Signal amplification in the KEAP1-NRF2-ARE antioxidant response pathway

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ABSTRACT

The KEAP1-NRF2-ARE signaling pathway plays a central role in mediating the adaptive cellular stress response to oxidative and electrophilic chemicals. This canonical pathway has been extensively studied and reviewed in the past two decades, but rarely was it looked at from a quantitative signaling perspective. Signal amplification, i. e., ultrasensitivity, is crucially important for robust induction of antioxidant genes to appropriate levels that can adequately counteract the stresses. In this review article, we examined a number of well-known molecular events in the KEAP1-NRF2-ARE pathway from a quantitative perspective with a focus on how signal amplification can be achieved. We illustrated, by using a series of mathematical models, that redox-regulated protein sequestration, stabilization, translation, nuclear trafficking, DNA promoter binding, and transcriptional induction – which are embedded in the molecular network comprising KEAP1, NRF2, sMaf, p62, and BACH1 – may generate highly ultrasensitive NRF2 activation and antioxidant gene induction. The emergence and degree of ultrasensitivity depend on the strengths of protein-protein and protein-DNA interaction and protein abundances. A unique, quantitative understanding of signal amplification in the KEAP1-NRF2-ARE pathway will help to identify sensitive targets for the prevention and therapeutics of oxidative stress-related diseases and develop quantitative adverse outcome pathway models to facilitate the health risk assessment of oxidative chemicals.

1. Introduction

1.1. Overview of the KEAP1-NRF2-ARE pathway

Cells, particularly those of the aerobic organisms, are constantly under oxidative and electrophilic insults from both internal metabolic and respiratory reactions and environmental exposures. To maintain cellular redox homeostasis and limit the damage to biomacromolecules, cells have evolved an elaborate molecular program that would be adaptively activated by reactive species to enhance their antioxidant and antielectrophilic capacity on demand [1–3]. Depending on the stress intensity and duration, this adaptive cytoprotective response can recruit both posttranslationally and transcriptionally-mediated programs [2, 4–6]. In mammalian cells, the transcriptional induction of antioxidant and cytoprotective enzymes involves several key signaling components and regulatory events. Here, the nuclear factor erythroid 2-related factor 2 (NRF2) serves as the master transcription factor, and Kelch-like ECH-associated protein 1 (KEAP1) functions as the molecular sensor for reactive species including electrophiles [7–9]. At basal conditions, KEAP1 readily sequesters NRF2 and tethers it for ubiquitination and proteasomal degradation such that NRF2 abundance is kept low. Under stress conditions, KEAP1 is modified on some specific cysteine moieties, which disables its E3 ligase adaptor activity. As a result, NRF2 is stabilized and increased via *de novo* protein synthesis. When the NRF2 abundance exceeds the KEAP1 abundance, it will escape the sequestration by KEAP1 and translocate into the nucleus [2]. In the nucleus, NRF2 binds to small musculoaponeurotic fibrosarcoma (sMaf) proteins to form transcriptionally active heterodimers [10,11]. The NRF2-sMaf heterodimer recognizes specific antioxidant response elements (ARE) in the promoters of a battery of target genes including antioxidant and phase II detoxification enzymes to induce their transcription [12–17].

1.2. Signal amplification (ultrasensitivity) is necessary for robust antioxidant response

While the KEAP1-NRF2-ARE signaling pathway is often described as a linear structure starting from oxidative stressors and culminating in antioxidant gene induction, in its wholeness, it is a complex molecular circuitry, the structure of which is primarily negative feedback and in some cases also involves incoherent feedforward [4,5]. The feedback and feedforward loops embedding the KEAP1-NRF2-ARE pathway are closed through the antioxidant and detoxification reactions that eliminate the reactive species, oxidative chemicals and their metabolites (Fig. 1).

While oxidative stress induced by transient exposure to reactive chemicals may resolve by itself overtime, persistent ones would need sustained induction of antioxidant enzymes to levels that are sufficient to counteract the oxidative impacts, wherein the intracellular reactive oxygen species (ROS) and electrophiles and redox state can be restored to near normal levels [4]. How can the antioxidant induction be maintained at such adequately high levels when the ROS/electrophiles, which triggered the induction in the first place, have returned to near normal levels? The maintenance of this adapted steady state requires some considerable signal amplification in the KEAP1-NRF2-ARE pathway, such that a small increase in the ROS/electrophile levels can sustain a larger increase in the expression of antioxidant genes. The terminology for biochemical signal amplification is ultrasensitivity, which refers to a quantitative signal transfer where a small percentage change in the input signal level leads to a larger percentage change in the output signal level [18–20]. An ultrasensitive input-output (dose-response) relationship is generally sigmoidal with the slope steeper than that exhibited by the classical Michaelis-Menten kinetics, and is often described empirically with Hill functions. At least six ultrasensitive response motifs (URM), which are specific molecular interactions that can amplify biochemical signals, have been discovered, including positive cooperative binding, homomultimerization, multistep signaling, molecular titration (or sequestration), saturable covalent modification cycle, and positive feedback regulation [19,20]. In the past two decades, a great amount of details of the molecular interactions in the KEAP1-NRF2-ARE pathway have been revealed. However, their quantitative signaling properties, i.e., whether and how they may amplify redox signals and function collectively to ensure sufficient cellular antioxidant induction, are still poorly explored [4,21,22].

1.3. Objectives

The KEAP1-NRF2-ARE pathway and its roles in various physiological and pathological processes have been extensively reviewed over the years as our knowledge in the molecular details continues to expand [1–3,8,23–25]. However, rarely was this pathway examined from a quantitative signaling perspective. Having a quantitative understanding of the KEAP1-NRF2-ARE pathway will help us to further ascertain not only the cellular physiology of oxidative stress response but also how they may be altered under pathological conditions to facilitate adverse outcomes. The KEAP1-NRF2-ARE pathway is exploited by many cancer cells to enhance their survival and chemoresistance [26,27]. Such survival advantages may be obtained through novel molecular circuits of ultrasensitivity arising out of genetic mutations [28]. The KEAP1-NRF2-ARE pathway can be also hijacked by viruses to facilitate their infection [29,30]. Therefore, a quantitative appreciation, such as for the degree of amplification and magnitude of NRF2 activation, can help to identify sensitive molecular targets to develop drugs that more precisely modulate intracellular antioxidant levels. In addition, quantitative knowledge of the KEAP1-NRF2-ARE pathway will also provide information for identifying sensitive and reliable biomarkers for chemical toxicity testing assays, as well as for developing quantitative systems biology models to support next-generation risk assessment. In this review article, we aim to examine several key molecular processes known to operate in this canonical KEAP1-NRF2-ARE pathway, in the context of signal amplification. These processes include the sequestration, stabilization, translation, translocation, and autoregulation of NRF2 and its interactions with other components such as KEAP1, sMaf, p62, and BACH1. We illustrated, with simple mathematical models (codes available in Berkeley Madonna and *R* format at https://github.com/pulsatility/2022-KEAP1-NRF2-ARE-Pathway-Review), that ultrasensitivity may arise out of these well-known molecular interactions.

2. Signal amplification in the KEAP1-NRF2-ARE pathway

The KEAP1-NRF2-ARE transcriptional pathway is an elaborately integrated dynamical system. It can be challenging to disentangle and reverse-engineer the system into recognizable building blocks of signal amplification. A number of URMs are believed to operate in the KEAP1-NRF2-ARE transcriptional pathway [4,21,22]. Here, we dissect this pathway into potential URMs by focusing on several key known molecular processes leading to NRF2-mediated transcriptional induction of cytoprotective genes.

2.1. KEAP1-dependent NRF2 sequestration and degradation

Background: Our understanding of how KEAP1 regulates NRF2 and oxidative stress leads to NRF2 activation has evolved since the discovery of these two proteins [2,31]. The activation of NRF2 was first thought to be similar to the NF-κB pathway, involving release of NRF2 sequestered by KEAP1 [32–34]. Subsequently it was recognized that electrophilic compounds or class I-V NRF2 activators, which conjugate or modify the cysteine residues of KEAP1, do not alter the binding between KEAP1 and NRF2 [2,31,35–37]. Rather, it is the stability, and thus half-life, of NRF2 that is increased as electrophiles inhibit KEAP1's function as E3 ligase adaptor to ubiquitinate NRF2 for proteasomal degradation [9,38,39]. Therefore, a floodgate model has emerged for NRF2 activation by
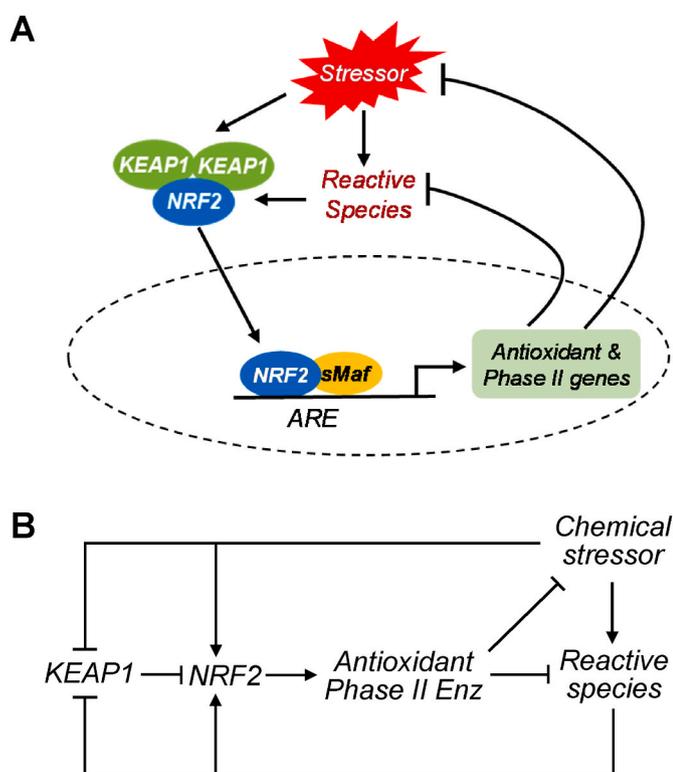

