*-nitrosylation facilitated by its flanking residues (K131, R135, K150) [70] and this idea of *S*-nitrosylation of Keap1 is supported by others [71,72]. Supplementary to these modifications, cysteine residues can also be glutathionylated by oxidised glutathione (GSSG) and this molecule can also cause the formation of disulfide bridges [66]. This is important as under conditions of oxidative stress, the balance between oxidised and reduced glutathione (GSSG:GSH) is altered, and it is possible that increased GSSG or depleted GSH can enhance the activation of Nrf2, as can be seen, for example after exposure of cells to buthionine sulfoximine (BSO), an inhibitor of the rate-limiting enzyme in the glutathione synthetic pathway [73,74]. These studies support the idea of a "multiple sensor mechanism" within Keap1 and imply that it is most likely the modification of a combination of cysteines that is responsible for the activation of Nrf2 as opposed to direct adduct formation with one individual residue. Further studies exploiting recent advances in techniques of mass spectrometry may allow better understanding of this mechanism.

Due to the low abundance of Keap1 in most cell lines, the approach of ectopically expressing various mutant forms of Keap1 has primarily been used to identify a battery of cysteines thought to be most important in this pathway, namely Cys151, Cys273 and Cys288. However, one must bear in mind when considering this data that whilst the mutation of one or a number of cysteine residues in Keap1 may prevent the up-regulation of an Nrf2 response, this does not necessarily mean that this cysteine residue is primarily responsible for eliciting the Nrf2 response. One major problem with this type of approach is that often it is not determined whether the mutation made within the Keap1 protein sequence causes any changes in the protein folding or structure that may result in a non-functional Keap1 protein and an up-regulation of Nrf2 independent of cysteine modification. Furthermore, the practice of ectopically expressing a protein in a cell line may divert the environment of the protein away from that of a truly physiological state, the importance of which is yet to be established. What one must also take into account is that adduct formation may not be primarily responsible for the activation of Nrf2 via Keap1. A number of adduct-forming electrophiles also induce the formation of reactive oxygen species which are capable themselves of activating Nrf2. Thus it is not yet plausible to claim that the modification of Keap1 is directly responsible for the induction of Nrf2. Ultimately, it is clear that further exploration of the importance of Keap1 cysteine modification will help increase our overall understanding of how activation and regulation of the Nrf2 pathway occurs, and therefore how we sense and respond to our chemical environment.

3. Keap1-independent regulation

Despite the aforementioned support for the regulation of Nrf2 via Keap1, a body of evidence is emerging showing that Nrf2 can be regulated independently of Keap1. A study by Li et al. showed that the Nrf2 inducer sulforaphane prevents the dissociation of Nrf2 from Keap1, supporting the view that there are alternative mechanisms of Nrf2 activation that do not rely on Keap1 [4]. The expression level and function of a protein can be controlled by regulation at various levels including; transcriptional, post-transcriptional, protein abundance, post-translational modification and subcellular localisation. The phosphorylation of Nrf2 by several signal transduction pathways, the involvement of epigenetic factors such as microRNAs or the interaction of Nrf2 with other proteins may also play a role in Nrf2 activation and this section will describe these in detail.

3.1. Transcription regulation and autoregulation

The core DNA sequences, ARE and XRE (xenobiotic-responsive element), are found in the promoter region of many cell defence genes known to be regulated by Nrf2. Nrf2 binds to the ARE to up-regulate gene expression whilst the XRE is activated by the transcription factor AHR (aryl hydrocarbon receptor). Ligands which activate AHR cause its heterodimerisation with ARNT (AHR

nuclear translocator) and this complex activates the XRE to induce the expression of many phase I enzymes such as cytochrome p450s [75] which promotes the generation of reactive intermediates from a parent compound and this reactive intermediate can potentially activate the antioxidant pathway via the ARE. A study by Miao et al. showed that TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), a potent inducer of AHR, also induces ROS as well as Nrf2 itself and can therefore activate both the ARE and XRE pathways directly [2]. The XRE and ARE elements are found in close proximity within the promoters of several Nrf2 regulated genes (e.g. GST) as well as Nrf2 itself [2,76] (Fig. 3). The Nrf2 promoter region contains one XRE-like element at position −712 and the Nrf2 mRNA initiation site contains two XRE-like elements at position +755 and +850 [2].

The presence of these DNA binding sites (ARE/XRE) within the promoter region of Nrf2 suggests the ability of Nrf2 to regulate its own transcription – i.e. autoregulation. In support of this, Kwak et al. showed that the potent ARE activator D3T (3*H*-1,2-dithiole-3-thione) increased Nrf2 protein and mRNA levels and that these increases were inhibited by co-treatment with the protein synthesis inhibitor cyclohexamide [38]. Furthermore, they demonstrated direct binding of Nrf2 to its own promoter region and that the over-expression of Nrf2 increased the activity of a luciferase reporter assay of the isolated proximal region of the Nrf2 promotor, whereas using mutant Nrf2 repressed the activity of the luciferase reporter [38]. This evidence suggesting that Nrf2 activates its own expression hints at a positive feedback loop within this pathway, leading to enhanced cell defence.

Since Nrf2 has been implicated in the pathogenesis of various diseases and it is known that polymorphisms have been also been associated with susceptibility to pathologies which could be associated with Nrf2 such as idiosyncratic drug reactions, it is plausible that the Nrf2 gene contains polymorphisms that may predispose individuals to certain health problems. A study by Yamamoto et al. identified the presence of three single nucleotide and one triplet repeat polymorphism in the regulatory region of the Nrf2 gene [77]. In this study, no link was established between the frequency of the polymorphism and the pathogenesis of diseases such as lupus (SLE) or COPD. Furthermore, the coding region of the gene showed no polymorphisms although a larger population screening may be warranted. In contrast, Marzec et al. identified multiple SNPs in the Nrf2 gene and saw a correlation between the −617 SNP and the susceptibility to acute lung injury (ALI) implicating Nrf2 in the development of this disease [78]. Since then, Nrf2 polymorphisms have been linked to diseases including vitiligo and nephritis with polymorphism seen at −650C/A and −635/A respectively [79]. What remains to be determined is what effects these polymorphisms have on the expression levels and activity of Nrf2 and whether this information can be used to tailor drug regimes to individuals and increase drug safety.

NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) is a transcriptional factor involved in cellular processes such as apoptosis, inflammation and development. NF-κB is associated with its inhibitor IκB in the cytoplasm and is activated upon the phosphorylation of IκB by IκB kinases (IKK), leading to the dissociation of the complex and nuclear translocation. Many chemopreventive agents are known to activate Nrf2 whilst repressing NF-κB activity, moreover NF-κB can suppress the transcription of ARE-dependent genes [80]. Cross-talk between