Fig. 1. Schematic illustration of the KEAP1-NRF2-ARE pathway embedded in negative feedback and incoherent feedforward circuitry. (A) Biologist's view. **(B)** Engineer's view.

electrophilic compounds, where NRF2 stabilization first leads to its cytosolic accumulation, and when its abundance rises past the abundance of the KEAP1 homodimer, it will escape from the sequestration by KEAP1 and translocate to the nucleus [23,40]. In contrast, non-electrophilic, class VI NRF2 activators compete with NRF2 for binding to KEAP1 by displacing the DLG motif of NRF2 from the KEAP1 dimer, thus a hinge-latch model explains how NRF2 is activated by this type of compounds [31]. Due to the high binding affinity between NRF2 and KEAP1 [32,34,41–43], at the juncture where NRF2 rises to a level that exceeds KEAP1 dimer, two underlying URMs start to operate simultaneously. (i) Zero-order degradation of NRF2 by KEAP1 – here, KEAP1, essentially functioning as an enzyme, is saturated by NRF2, therefore NRF2 degradation through this route becomes zero order. (ii) Molecular titration (protein sequestration) – here, the free NRF2 abundance will increase dramatically as no more free KEAP1 is available to sequester NRF2. We have recently thoroughly examined the two ultrasensitive mechanisms through computational models based on detailed molecular interactions known between KEAP1 and NRF2 [22]. Here, we use a much-simplified version of the model (denoted as Model I) to illustrate the main mechanism of ultrasensitivity.

Model I: The structure of the dynamic model is presented in Fig. 2A, which includes saturable NRF2 binding to KEAP1, KEAP1-dependent NRF2 degradation, and inhibition of NRF2 degradation by electrophilic stressor S. Association of NRF2 with KEAP1 has dual consequences: it (i) titrates/sequesters and (ii) degrades NRF2. Simulations show that with a high binding affinity between KEAP1 and NRF2, free NRF2 ($NRF2_{free}$) can exhibit ultrasensitivity, i.e., a steep steady-state dose-response with respect to S (Fig. 2B). The degree of ultrasensitivity is enhanced (reduced) when the binding affinity is increased (decreased) as illustrated by changing the association rate constant k_f (Fig. 2C). Consistent with the role of molecular titration in generating ultrasensitivity, the abundance of KEAP1 also modulates the degree of ultrasensitivity. Although resulting in a higher basal $NRF2_{free}$ level, decreasing KEAP1 abundance reduces the ultrasensitivity of the $NRF2_{free}$ response (Fig. 2D). In summary, when electrophilic chemicals block KEAP1-mediated NRF2 degradation, accumulating NRF2 from *de novo* synthesis can escape KEAP1 sequestration, resulting in an abrupt increase in free NRF2.
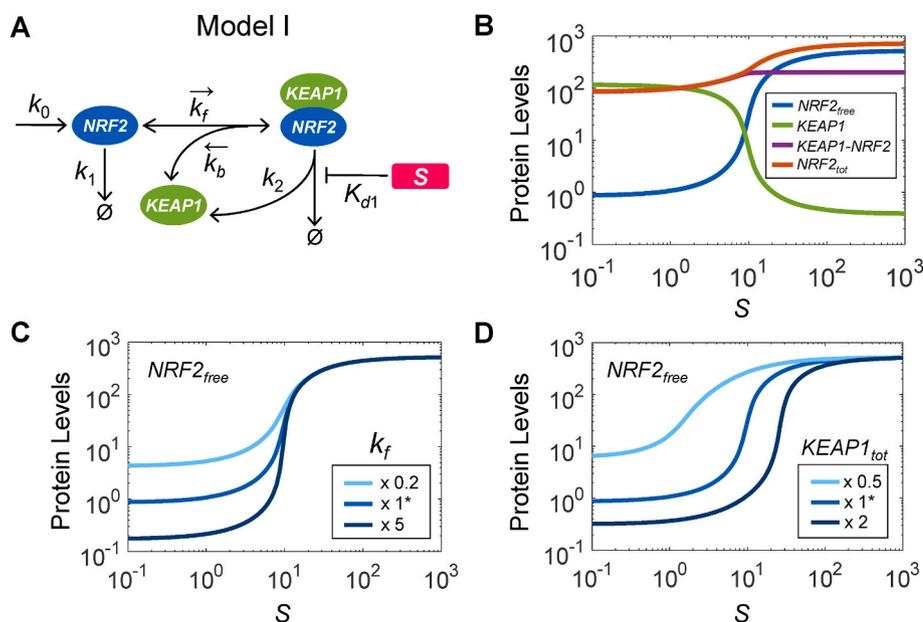
2.2. Multistep signaling leading to nuclear NRF2 accumulation

Background: Inhibition of KEAP1-dependent destabilization of NRF2 is not the only way by which the cellular and particularly nuclear NRF2 abundance is increased. Additional mechanisms have evolved, including enhancement of NRF2 translation and nuclear retention. Augmented NRF2 translation was first observed in rat cardiomyocytes treated with H_2O_2, in the absence of increased mRNA level and protein half-life of NRF2 [44]. Subsequently, a functional internal ribosome entry site (IRES) was identified in the 5′-UTR of the NRF2 mRNA sequence [45]. This IRES activity is inhibited at basal conditions, but under oxidative stress such as by H_2O_2 and sulforaphane, it becomes the major translation initiation site, resulting in enhanced NRF2 translation despite that the 5′ cap-dependent global translation is inhibited. Enhanced NRF2 translation through the cap-independent, IRES-dependent mechanism was further demonstrated in HepG2 cells treated with (R)-α-lipoic acid, a dithiol redox-active compound that can induce phase II enzymes [46]. The enhancement of NRF2 translation is facilitated by multiple underlying mechanisms. Redox-sensitive phosphorylation of eIF2α may be involved in the switching of the ribosome entry site [45]. It was also demonstrated that under H_2O_2 treatment, La autoantigen La/SSB translocated from the nucleus into the cytosol where it binds to the IRES of NRF2 mRNA to enhance its translational activity [47]. A G-quadruplex structure was recently identified in the 5′ UTR of NRF2 mRNA which plays a crucial role in mediating H_2O_2-induced translation enhancement, and it appears to involve elongation factor 1α, whose association with NRF2 mRNA is increased by H_2O_2 [48].

A 3′ open reading frame (ORF) of the NRF2 mRNA sequence has been identified as a repressive mechanism suppressing NRF2 translation under basal conditions [49]. This repression can be released by certain natural compounds such as apigenin, resveratrol, and piceatannol that induce enhanced NRF2 translation [50]. The release of translational repression may involve calcium/calmodulin-dependent protein kinase kinase 2 and AMP-activated protein kinase (AMPK). However, classical NRF2 activators such as H_2O_2, *tert*-butyl hydroperoxide, or paraquat do not affect the 3′ ORF-mediated translation suppression, suggesting that this mechanism of translation enhancement is compound-specific. Lastly, a variety of microRNA species, including miR153, miR27a, miR142-5p, miR144, and miR28, are also involved in regulating NRF2 posttranscriptionally through interacting with the 3' UTR of NRF2

Fig. 2. Model I: NRF2 ultrasensitivity arising from saturable NRF2 sequestration and degradation by KEAP1. (A) Structure of Model I, containing constitutive synthesis of $NRF2$ (k_0 step), $KEAP1$-independent $NRF2$ degradation (k_1), reversible binding of $NRF2$ and $KEAP1$ (k_f and k_b), and $KEAP1$-dependent $NRF2$ degradation and concurrent recycling of $KEAP1$ (k_2) which can be inhibited by electrophilic stressor S (K_{d1}). **(B)** Simulated steady-state dose-response of various state variables as indicated, with free NRF2 ($NRF2_{free}$) exhibiting ultrasensitivity. **(C)** The degree of ultrasensitivity of the $NRF2_{free}$ response is modulated by the binding affinity between $NRF2$ and $KEAP1$ as indicated by different k_f values. **(D)** The degree of ultrasensitivity of the $NRF2_{free}$ response is modulated by the total abundance of $KEAP1$ ($KEAP1_{tot}$) as indicated. x 1* denotes using the default parameter values, x 0.5 and x 2 denote using half and twice the default values respectively.

mRNA, which may be subject to regulation under oxidative stress [51, 52].

Although KEAP1 is the canonical redox sensor for NRF2 activation, the NRF2 protein itself is also redox-sensitive. The Neh5 transactivation domain of NRF2 contains a chromosomal maintenance 1 (CRM1 or exportin 1)-dependent nuclear export signal (NES_{TA}), which hosts a redox-reactive cysteine residue at position 138 [53]. Under oxidative and electrophilic stress, the NES_{TA} activity is lost due to the modification of the cysteine residue. As a result, the NRF2 cytosol-nucleus equilibrium shifts to disfavor NRF2 nuclear exportation, leading to increased NRF2 retention in the nucleus even in the absence of increased total cellular NRF2 abundance. The increased nuclear retention of NRF2 is also facilitated by the colocalization of another NES in the leucine zipper (ZIP) domain (NES_{ZIP}), which is masked upon NRF2 heterodimerization with MafG, a small Maf protein, via the ZIP–ZIP interface as the masking precludes NRF2 and CRM1 binding [54]. It has also been reported that nitrosative stress can result in S-nitrosylation of CRM1 at C528 or C585 in its NES-binding domain, which inhibits its interaction with NES-bearing proteins including NRF2, contributing to NRF2 accumulation in the nucleus [55].