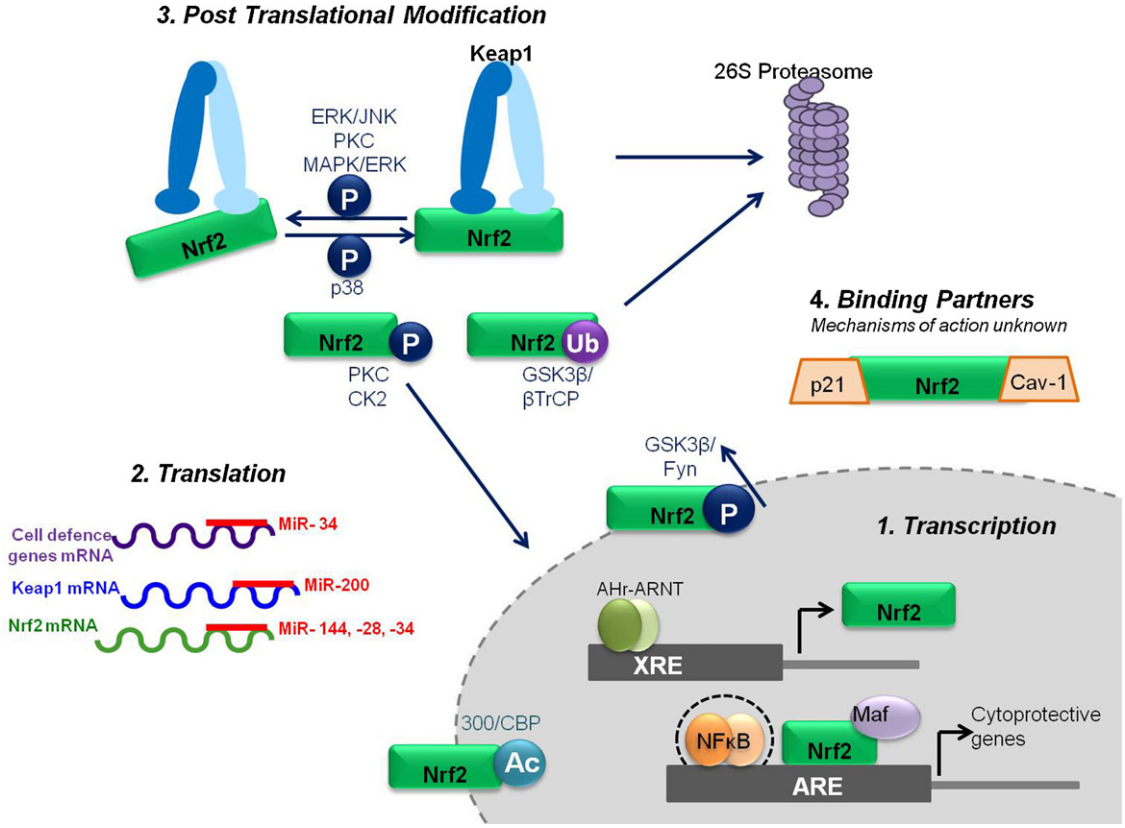


Fig. 3. Schematic overview of Keap1-independent regulation of Nrf2. Nrf2 has been shown to be regulated by a number of mechanisms independent of Keap1. These include regulation at the transcriptional level (1) by AHr-ARNT inducing Nrf2 expression and NF-κB which has been proposed to bind to an ARE with in the Nrf2 promotor region (dotted circle highlights that this is a suggestion and has not been proven experimentally). At the post-transcriptional level (2), components of the Nrf2 pathway are regulated by several micro-RNAs (miR-28, 34, 144, 200). Post translationally (3), Nrf2 is phosphorylated (P), ubiquitinated (Ub) and acetylated (Ac) by a variety of enzymes. Each post-translational modification affects Nrf2 differently by altering; Nrf2's interaction with Keap1 (ERK/JNK, PCK, MAPK/ERK, p38); Nrf2 localisation (PKC, CK2, GSK3β/Fyn); Nrf2 protein degradation (GSK3β/βTrCP) and Nrf2 DNA binding (300/CBP, Maf). A number of proteins have been identified as Nrf2 binding partners (4) (p21, caveolin-1) however their mechanisms of action are unknown.

Nrf2 and NF-κB and the identification of an NF-κB binding region in the promoter region of Nrf2 suggests that Nrf2 could be regulated by NF-κB (Fig. 3) [80]. Interestingly, Keap1 has been shown to bind to IKKβ, a member of the IKK complex, promoting its ubiquitination and degradation such that Keap1 plays a role in the negative regulation of the NF-κB pathway [81,82]. The regulation and interaction of these two transcription factors requires further study to really establish where the crossover may lie.

3.2. Post-transcriptional regulation: microRNAs

MicroRNAs (miRs) are short, single-stranded non-coding RNAs of approximately 21–23 nucleotides in length. They are transcribed from genetic loci by RNA polymerase II and processed before being exported from the nucleus as short hairpin loops for maturation and cleavage [83]. Upon maturation, these microRNAs form a complex called the RNA-induced silencing complex (RISC) which binds to target mRNAs at the 3′UTR region and exerts its function through mRNA degradation or protein translation inhibition to inhibit protein expression [83]. MicroRNAs have been the centre of a vast amount of research in the past few years due to their ability to fine-tune the regulation of various proteins and processes, including the Nrf2 pathway. Micro-RNAs that have been shown to be involved in the regulation of Nrf2 include miR-144 [84], miR-28 [83], miR-200 [85] and miR-34 [86] (Fig. 3). The Songkoya et al. study showed an inverse association between miR-144 and Nrf2; an increase in miR-144 expression reduces Nrf2 protein levels, decreases glutathione regeneration and alters the antioxidant capacity of erythroid cells, an important mobile detoxification system in the body [84]. Interestingly, an abnormal expression of miR-144 has been associated with the sickle cell disease (SCD) and this implicates a role for Nrf2 in this disease [84].

A similar relationship was seen between Nrf2 and miR-28 in breast epithelial cells [83]. MiR-28 was shown to regulate Nrf2 by binding facilitating the degradation of Nrf2 mRNA as well as promoting the degradation of Nrf2 protein [83]. MiR-28 had no effect on either Keap1 protein expression or the Keap1/Nrf2 interaction highlighting a mechanism by which Nrf2 is regulated independently of Keap1 [83]. The aberrant expression of miR-28 has been seen in various cancers including lymphoma, glioma and squamous carcinoma supporting previous evidence for a role of Nrf2 in cancer. Other microRNAs shown to regulate Nrf2 include miR-200 which targets Keap1 mRNA [85] and miR-34 which not only targets Nrf2 but also downstream genes involved in the oxidative stress response (Mgst1) suggesting a double-dampening effect [86]. The actual mechanism by which micro-RNAs regulate Nrf2 and other proteins requires further elucidation but current hypotheses are reviewed by Filipowicz et al. [87].

3.3. Post-translational modification: phosphorylation/acetylation

There have been several studies suggesting that phosphorylation of Nrf2 may contribute to its nuclear exclusion and degradation. Nrf2 contains many serine, threonine and tyrosine residues, which may provide sites for phosphorylation by different kinases [88] and a number of different pathways have been explored including mitogen-activated protein kinase cascades (MAPK), the phosphatidylinositol 3-kinase (PI3K/AKT) pathway, protein kinase C (PKC), GSK3β pathway and the ERK signalling pathways (Fig. 3).

Protein kinase C has been shown to phosphorylate Nrf2 in its Neh2 domain at Ser-40, disrupting the association between Nrf2 and Keap1 thus promoting the translocation of Nrf2 into the nucleus [89]. Reduced nuclear translocation of Nrf2 was seen in mutant $Nrf2^{S40A}$ compared to wild type Nrf2. PKC are a family of serine/threonine kinases which can be subdivided into 3 classes; classical, novel and atypical [90]. The isoforms require different co-factors for activation and play different roles in growth, differentiation, apoptosis, survival and carcinogenesis. They can be activated upon oxidative stress, a principal activator of the Nrf2 antioxidant pathway and a study by Numazawa et al. showed that the atypical isoform is responsible for the phosphorylation and nuclear translocation of Nrf2 to induce phase II cytoprotective proteins [90]. In contrast to this, Bloom et al. showed that Ser-40 is required for the release of Nrf2 from its repressor but not for its nuclear accumulation or increased stability [91]. The identification of nuclear localisation sequences (NLSs) and nuclear export sequences (NESs) in Nrf2 suggests that in order to facilitate its translocation, NLS motifs in Nrf2 are identified by adaptor proteins such as importins forming a complex with Nrf2 to transport it to the nuclear membrane through the nuclear pore complex [92].