In summary, oxidative stress-induced nuclear NRF2 accumulation is a consequence of multiple independent processes – (i) stabilization and (ii) enhanced translation of NRF2, both resulting in increased cellular NRF2 abundance, and (iii) repressed nuclear exportation. In theory, these multistep signaling processes can generate a synergistic effect, leading to an ultrasensitive increase of NRF2 in the nucleus. Below we use a simple mathematical model to illustrate this potential ultrasensitivity through multistep signaling.

Model II: The structure of the model is presented in Fig. 3A, which includes the 3 steps described above leading to nuclear NRF2 ($NRF2_n$) accumulation. Simulations show that when the 3 steps are all operating, $NRF2_n$ exhibits ultrasensitivity, with the maximal slope, or local response coefficient (LRC_{max}), slightly greater than 2 (Fig. 3B). Based on the convergence theory of signal transfer, 3-step synergistic signaling can achieve an LRC_{max} of 3 in ideal situations when each step mediates a proportional response [56]. In comparison, cytosolic NRF2 ($NRF2_c$) also exhibits a slightly ultrasensitive response with LRC_{max} greater than 1. This is because only signaling steps (i) and (ii) impinge on $NRF2_c$, so in theory LRC_{max} is 2 at best. When only one step of NRF2 activation is present, the $NRF2_n$ ultrasensitivity is lost (Fig. 3C). When two steps are present, some $NRF2_n$ ultrasensitivity still remains (Fig, 3D). As mentioned above, the degree of ultrasensitivity can be affected by many factors and ideal conditions may not be present in cells. For example, the translational enhancement induced by stressors may not mediate a proportional response if there still exists IRES-independent translation, which will reduce the sensitivity in this step. The inhibitory effect on nuclear exportation would require the fluxes between cytosolic and nuclear NRF2 to establish a near equilibrium condition to be optimal for a proportional nuclear accumulation [22].

2.3. Positive autoregulation of NRF2 and sMaf via transcriptional self-induction

Background: While the cellular NRF2 abundance can increase under oxidative stress through protein stabilization and enhanced translation as described above, it is also regulated at the transcriptional level. The proximal region (1 kb) of the NRF2 gene promoter contains two ARE-like sites and chromatin immunoprecipitation assays demonstrated direct binding of NRF2 protein to its own promoter [57]. Treatment with oxidative chemicals such as 3H-1,2-dithiole-3-thione (D3T), arsenic, and hypochlorous acid can indeed increase the NRF2 mRNA level in a variety of cell types including keratocytes and macrophages [57–59]. These results indicate that NRF2 forms a transcriptionally-mediated positive autoregulatory feedback loop to enhance its own expression.

To be transcriptionally active, NRF2 has to partner with members of the sMaf protein family, including MafF, MafG, and MafK, to form a heterodimer that can effectively recognize and bind to the ARE (synonym for CNC-sMaf binding element, i.e., CsMBE) sites of target genes with high affinity [10,11,60]. As a basic region leucine zipper (bZIP)-type protein, sMafs can homodimerize, but the homodimer lacks the transactivational domain thus it can only act as transcriptional repressors [61]. The sMaf homodimer binds to Maf recognition elements (MAREs) that are similar but different than the AREs/CsMBEs commonly found on the antioxidant genes [62–64], therefore it is not expected that the homodimer will compete with NRF2-sMaf to regulate the promoter activity of these genes. The gene expression of these sMaf proteins themselves is also subject to regulation by the NRF2-sMaf heterodimer. For instance, in HepG2 cells all three sMaf proteins can be transcriptionally induced by treatment with pyrrolidinedithiocarbamate (PDTC) and phenylethyl isothiocyanate (PEITC), with the
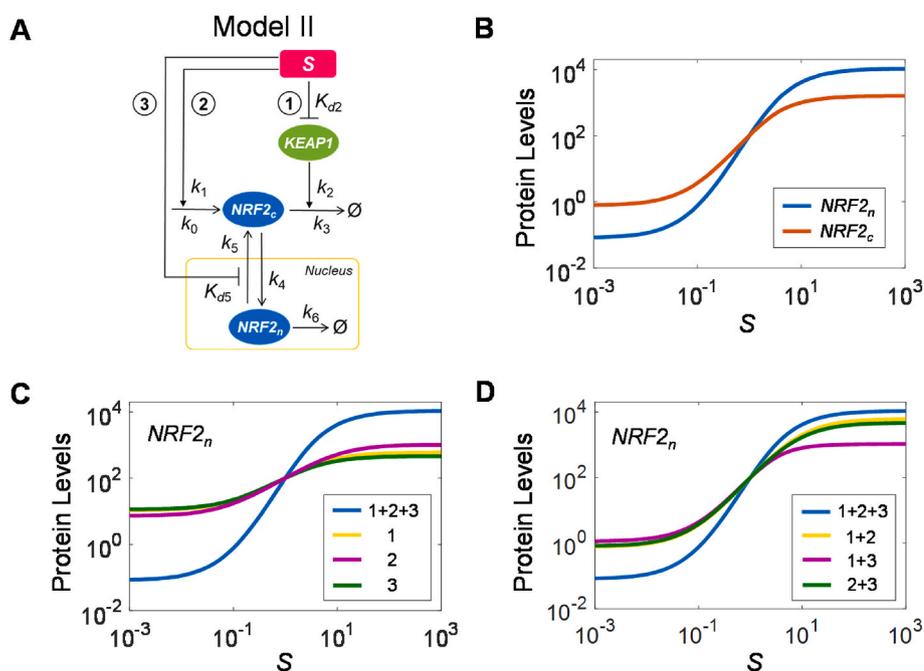

Fig. 3. Model II: Nuclear NRF2 ultrasensitivity arising from multistep signaling. **(A)** Structure of Model II, containing basal synthesis of cytosolic NRF2 ($NRF2_c$) (k_0) and stressor *S*-induced synthesis due to translation enhancement (k_1), *KEAP1*-independent $NRF2_c$ degradation (k_3), *KEAP1*-dependent $NRF2_c$ degradation (k_2), inhibition of *KEAP1* activity by *S* (K_{d2}), importation of $NRF2_c$ into the nucleus (k_4), exportation of nuclear NRF2 ($NRF2_n$) (k_5) which is inhibited by *S* (K_{d5}), and $NRF2_n$ degradation (k_6). **(B)** Simulated steady-state dose-response of $NRF2_c$ and $NRF2_n$, with the latter exhibiting a higher degree of ultrasensitivity. **(C)** Loss of ultrasensitivity of $NRF2_n$ when only one of the three steps, (i) $NRF2_c$ stabilization, (ii) enhanced $NRF2_c$ translation, and (iii) inhibited $NRF2_n$ exportation, is present as indicted. **(D)** Reduced ultrasensitivity of $NRF2_n$ when two of the three steps are present as indicted.

induction of MafF the strongest [65]. PDTC also induced these sMafs in a variety of other human cell types. However, t-butylhydroquinone (tBHQ), a strong NRF2 activator, failed to induce any sMaf expression. Diethyl maleate (DEM) can induce MafG expression in mouse embryonic fibroblasts [12,13]. In the same study it was further demonstrated that one ARE motif is located upstream the first exon Ic of the MafG gene, which is conserved in mice and humans. Chromatin immunoprecipitation demonstrated that NRF2 and sMafs can indeed bind to this ARE site. H_2O_2 can induce Adapt66, an MafG homolog, in HA-1 hamster fibroblasts although its role in NRF2-mediated oxidative stress response is unclear [66]. Taken together, these results indicate that depending on the cell types and oxidative chemicals, a transcriptionally-mediated autoregulatory loop also exists for sMaf proteins.

The autoregulation of both NRF2 and its partner sMaf protein forms coupled positive feedback loops, which can generate potential ultrasensitivity for their transcriptional activity. In addition, the convergence of transcriptional upregulations of NRF2 and sMaf into an enhanced formation of NRF2-sMaf dimer is also a multistep signaling URM. Therefore, in this case two URMs, positive feedback and multistep signaling, are intertwined to generate potential strong signal amplification. Below we use a simple mathematical model to illustrate this complex ultrasensitivity.