Protein kinase CK2 is a highly conserved protein with a broad range of substrates with functions ranging from signal transduction, gene transcription, replication and survival. The Nrf2 sequence has approximately 13 potential CK2 target phosphorylation sites [93] and the transcription activation domains Neh4 and Neh5 specifically have been identified as target regions which can be phosphorylated by CK2 in vitro [94]. The phosphorylation of these sites correlates with the translocation of Nrf2 into the nucleus and this translocation is reduced in the presence of a CK2 inhibitor [94].

The tyrosine kinase Fyn has been shown to phosphorylate tyrosine-568 in Nrf2, conferring its nuclear export and degradation. In the presence of the mutant $Nrf2^{Y568A}$, nuclear accumulation of Nrf2 was observed, resulting from a loss of phosphorylation at tyrosine-568 and a loss of the interaction with exportin Crm1 [95]. Furthermore, Jain and Jaswal showed that GSK3β (glycogen synthase kinase 3β) acts upstream of Fyn activating its phosphorylation and resulting in its nuclear accumulation. Having accumulated in the nucleus Fyn is perfectly positioned to phosphorylate Nrf2 and cause its nuclear export, ubiquitination and subsequent proteasomal degradation [96]. Additionally, several serine/threonine residues in Nrf2 have been identified to be phosphorylated by a panel of MAP kinases. Keum et al. showed that p38 phosphorylates Nrf2 and promotes its association with Keap1 thereby preventing its nuclear translocation. Interestingly, this effect was reversed by sulforaphane and this is a proposed mechanism of action for sulforaphane-mediated induction of Nrf2 [97]. Recent studies have highlighted the involvement of JNK (c-jun N-terminal kinase 1/2) and ERK (extracellular signal-regulated kinase) in the activation of Nrf2. Butylated hydroxyanisole (BHA) was shown to increase the phosphorylation of both ERK1/2 and JNK1/2 to activate Nrf2 which was released from Keap1 and translocated to the nucleus under the control of ERK and JNK signalling pathways [98]. In addition, PERK kinase has also been shown to phosphorylate Nrf2, but the exact site of phosphorylation is yet to be identified [99].

The majority of known Nrf2 activators also activate a number of other kinase pathways for example, tBHQ activates the PI3K/Akt pathway while BHA activates the MAP kinases [37,98]. The molecular mechanism underlying their roles in the activation of these kinase pathways remains poorly understood as activation of specific pathways is dependent on a number of factors such as the chemical characteristics of the inducing agent, the cell type used, and the sequence of the ARE, ultimately adding to the complexity of the regulation of Nrf2 by the previously described signalling pathways.

Despite evidence showing the effect of several Nrf2 activators on a wide variety of signalling pathways, little is known about the interplay between these pathways and how they may coordinate to contribute to the regulation of the Nrf2 pathway. The

identification of GSK3β as a key regulator of Nrf2 stability has provided insight to the activation of Nrf2 by phosphorylation and it may act as a "common downstream effector" for a number of Nrf2 inducers [88]. GSK3β is an important regulator of several metabolic processes including glycogen metabolism, Wnt signalling and apoptosis [100]. GSK3β can stabilise Nrf2 by phosphorylating the Neh6 region which in turn is proposed to facilitate its ubiquitination by adaptor protein β-TrCP which forms a complex with Cullin-1 to form a complete E3 ligase [101]. GSK3β is a downstream target of multiple kinase cascades i.e. Akt and MAPK, and the activation of these pathways inhibits GSK3β through phosphorylation at multiple sites. It is proposed that the Nrf2 activator nordihydroguaiaretic acid (NDGA) targets these kinase cascades, inhibiting GSK3β, in turn stabilising Nrf2 via its reduced phosphorylation and ubiquitination [88]. Evidence [100] and knowledge of GSK3βs relationship with other transcription factors NF-AT [102] and cyclin-1 [103] supports the latter. GSK3β inhibition promotes Nrf2 stabilization in Keap1-deficient cells [88], in Keap1$^{-/-}$ mice and in the presence of the mutant Nrf2$^{\Delta ETGE}$ whereby Keap1 cannot bind to Nrf2 [101] thereby suggesting that GSK3β degrades Nrf2 in a Keap1-independent manner. It is possible that GSK3β phosphorylates these residues with/without other kinases but this requires further investigation.

Knowing that both ubiquitination and phosphorylation play a role in the regulation of Nrf2, it comes as no surprise that the acetylation of Nrf2 also plays a role in the regulation of this pathway. The transcriptional co-activators p300/CBP acetylate histones to facilitate chromatin decondensation and recruit RNA polymerase machinery [104,105], have been shown to associate with Nrf2 [106,107] and other transcription factors such as NF-κB [108]. p300/CBP binds to Nrf2 in response to oxidative stress induced by arsenite and acetylates a number of lysine residues within the Neh1 DNA binding region of Nrf2. Mutations of these lysine sites to arginine results in no changes in Nrf2 protein stability but does compromise the ability of Nrf2 to bind to DNA [106]. It is probable that this mechanism exerts itself downstream of the Nrf2/Keap1 complex and enhances the ability of Nrf2 to bind to DNA.

The number of factors which have been shown to be involved in the post translational modification of Nrf2 highlights just how complicated and high regulated this pathway is. The clarification of the interplay between each of the pathways involved will help to further our understanding of cell defence and may highlight potential therapeutic targets.

3.4. Protein stability and binding partners

A number of Nrf2 activators such as tBHQ and sulforaphane have been associated with increases in Nrf2 protein stability in what has been ascribed to an increase in the half-life of Nrf2 rather than an increase in the rate of protein translation. Whilst most of the proteins of the BTB-Kelch super-family are yet to be understood, a few of them have been shown to function in E3 ubiquitination and proteasomal degradation by acting as a substrate adaptor for the Cul3-based E3 ubiquitin ligase [3]. Following the identification of Keap1, a member of this family, to function as a substrate for Cul3-based E3 ubiquitin ligase, other BTB-Kelch proteins have been identified including ectoderm-neural cortex protein 1 (ENC1) [109]. ENC1 has a similar sequence and domain organization to Keap1 suggesting that their functions may be similar. A nuclear matrix protein found in the nervous system, ECN1 has an important role in neuronal differentiation and its ectopic expression has been linked to brain tumorigenesis by augmenting cell proliferation and inhibiting apoptosis [3]. ENC1 can form a complex with Cul3–Rbx1 and facilitate its own ubiquitination however no ubiquitination of Nrf2 has been seen. Interestingly, it can reduce the protein levels of Nrf2 as well as the transcription of Nrf2 dependent genes (NQO1, HO-1) [3]. As ECN1 had no effect on Nrf2 mRNA nor its stability it is proposed that it down regulates the rate of Nrf2 protein synthesis [3] the mechanism of which remains to be understood.

Okadaic acid, a protein phosphatase inhibitor, which induces intracellular hyper-phosphorylation, increases Nrf2 stability thereby further supporting increased stability as a mechanism of Nrf2 activation [37]. tBHQ stabilization of Nrf2 is dependent on the MAPK/ERK signalling cascade as Nrf2 induction by tBHQ is inhibited in the presence of MAPK/ERK inhibitors suggesting that the MAPK/ERK signalling cascade drives this stability through phosphorylation [37]. DJ-1 – a protein associated with Parkinson's and cancer belonging to the Thi/PfpI superfamily – stabilizes Nrf2 by impeding its association with Keap1, thus reducing Nrf2 ubiquitination and subsequent degradation [110]. Thus far, no physical interaction has been seen between DJ-1 and Nrf2, Keap1 or Cullin3 and this interaction seems to be cell type specific [110,111]. As for direct binding partners other than Keap1, Nrf2 has been shown to interact with p21, a cyclin dependent kinase (CDK) (Fig. 3) which binds with Nrf2 at the DLG and ETGE motifs through its C-terminal KRR motif to stabilize Nrf2 and thus confer protection against oxidative stress however the exact mechanism remains to be elucidated [112].