Model III: The structure of the model is presented in Fig. 4A, which includes the dual transcriptional induction of *NRF2* and *sMaf* by the *NRF2-sMaf* dimer and the reversible formation of the heterodimer. As the stressor *S* level increases to stabilize *NRF2* through inhibiting its degradation, both the total *NRF2* and total *sMaf* abundances increase and exhibit strong ultrasensitivity (Fig. 4B). The *NRF2-sMaf* dimer, which is the output here, exhibits the highest degree of ultrasensitivity compared with free *NRF2* and free *sMaf* which decreases at high S levels due to sequestration by *NRF2* that continues to rise. When one of the two positive feedback loops is absent, the degree of *NRF2-sMaf* ultrasensitivity is considerably reduced, but still stronger than when both loops are absent (Fig. 4C). The two autoregulatory loops do not necessarily function in all cell types, suggesting that their availability is a regulated process.
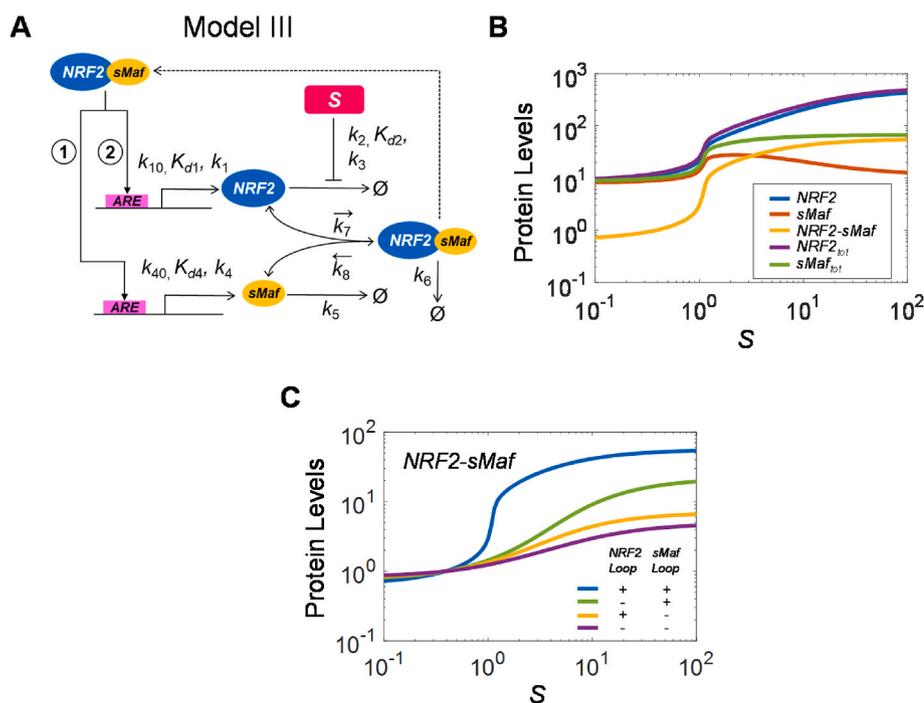
2.4. Positive autoregulation of NRF2 via transcriptional induction of p62

Background: While KEAP1 represses NRF2 through protein sequestration and destabilization, lines of evidence indicate that KEAP1 itself can also be regulated by NRF2, forming a complex regulatory feedback loop between the two [67–70]. The regulation of KEAP1 by NRF2 is primarily mediated through the p62/SQSTM1 (sequestosome 1) protein, which is upregulated under various stresses and is a cargo receptor for autophagic degradation of ubiquitinated proteins, including KEAP1. It has been demonstrated that p62 is physically associated with KEAP1, via a KEAP1-interacting region (KIR) containing a DPSTGE motif that is structurally similar to the KEAP1-binding ETGE motif of NRF2 [67,71–73]. Depending on the phosphorylation status of the DPSTGE motif, its binding affinity for KEAP1 is comparable to or even higher than the DLG motif, which is the weaker KEAP1-binding motif of NRF2 [43,74].

p62 represses KEAP1 activity/abundance in two ways. First, by competing with NRF2 for KEAP1, p62 can disrupt the relatively weak, DLG-mediated association of NRF2 with the KEAP1 dimer, and higher p62 levels can further disrupt the stronger ETGE-mediated association of NRF2 with KEAP1 [75]. The "latched", closed state of the KEAP1-NRF2 complex, i.e., when both the DLG and ETGE motifs are bound to the two monomeric subunits of the KEAP1 dimer, is absolutely required for KEAP1-mediated NRF2 ubiquitination and subsequent proteasomal degradation [76,77]. Therefore, by forcing the KEAP1-NRF2 complex into the "unlatched", open state, i.e., disengaging the DLG binding, p62 inhibits the E3-ligase adaptor activity of KEAP1, resulting in NRF2 protein stabilization. Second, p62 downregulates KEAP1 abundance by promoting its degradation via autophagy. p62-mediated autophagy, as opposed to the proteasomal pathway, is the primary mechanism of KEAP1 degradation [78,79]. It has been demonstrated in a variety of cell types that by manipulating p62 levels through either ectopic expression or RNAi knockdown, KEAP1 protein abundance can be decreased or increased, respectively, without changes in its mRNA levels [71]. When p62 was depleted with RNAi in Hepa-1c1c7 cells, the half-life of KEAP1 nearly doubled, extending from 11.3 to 21.1 h [71]. The half-life of KEAP1 is regulated by oxidative and electrophilic stressors. For instance, in HepG2 cells, the half-life of KEAP1 is 12.7 h, but under treatment with

Fig. 4. Model III: Ultrasensitivity arising from coupled NRF2 and sMaf positive autoregulation and heterodimerization. (A) Structure of Model III, containing basal synthesis of *NRF2* (k_{10}) and *sMaf* (k_{40}), *NRF2-sMaf* dimer-induced synthesis of *NRF2* (k_1 and K_{d1}) and *sMaf* (k_4 and K_{d4}), stressor *S*-inhibited degradation of *NRF2* (k_2 and K_{d2}) and *S*-independent degradation of *NRF2* (k_3), degradation of *sMaf* (k_5), association (k_7) and dissociation (k_8) between *NRF2* and *sMaf*, and degradation of *NRF2-sMaf* dimer (k_6). **(B)** Simulated steady-state dose-response of *NRF2*, *sMaf*, and *NRF2-sMaf* as indicated, exhibiting strong ultrasensitivity. **(C)** The degree of ultrasensitivity of *NRF2-sMaf* is altered when the *NRF2* and *sMaf* autoregulatory loops are both present, only one is present, and both are absent.

electrophiles such as tBHQ, 1,2-naphthoquinone (1,2-NQ), or DEM, the half-life was shortened to 3.4, 10.4, and 7.1 h, respectively [79].

While p62 activates NRF2 through repressing KEAP1, p62 itself is also a transcriptional target of NRF2, thus forming a positive autoregulatory loop among the three proteins: KEAP1 –| NRF2 → p62 –| KEAP1. An ARE has been identified in the p62 promoter and immunoprecipitation and gel shift assays demonstrated that NRF2-MafG dimer can indeed bind to this sequence and induce transcriptional activity [72]. The endogenous p62 level was found to be positively correlated with that of endogenous NRF2 in various human cells [72]. In two cancer cell lines H1299 and A549, it was demonstrated that isodeoxyelephantopin can induce p62 and enhance autophagic flux in an NRF2-dependent manner [80]. Resveratrol, a plant-derived NRF2 activator, can also induce p62 with concomitant downregulation of KEAP1 in testicular cells of diabetic mice [81].

As an oxidative chemical, arsenic is a strong NRF2 activator, but recent studies revealed that the mechanism of NRF2 activation by arsenic appeared to be more complex than previously thought [67]. Arsenic can induce p62 in two ways depending on the dose level. Low-level arsenic exposures can somehow inhibit the autophagic flux [82,83]. As a cargo receptor for autophagy, p62 is normally incorporated into the autophagosome and degraded during the autophagy process [84]. By inhibiting autophagy, low-level arsenic exposure leads to accumulation of p62, which has been observed in lung epithelial cells [82]. Although the KEAP1 level will increase as a result of reduced autophagy under arsenic exposure, increased p62 will sequester more KEAP1 molecules, therefore NRF2 will be still activated despite the higher KEAP1 levels. Acute treatment with low-level arsenic also inhibited autophagic flux in HaCaT cells, leading to upregulation of p62 [85]. In contrast to low-level arsenic-induced p62 protein accumulation through inhibiting autophagy-mediated p62 degradation, high-level arsenic induced p62 transcriptionally as an oxidative stressor in an NRF2-dependent manner, which was observed in HaCaT cells *in vitro* and the epidermis of arsenic-treated mouse *in vivo* [86]. Long-term exposure of HaCaT cells to arsenic leading to malignant transformation also resulted in an NRF2-dependent p62 upregulation [85].

In summary, a positive autoregulatory loop exists between KEAP1, NRF2, and p62, involving (i) sequestration and destabilization of NRF2 protein by KEAP1, (ii) transcriptional induction of p62 by NRF2, and (iii) sequestration and autophagy of KEAP1 by p62. Ultrasensitivity of NRF2 activation may arise due to the positive feedback nature of this autoregulatory loop and the sequestration and autophagy of KEAP1 by p62 which may double as a multistep signaling URM. Below we use a simple mathematical model to illustrate potential ultrasensitivity through the coupled positive feedback and multistep signaling.

Model IV: The structure of the model is presented in Fig. 5A, which includes transcriptional induction of *p62* by *NRF2*, *p62* binding to *KEAP1*, increased *KEAP1* autophagic degradation, and *KEAP1*-dependent *NRF2* degradation. As the stressor *S* level increases to inhibit *KEAP1*-dependent *NRF2* degradation, the total *p62* abundance increases and total *KEAP1* abundance decreases (Fig. 5B). *NRF2*, which is the output here, exhibits strong ultrasensitivity. When the *p62*-mediated *KEAP1* autophagic degradation is disabled or induction of *p62* by *NRF2* is disabled, the ultrasensitivity is reduced or lost (Fig. 5C).