Recently, caveolin-1 has been identified as an Nrf2 binding partner (Fig. 3). Caveolin-1 is a scaffold protein in caveolar membranes and is involved in signal transduction and the uptake of lipophilic compounds [113]. Caveolin-1 interacts with a number of proteins such as; Toll-like receptor 4; LC3B, a constituent of the autophagy machinery; Fas and survivin to regulate various biological processes such as cholesterol homeostasis and apoptosis [113,114]. A study by Zheng et al. highlighted a possible association between Caveolin-1 and Nrf2 [114] and this was further confirmed by Li et al. who used siRNA to knock-down Caveolin-1 which resulted in a dissociation of Nrf2 and Keap1. On the other hand, ectopic expression of Caveolin-1 did not cause any changes in the Nrf2/Keap1 association but further reduced the transcriptional activity of Nrf2. They went on to show that Nrf2 binds Caveolin-1 via a "caveolin-1 binding motif" and mutagenesis of this motif reduced the association between Caveolin-1 and Nrf2 whist enhancing the association between Keap1 and Nrf2. They propose that Caveolin-1 may compete with Keap1 for binding to Nrf2 [113].

3.5. Nrf2 cysteine modification

Although much attention has been focused on the modification of reactive cysteine residues in Keap1, the modification of cysteines in Nrf2 is another possible mechanism for its regulation. Li et al. characterised a nuclear export sequence in the Neh5 transactivation domain which contains a reactive cysteine residue at position 183. Mutating this residue (C183A) resulted in reduced translocation rates of Nrf2 compared to wild type following the activation of Nrf2 by tBHQ and H_2O_2 while no significant effect was seen on Keap1 [115]. Under the conditions of oxidative stress or in the presence of electrophiles, it is possible that modification at Cys-183 prevents the binding of exportin Crm1 to this Neh domain resulting in nuclear accumulation Nrf2. As of yet, no mass spectrometry techniques have been performed to detect the formation of any sulfydryl adducts on this residue and it should be noted that there is currently no strong evidence to support the modification of Nrf2 protein itself.

4. Conclusion

In this commentary, we have provided an overview of the current understanding of Keap1-dependent and -independent

regulation of Nrf2. Several studies have pointed to the importance of Nrf2 in the regulation of multiple biological processes including the pathogenesis of a number of conditions ranging from cancer, autoimmune disorders and chronic diseases. Understanding the molecular mechanisms underlying the regulation of Nrf2 and its activation by electrophiles and xenobiotics will be key to both the improvement of current and the development of novel strategies for the therapeutic manipulation of this pathway. It is safe to say that there are a multitude of mechanisms playing a role in the regulation of this pathway however it is not yet possible to say which may be the most important. What is interesting is that there are so many alternative pathways for the activation of Nrf2 and these potentially act as fail safe mechanisms to ensure the sufficient activation of Nrf2 in times of stress or prevent its constitutive activation. Under basal intracellular redox conditions, Keap1 continuously drives the regulation of Nrf2 maintaining low cellular levels of the protein however under conditions of stress (chemical/oxidative), the regulation of Nrf2 becomes complex involving both Keap1-dependent and -independent mechanisms. In the presence of electrophiles and oxidants, it is proposed that the modification of an array of cysteine residues in Keap1 is primarily responsible for the obstruction of Nrf2 proteasomal degradation and thus an increase in its transcriptional activity however changes in Nrf2s mRNA levels, intracellular localisation, transcriptional activity, and stability for the most part mediated by post-translational modifications also seem to be involved in the activation of this pathway. These mechanisms of Keap1-independent regulation may occur under both redox sensitive and insensitive conditions and may aid in the fine tuning of the regulation of Nrf2 levels under basal conditions.