2.5. Activation of NRF2 via nuclear exclusion of BACH1

Background: Containing a BTB domain at the N terminal, BACH1 belongs to the Cap'n'collar (CNC) basic leucine zipper (b-Zip) family of transcription factors which also include NRF2 [87]. Like NRF2, BACH1 can bind to sMaf and form a heterodimer [87,88]. Also like the NRF2-sMaf heterodimer, the BACH1-sMaf heterodimer recognizes CsMBEs, including the multiple AREs in the NAD(P)H quinone dehydrogenase 1 (NQO1) and heme oxygenase (HO-1) gene promoters [89–91]. Therefore, BACH1 competes with NRF2 in two fronts. First, it competes with NRF2 for binding to sMaf to form respective heterodimers. Second, the two heterodimers compete against each other for binding to AREs. However, unlike the NRF2-sMaf heterodimer which acts as a transcriptional activator for antioxidant genes, BACH1-sMaf functions primarily as a transcriptional repressor, albeit in certain conditions also as an activator [87,88]. It has been demonstrated in a variety of cell types that BACH1-MafK inhibits NQO1 and HO-1 gene expression [89–91]. Using siRNA to silence BACH1, it was demonstrated in Huh-7 hepatocytes that both the basal and heme-stimulated HO-1 expression can be upregulated [92]. But unlike sMaf, which does not have a domain to recruit corepressors and thus in its homodimeric form
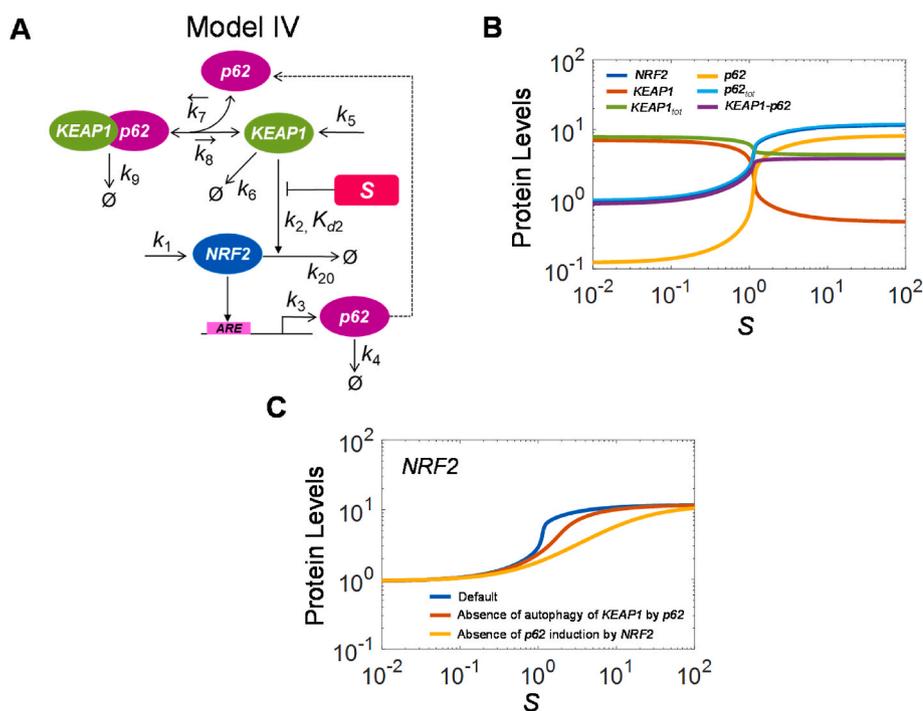

Fig. 5. Model IV: Ultrasensitivity arising from positive autoregulation of NRF2 through p62-mediated sequestration and autophagy of KEAP1. (A) Structure of Model IV, containing basal synthesis of *NRF2* (k_1), *KEAP1*-independent *NRF2* degradation (k_{20}), *KEAP1*-dependent *NRF2* degradation which can be inhibited by stressor *S* (k_2, K_{d2}), *NRF2*-induced synthesis of *p62* (k_3), basal degradation of *p62* (k_4), synthesis of *KEAP1* (k_5), *p62*-independent degradation of *KEAP1* (k_6), association (k_7) and dissociation (k_8) between *KEAP1* and *p62*, and autophagic degradation of *KEAP1*-*p62* complex (k_9). **(B)** Simulated steady-state dose-response of *NRF2*, *KEAP1*, and *p62* variables as indicated, exhibiting ultrasensitivity. **(C)** Ultrasensitivity of *NRF2* when (i) the *p62* autoregulatory loop and *p62*-mediated *KEAP1* sequestration and degradation are intact, (ii) *p62*-mediated *KEAP1* autophagy is absent (by setting $k_9=k_6$, and $k_5=1.27573E-4$ so that free *KEAP1* remains at the same basal level as in the intact model), and (iii) *p62* induction by *NRF2* is disabled such that *p62* remains at the same basal level as in the intact model.

can function only as passive repressors by occupying cognate MAREs [93], BACH1 has a corepressor-recruiting domain and can function as an active transcriptional repressor [94].

Under oxidative stress, the BACH1-mediated repression of antioxidant genes is released primarily through its nuclear exclusion by a variety of molecular mechanisms that appear to be chemical-specific. Cadmium-induced BACH1 nuclear exclusion involves its C-terminal conserved cytoplasmic localization signal (CLS), a non-canonical nuclear export signal, in a CRM1-dependent manner [95,96]. Phosphorylation of tyrosine 486 also seems to be essential for cadmium-induced nuclear exportation of BACH1 [97]. Arsenic-induced nuclear exclusion of BACH1 can occur as early as 30 min in HaCaT cells, which was accompanied by reduced DNA binding of BACH1 to the HO-1 promoter [91]. Accumulation of nuclear NRF2 didn't occur to appreciable levels till 3 h later and was not required in this case to induce HO-1 gene expression as the basal level of NRF2 is sufficient as long as BACH1 is inactivated [91]. tBHQ induced BACH1 nuclear exclusion within 2 h in HepG2 cells [97]. This exportation requires phosphorylation of tyrosine 486 near an NES and the C-terminal CLS and is CRM1-dependent. Redox-sensitive cysteine residues in BACH1 protein also play a role in the exclusion of BACH1 by chemical oxidants. Human BACH1 is a thiol-rich protein containing 34 cysteine residues, of which cysteine 574 and 557 seem to play a key role in the oxidative inactivation of BACH1 [98]. When the conserved cysteine 574 in the DNA-binding domain and cysteine 557 were substituted to serine, activation of BACH1-inhibited ARE transcriptional activity by sulfhydryl oxidizing agents diamide and 4-Hydroxy-2-nonenal can no longer occur; and the C574S substitution also inhibited diamide-induced cytoplasmic translocation of the GFP-BACH1 fusion protein [98].

In summary, oxidative stress induces BACH1 nuclear exportation through multiple mechanisms. As BACH1 exits the nucleus, it releases sMaf that was previously sequestered, which may cause a steep rise in the free nuclear sMaf concentration through reverse molecular titration, leading to an ultrasensitive increase of NRF2-sMaf formation. In addition, as the BACH1-sMaf level recedes, it will also give away more AREs to NRF2-sMaf that is simultaneously increasing in abundance. The simultaneous rise and fall of BACH1-sMaf and NRF2-sMaf levels respectively may add a multistep signaling mechanism for further

ultrasensitivity. Below we use a simple mathematical model to explore whether ultrasensitive NRF2 activation may arise through molecular titration and multistep signaling in the framework of NRF2, BACH1, and sMaf interactions.

Model V: The structure of the model is presented in Fig. 6A, which includes stressor S stimulated-nuclear exportation of $BACH1_n$, competition between $NRF2$ and $BACH1_n$ for binding to $sMaf$, and competition between $NRF2$-$sMaf$ and $BACH1_n$-$sMaf$ heterodimers for binding to ARE. In disagreement with the intuition above, we found that no ultrasensitivity can arise from this signaling scheme (Fig. 6B), even when the $sMaf$ abundance is limiting and the binding affinity between $BACH1_n$ and $sMaf$ is high, which are conditions that favor generation of ultrasensitivity through molecular titration. For molecular titration-mediated ultrasensitivity to occur, the system has to be able to reach a state that the total nuclear $BACH1$ abundance is close to the total $sMaf$ abundance, such that both free $BACH1_n$ and $sMaf$ are very low. At this point, a small decrease in the total $BACH1$ abundance would release $sMaf$ to join the very low, free $sMaf$ pool, producing a steep rise in the free $sMaf$ abundance. In the signaling scheme in Fig. 6A, only the free, not $sMaf$-bound, $BACH1_n$ is exported out of the nucleus. Since the free $BACH1_n$ is very low at the point of expected ultrasensitivity, the impact of S-stimulated $BACH1_n$ exportation on reducing total nuclear $BACH1$ abundance will be very limited. As a result of this subsensitive decrease in the total nuclear $BACH1$ in response to S, no ultrasensitivity arises through the operation of molecular titration. Although molecular titration does not produce ultrasensitivity here, we further argue that multistep signaling by $NRF2$-$sMaf$ and $BACH1_n$-$sMaf$, which compete for the ARE sites, may lead to some degree of ultrasensitivity, as suggested above. Interestingly, no ultrasensitivity arises either, even under the condition that free $BACH1_n$ is the dominant nuclear form (Fig. 6C). This lack of multistep ultrasensitivity is because as $BACH1_n$ moves out of the nucleus, the receding of $BACH1_n$-$sMaf$ and rising of $NRF2$-$sMaf$ never occur in a linear range simultaneously, therefore no synergy comes out of the competition of the two heterodimers for ARE. In summary, the BACH1 signaling scheme as in Fig. 6A is a case that intuition fails to correctly make even qualitative predictions in the absence of computational analysis of the underlying structure. If the subsensitive decrease in the total nuclear BACH1 abundance is the main reason that molecular
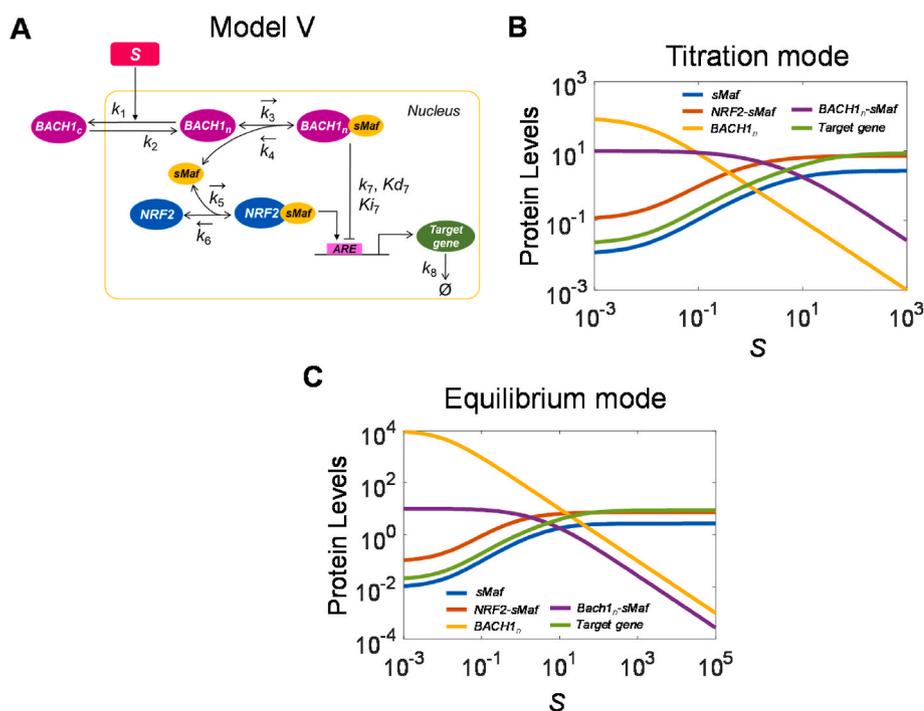