The major limitation on the understanding of the function of Keap1 modifications in the activation of Nrf2 is the current technologies employed to study it. Recent advances in proteomic technologies (shot gun proteomics, LC–ESI-MS/MS, MRM) have allowed the detection of direct protein adducts and have facilitated the detection of a multitude of drug protein targets resulting in the development of an online database (Target Protein Database) of the common protein alkylation patterns of a variety of drugs. Whilst there is much evidence supporting Keap1 modification as a mechanism for regulating Nrf2 in vitro, this is not yet unequivocal, and certainly this is yet to be proven in vivo [116]. Combining proteomics with functional protein assays as well as knockout animals and RNA interference (RNAi), will hopefully lead to an understanding of the functional relevance of direct protein adduct formation and protein interactions which will be of particular interest to those working in the Nrf2/Keap1 field. Additionally, the development of cell lines containing bacterical artificial chromosomes (BACs) expressing the protein of choice with a fluorescent tag at close to physiological levels will help us understand the interaction between different members of this pathway.

The publication of the Nrf2 interactome and regulome in 2012 highlights a vast array of potential proteins involved in the regulation of this pathway including NF-κB [117]. The fact that there is interplay between these two cell defence pathways provides evidence for a coordinated response to insult to confer cell protection, however the exact mechanisms by which they each may play a role in the regulation of the other pathway remains to be determined. The activation of the NF-κB pathway has been associated with the previously mentioned Sequestosome1 (p62). Sequestosome-1 contains a number of protein binding domains which allows it to homodimerise and form aggregates or speckles. Within these speckles, sequestosome-1 associates with and activates the E3 ligase TRAF6 which has been linked to the activation of NF-κB [118]. The bone disorder Pagets disease characterised by increased osteoclastogenic activity resulting in enlarged and misshapen bones is associated with various mutations in the Sequestosome-1 gene and the phenotype is synonymous with mice deficient in TRAF6 [118]. Taken together, the link between the Nrf2 and NF-κB pathways via both Keap1 and p62 highlights the importance of the regulation and coordination of these pathways in human health and disease.

There is a great deal of research being undertaken in order to fill the knowledge gaps addressed in this commentary and to fully characterize this pathway in terms of the multiple functions of this transcription factor, its binding partners and co-factors, the various signalling cascades which co-ordinate to regulate it and the downstream effects. Whilst it is evident that Nrf2 is regulated by both Keap1-dependent and Keap1-indpendent mechanisms, there are still questions to be answered. What will be very interesting to uncover is the relative importance of each of these mechanisms in the activation and regulation of the Nrf2 pathway. For example, of the numerous kinase pathways discussed in this commentary, are they all of importance physiologically? Are they under the control of endogenous factors or only via exogenous Nrf2 modulators?

The role of Nrf2 in a number of pathological states and its links to cancer is clearly very important, and a full exploration and understanding of Nrf2 regulation will likely be of value in designing better therapies in the future. With a better understanding of the exact mechanisms of regulation of this pathway, it may be useful and possible to design therapies that do not target Nrf2 directly but influence other members of the pathway and to fine-tune the activation of the pathway to cater to individual needs. As Nrf2 has been shown to be up-regulated by both non-hepatotoxic and hepatotoxic doses of acetaminophen in vivo [119] and $Nrf2^{-/-}$ mice are more susceptible to drug induced toxicities, it is evident that Nrf2 contributes to the cell's attempt to defend itself against toxicity and potentially drug induced liver injury (DILI) and adverse drug reactions (ADRs) which are – for the most part – responsible for drug attrition [120]. A more complete understanding of this pathway may help us to further our knowledge of these phenomena and potentially design novel assays to discern drug safety.

Acknowledgements

The authors would like to acknowledge that there is a vast amount of literature regarding the potential mechanisms of activation of the Nrf2 pathway that has not been included here due to limitations in the space and the scope of this commentary. The authors would also like to acknowledge the funding bodies supporting the ongoing research into this pathway at the MRC Centre for Drug Safety Science at the University of Liverpool; The Medical Research Council and The Biotechnology and Biological Sciences Research Council.

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