Fig. 6. Model V: Release of sequestration of sMaf and ARE by nuclear BACH1 exportation. (A) Structure of Model V, containing stressor S-induced nuclear exportation of $BACH1_n$ (k_1), nuclear importation of $BACH1_c$ (k_2), reversible binding between $BACH1_n$ and $sMaf$ (k_3 and k_4), reversible binding between $NRF2$ and $sMaf$ (k_5 and k_6), transcriptional activation of an ARE $Target$ $gene$ by $NRF2$-$sMaf$ and its competitive inhibition by $BACH1_n$-$sMaf$ (k_7, K_{d7}, K_{i7}), and degradation of the protein product of the $Target$ $gene$ (k_8). **(B-C)** Simulated steady-state dose-response of free $sMaf$, free $BACH1_n$, $NRF2$-$sMaf$, $BACH1_n$-$sMaf$, and $Target$ $gene$ expression as indicated, when the model operates in titration mode (B) where $k_3 = 0.1$, $k_4 = 0.01$, and total $BACH1 = 100$, thus $BACH1_n$-$sMaf$ is the dominant form of nuclear $BACH1$, or in equilibrium mode (C) where $k_3 = 0.01$, $k_4 = 0.1$, and total $BACH1 = 10000$, thus free $BACH1_n$ is the dominant form of nuclear $BACH1$, respectively. None of the variables exhibit ultrasensitivity.

titration fails to produce ultrasensitivity here, then in situations where the stressor can reduce the total BACH1 abundance readily, ultrasensitivity may occur. This scenario is demonstrated by the heme-induced BACH1 inhibition in the section below.

2.6. Multistep inhibition of BACH1 by heme

Background: As a prosthetic group in many hemoproteins, heme, once released from its host proteins, can be highly cytotoxic because its iron atom may catalyze the Fenton reaction, producing free radicals. Oxidative stress can accelerate the release of heme from its host proteins, thus worsening the stress condition. The detoxification of heme is mediated by HO-1, which breaks down heme into ferrous iron, carbon monoxide, and biliverdin. Therefore, HO-1 can be induced by both oxidative stressors and heme itself [99]. As a matter of fact, heme is a ligand of BACH1, which inhibits its transcriptional function [100]. As a result, there exists a negative feedback between heme and HO-1, i.e., heme –| BACH1 –| sMaf → NRF2 → HO-1 –| heme, which regulates heme homeostasis. NRF2 needs to be present but does not have to rise significantly above the basal level for the induction of HO-1 [91]. The heme-binding region of BACH1 is located in its C-terminal, which contains four evolutionarily conserved cysteine-proline (CP) dipeptide motifs [100]. Accumulating evidence revealed, as detailed below, that once bound to BACH1, heme inhibits BACH1 through 3 independent mechanisms: it (i) induces BACH1 nuclear exportation, (ii) inhibits BACH1 binding to the enhancers of repressed genes, and (iii) destabilizes BACH1 protein.

Heme induces BACH1 nuclear exportation: Just as described above for oxidative stressors, nuclear exclusion of BACH1 is also a key regulatory step induced by heme. In HaCaT cells treated with hemin, nuclear exclusion of BACH1 can be induced as early as 30 min [91]. In 293T cells, reducing intracellular heme levels by inhibiting heme synthesis with succinylacetone resulted in nuclear accumulation of BACH1, while conversely, hemin caused nuclear exclusion of BACH1 [96]. Heme induces the nuclear exportation of BACH1 through a novel CRM1-dependent NES on BACH1, which involves two heme-binding regulatory motifs, CP3 and CP4, and a hydrophobic region surrounding CP3 [96].

Heme inhibits DNA binding of BACH1-sMaf heterodimer: Heme binding to BACH1 inhibited the MARE-binding activity of BACH1-sMaf heterodimer in the enhancers of genes such as β-globin and HO-1, without affecting the formation of the dimer itself [89,96,100]. The inhibition of DNA binding induced by heme involves the four heme-binding motifs CP3-CP6 [100].

Heme promotes BACH1 degradation: Degradation of BACH1 by heme is another important regulatory mechanism. In NIH3T3 cells, hemin treatment destabilized BACH1, reducing its half-life from 2.8 to 0.6 h [101]. The enhanced instability requires ubiquitination of BACH1 by the E3 ubiquitin ligase HOIL-1 (heme-oxidized IRP2 ubiquitin ligase-1) followed by proteasomal degradation. The heme regulatory motifs involving the CP3-CP5 regions appear to be important for mediating the interaction between BACH1 and HOIL-1. Heme-regulated BACH1 degradation is exploited by cancers to enhance metastasis. It was recently demonstrated in lung cancer cells, adding hemin or increasing heme by adding H_2O_2 and diamide to the cell culture can reduce BACH1 protein levels whereas reducing heme by adding NAC increased BACH1 protein levels [102]. Hemin-induced BACH1 degradation was blocked by inhibiting the proteasome. Alternatively, it was also demonstrated in lung cancer cells that constitutive activation of NRF2 due to loss of KEAP1 function, which induced HO-1 and reduced the heme level, can stabilize BACH1 [103]. The heme-promoted BACH1 degradation is mediated by ubiquitin ligase Fbxo22 in this case. Enhanced BACH1 expression in these cancer cells accelerates tumor metastasis by activating a variety of metastasis-promoting pathways including glycolysis [102–104].

In summary, heme binding to BACH1 as a ligand causes (i) its nuclear exclusion, (ii) its loss of DNA (MARE) binding capacity when in the heterodimeric complex with sMaf, and (iii) its destabilization by promoting its ubiquitination. These regulations may function as multistep signaling to allow ultrasensitive de-repression of ARE-mediated target genes, which may explain, in part, the high-fold induction of HO-1 often observed in many oxidative conditions [59,91,105]. Below we use a simple mathematical model to explore whether ultrasensitivity may arise through the heme-regulated multistep repression of BACH1.

Model VI: The structure of the model is presented in Fig. 7A, which includes inhibition of BACH1 by heme via the 3 steps described above. For simplicity, sMaf and NRF2 are not included as the focus here is on the regulation of BACH1 itself by heme. Simulations show that when all the 3 steps are operating, both free *ARE* and $BACH1_n$-occupied *ARE* ($BACH1_n$-*ARE*) exhibit ultrasensitivity, with the maximal slope, or LRC_{max}, greater than 2 but less than 3 (Fig. 7B). When only one step of $BACH1_n$ inhibition is present, the ultrasensitivity is lost for free *ARE* (Fig. 7C) and also for $BACH1_n$-*ARE* when promotion of nuclear exportation or inhibition of *ARE* binding of $BACH1_n$ are the only regulated step (Fig. 7D). Interestingly, however, some ultrasensitivity of free *ARE* and $BACH1_n$-*ARE* remains when *BACH1* is destabilized by *Heme*. This occurs because the degradation of cytosolic BACH1 ($BACH1_c$) promoted by *Heme* causes the cytosol-nucleus *BACH1* balance to shift toward more $BACH1_n$ moving out of the nucleus, while $BACH1_n$ in the nucleus is also being degraded simultaneously, therefore rendering a multistep signaling scheme, although *Heme*-promoted degradation is seemingly the only signaling step. When two steps of *BACH1* inhibition by *Heme* are present, the ultrasensitivity remains but is generally weakened compared with when all 3 steps are present (Fig, 7E and 7F).

2.7. Other potential ultrasensitive mechanisms

While we focused above on a multitude of signal-amplifying, ultrasensitive mechanisms unique to the core of the KEAP1-NRF2-ARE pathway, there are many other molecular regulations in the pathway that may also contribute to ultrasensitive NRF2 activation and target gene induction, which we briefly summarize below.

Phosphorylation of NRF2: Phosphorylation of NRF2 in multiple Neh domains plays an important role in regulating NRF2 under both physiological and stress conditions. The phosphorylation is mediated by a variety of kinases, including PKC, GSK-3, AMPK, CK2, PERK, CDK5, and MAPKs, and depending on the sites of phosphorylation, these phosphorylation events regulate the stability, activity, and subcellular distribution of NRF2 [106]. In theory, phosphorylation and dephosphorylation of a protein substrate form a covalent modification cycle (CMC), which is a robust URM that can produce highly ultrasensitive responses [107]. According to the parameter conditions required for CMC-mediated ultrasensitivity, ultrasensitive NRF2 activation by phosphorylation would occur when the kinases operate near saturation by NRF2, and the accumulation of NRF2 due to phosphorylation-induced stabilization may further enhance the degree of CMC-mediated ultrasensitivity [108].

Cooperative NRF2 binding to multiple AREs: Multiple AREs are present in the promoters of many genes of the NRF2 regulon [109,110]. When these AREs are closely clustered or near each other through DNA looping, positive cooperative binding of the NRF2-sMaf heterodimer may occur. For instance, in the HO-1 promoter, the proximal E1 and distal E2 sites contain 2 and 4 closely clustered AREs, respectively, which were demonstrated to have strong binding with NRF2 in stressed conditions or with BACH1 in nonstressed conditions [91]. These multiple AREs provide opportunities of positive cooperative binding by the NRF2-sMaf heterodimer, which is another important mechanism of ultrasensitivity [19]. Recently it has been further demonstrated that localized transcription factor clustering through cooperative interactions with coactivators or mediator proteins can form phase-separated condensates on enhancers, which can render emergent behaviors including ultrasensitivity [111–113]. It is worth noting that
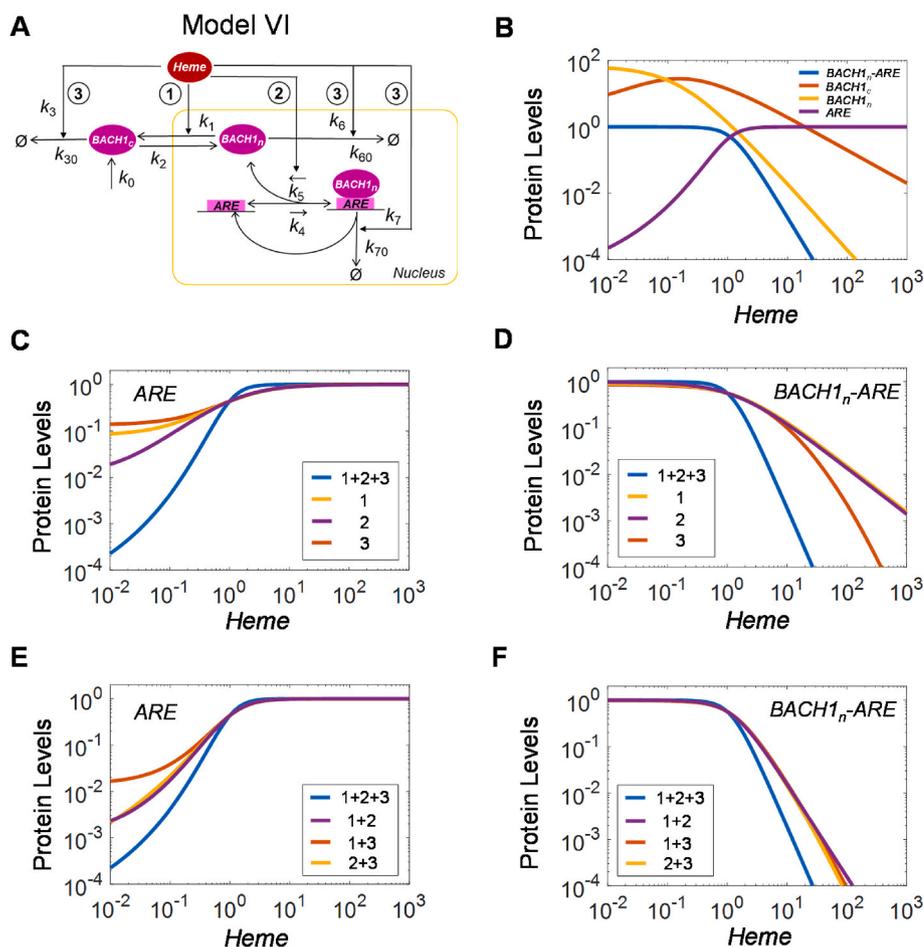
Fig. 7. Model VI: Ultrasensitivity arising from multistep inhibition of BACH1 by heme. (A) Structure of Model VI, containing basal synthesis of *BACH1* (k_0), basal degradation of *BACH1* (k_{30}, k_{60}, and k_{70}), *Heme*-stimulated degradation of *BACH1* (k_3, k_6, and k_7), *Heme*-stimulated nuclear exportation of $BACH1_n$ (k_1), nuclear importation of $BACH1_c$ (k_2), *Heme*-inhibited reversible binding between $BACH1_n$ and ARE (k_4 and k_5). **(B)** Simulated steady-state dose-response of $BACH1_c$, free nuclear $BACH1_n$, free *ARE*, and $BACH1_n$-*ARE* complex. Both free *ARE* and $BACH1_n$-*ARE* exhibit ultrasensitivity. **(C–D)** Ultrasensitivity of free *ARE* and $BACH1_n$-*ARE*, respectively, is lost or weakened when only one of the three *Heme*-regulated steps, (i) $BACH1_n$ nuclear exportation, (ii) $BACH1_n$ binding to *ARE*, and (iii) $BACH1_n$ degradation, is present as indicted. **(E–F)** Ultrasensitivity of free ARE and *BACH1-ARE*, respectively, is weakened when two of the three steps are present as indicted.

clustered homotypic DNA binding sites do not necessarily always lead to cooperative binding by transcription factors, which requires steric interactions between neighboring DNA elements and bound proteins. Noncooperative, independent binding of transcription factors to clustered sites clearly occurs, which can generate graded responses, as demonstrated in the NFκB system [114]. Such noncooperative interactions are also likely to occur between NRF2-sMaf and clustered AREs.

Homomultimerization of mature antioxidant proteins: Another important ultrasensitive mechanism is homomultimerization of proteins, including homodimerization, homotrimerization, and homotetramerization, etc. [19]. As a reversible process, homomultimerization takes two or more identical protein monomers to form a mature, functional protein multimer. In general, the degree of ultrasensitivity is positively correlated with the order of homomultimers, i.e., 2, 3, or 4 for homodimers, homotrimers, and homotetramers, respectively, especially when the multimers are proteolytically more stable than the monomers. It is thus perhaps not surprising that most of mature antioxidant enzymes, including glutathione reductase, glutaredoxin, peroxiredoxin, glutathione peroxidase and catalase, exist as either homodimers or homotetramers rather than as monomers [4]. Homomultimeric formation of these antioxidant enzymes likely plays a key role in enhancing the overall signal amplification in the adaptive oxidative stress response.

3. Discussion and conclusions

3.1. Integration of multiple ultrasensitive modules in the KEAP-NRF2-ARE pathway

In this review, we examined a number of key events in the canonical KEAP-NRF2-ARE signaling pathway from a quantitative perspective with a focus on signal amplification. With mathematical models, we illustrated that regulated protein sequestration, degradation, translation, nuclear trafficking, DNA promoter binding, and transcriptional induction – which are embedded in the molecular network containing KEAP1, NRF2, sMaf, p62, and BACH1 – may generate highly ultrasensitive NRF2 activation and hence antioxidant gene induction. The emergence of ultrasensitivity depends on the parameter conditions, which ultimately depend on the strengths of protein-protein and protein-DNA interaction and protein abundances. With the example of nuclear BACH1 exportation, we demonstrated that intuitions can be sometimes inadequate to make qualitative predictions without resorting to mathematical analysis.

Although we analyzed each of the potential ultrasensitive modules individually (Figs. 2–7), in cells they do not operate alone. Rather, they are intertwined with one another and work together as a complex system (Fig. 8). Each of the ultrasensitive module may provide only a small degree of signal amplification, but together these small amplifications can add up or even synergize, producing a much higher degree of amplification. Highly amplified NRF2 activation provides a high loop gain to the overall negative feedback circuitry of the antioxidant response (Fig. 1), which is crucially important in inducing adequate levels of antioxidants to limit oxidative damage and restore redox homeostasis under fluctuating oxidative and electrophilic conditions [4, 21]. It has been observed that basal intracellular ROS, especially H_2O_2, levels are maintained within a very narrow range (<2 fold) across multiple cell types, while the NRF2 abundance varies greatly (>20 fold) [115]. This pattern of low ROS variation and high NRF2 variation is consistent with the properties of a high-loop-gain negative feedback underpinning adaptive biological responses. For instance, in the
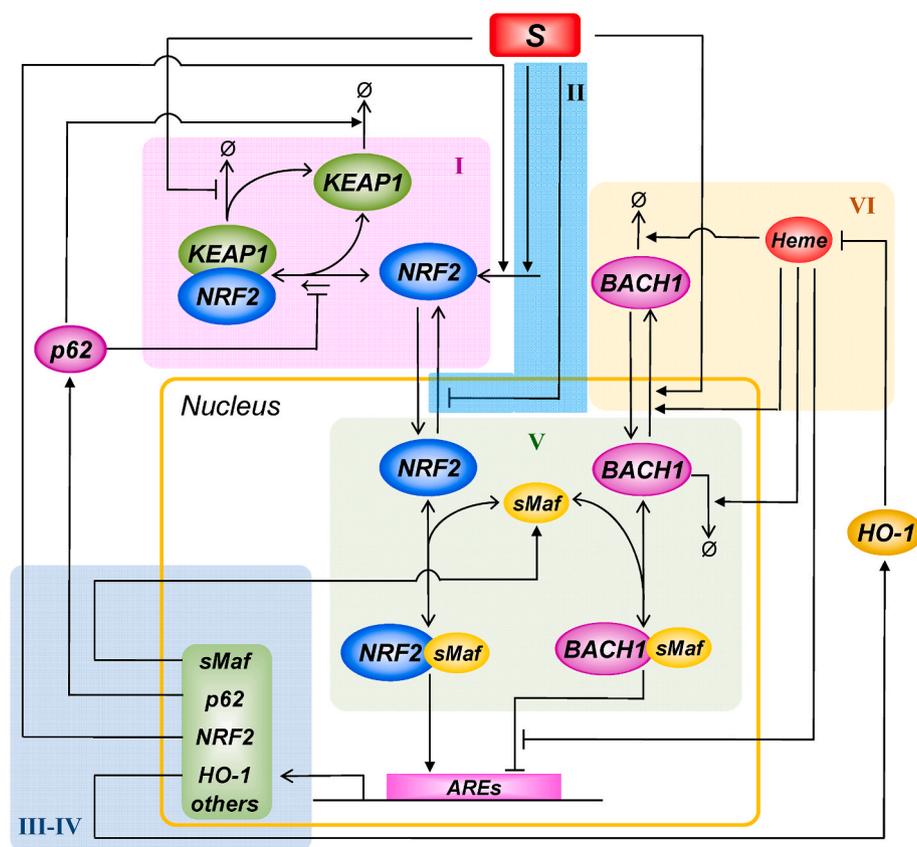
Fig. 8. A global view of the KEAP1-NRF2-ARE signaling pathway integrating multiple ultrasensitive modules of molecular interactions. The modules, corresponding approximately to models I-VI, are shaded and numerically labeled. *S*: oxidative or electrophilic species.

hypothalamic-pituitary-thyroid (HPT) axis where the thyroid hormone hemostasis is maintained by the HPT negative feedback, a similar pattern of variations exists between the plasma thyroxine (T4) level, which is narrowly ranging, and the thyroid-stimulating hormone (TSH) level, which is widely ranging [116].

Multiple regulations of a signaling protein or cascade are often viewed as redundancies evolutionarily accumulated for biological robustness such that the organisms can better cope with genetic mutations or environmental stresses. One example in the KEAP1-NRF2-ARE pathway is the multistep signaling leading to nuclear NRF2 accumulation (Fig. 3), where oxidative stressors may simultaneously increase NRF2 stability, translation and nuclear retention [38,45,53]. Another example is the multistep signaling of heme-induced release of nuclear BACH1 repression (Fig. 7), where BACH1 stability, nuclear localization, and ARE binding are inhibited by heme [96,100,101]. In the context of signal amplification, these multistep regulations are more than redundancy. As illustrated with the mathematical models, each step actively contributes to ultrasensitivity by some varying extent. An advantage of such cumulative augmentation of ultrasensitivity is that when mutations occur disabling a nonessential regulatory step, its impact on the overall signal amplification can be still limited. At such compromised conditions, although the operation of the antioxidant response may no longer be optimal, it can still be strong enough for cells to withstand most oxidative challenges. As our knowledge in the molecular details of the KEAP1-NRF2-ARE pathway continues to expand, more potentially ultrasensitive interactions are expected to emerge.

It is worth noting though that not all regulations of the KEAP1-NRF2-ARE pathway are equal and dispensable without major impact. There is a clear hierarchy of importance and the relative importance of each regulation can be physiologically and cell-dependent. The stabilization of NRF2 via inhibition of KEAP1 still plays the major signaling role, as demonstrated by the failed NRF2 accumulation in cells under oxidative stress when the sensing cysteines of KEAP1 are mutated [117], and by constitutive activation of NRF2 in malignant cancer cells due to mutations in KEAP1 and/or NRF2, including their binding motifs [118]. Therefore, centering around the stabilization of NRF2, many other regulations described here may play complementary roles, by enhancing ultrasensitivity in a progressive manner.

The multiple signaling events leading to NRF2 activation do not necessarily operate in all cells in all conditions. The dose-response range of each URM module, within which actual signal amplification occurs (i. e., the steep segment of the dose-response curve), is unlikely to be perfectly aligned to each other. As a result, the enhancement of ultrasensitivity may not be as strong as would be expected when these modules are simply put together. The interaction strengths between KEAP1, NRF2, sMaf, p62, BACH1 and ARE, as well as the protein abundances vary such that some of the URM modules operate only in certain cells under certain conditions. For instance, the cellular abundance of KEAP1 can be regulated transcriptionally by multiple actors [70]. The protein abundances of KEAP1 and NRF2 also fluctuate with a circadian rhythm, which may modulate the ultrasensitivity of NRF2 through the day [119]. Therefore, different combinations of these URM modules can exist in different cells, producing specific spatiotemporal activation of NRF2 and transcriptional and cellular responses to oxidative insults.

While we examined the ultrasensitivity of the KEAP1-NRF2-ARE pathway output in the context of a single ARE or promoter in isolation, it is worth noting that the competition of all accessible AREs in the entire genome for the binding of NRF2-sMaf dimer may play an important role in the transcriptional output of antioxidant genes. Base on sequence alone, it has been estimated that there are approximately 2.4 million putative AREs in the human genome [120], among which over 10 thousand may have strong binding affinity with NRF2-sMaf [121]. Because of epigenetic modifications and local chromatin

conditions, many of these potential binding sites are likely to be inaccessible. But a number of chromatin immunoprecipitation sequencing studies demonstrated that NRF2 can still bind to hundreds to thousands of chromosomal regions each containing one or multiple AREs [62,120, 122–124]. In the presence of a high number of non-cooperative, low-affinity ARE sites, a stoichiometric titration effect may arise for nuclear NRF2-sMaf heterodimers. Such configuration is known to produce linear transcriptional output [125,126]. In addition, when the amount of NRF2-sMaf is limiting, abundant high-affinity AREs may titrate the heterodimer away from low-affinity ARE sites, producing stress dose-delayed, but sigmoidal induction of genes harboring the low-affinity AREs, as demonstrated with other transcription factors [127]. Therefore, at the NRF2/ARE interface, depending on the binding affinity, and relative abundance of NRF2-sMaf dimer and accessible AREs in the genome, graded or ultrasensitive gene induction may be driven by nuclear NRF2.

The molecular events leading to NRF2 activation can be exploited or rewired to generate novel signaling by cancer cells to gain survival and growth advantages. Nestin, an intermediate filament (IF) protein transiently expressed during embryonic development, is also highly expressed in many cancer cells [128]. It was recently demonstrated that the Nestin protein possesses an ESGE motif in the C-terminal, which can competitively bind to the Kelch domain of KEAP1 and displace DLG-mediated NRF2 binding, thus acting as a class VI NRF2 activator [28]. Moreover, the Nestin gene promoter contains several AREs and it is a downstream target directly upregulated by NRF2 in non-small cell lung cancer (NSCLC). Therefore, a positive feedback is formed between Nestin and NRF2 in these cancer cells. Similar to the autoregulatory loop mediated by p62, this Nestin-mediated feedback may boost the ultrasensitivity of NRF2 activation and antioxidant induction, enhancing cancer malignancy and resistance to chemotherapy. Indeed, deleting Nestin sensitized NSCLC cells to oxidative stressors [28]. Heme-regulated BACH1 repression is also utilized by cancer cells [104]. KEAP1 mutation-induced NRF2 activation or treatment with exogenous antioxidants can lead to elimination of heme and stabilization of BACH1, which promotes metastasis of lung cancer [102,103]. The KEAP1-NRF2-ARE pathway can be hijacked by viruses to facilitate viral replication. In HCV-infected hepatocytes, the expressed viral nonstructural (NS) protein NS3 can specifically sequester sMaf in the cytosol, which in turn sequesters NRF2 in the cytosol, preventing its nuclear translocation and antioxidant induction [129]. As a result, the oxidative stress induced by viral invasion is untamed, which will further facilitate viral replication and cell death.

3.2. Post-NRF2 activation

In the present article we reviewed multiple signaling events in the KEAP1-NRF2-ARE pathway by focusing on signal amplification at steady state. The adaptive antioxidant response is a complex dynamic process, however. When the oxidative stressor goes away, deactivation of NRF2 will ensue. The unwinding of the induced antioxidant state does not appear to be a passive relaxation process, rather, it seems to be facilitated. KEAP1 may play an important role in this aspect. KEAP1 contains an NES in the IVR domain, which shuttles KEAP1 out of the nucleus in a CRM1-dependent manner [130,131]. Oxidative stress can inhibit the NES activity leading to increased nuclear localization of KEAP1 and thus that of sequestered NRF2. Increased nuclear KEAP1 abundance has been observed in cultured cells under oxidative stress conditions [132]. In the nucleus, oncoprotein Prothymosin α can compete with NRF2 for binding to KEAP1, releasing NRF2 to activate target genes [130]. Upon recovery from oxidative stress, the CRM1-dependent nuclear exportation of KEAP1 can escort KEAP1-sequestered nuclear NRF2 back into the cytosol, helping to reset the transcriptionally induced cytoprotective genes to the basal state [133]. While both KEAP1 abundance and activity are regulated by p62 posttranslationally, KEAP1 can also be regulated transcriptionally by

NRF2 under certain conditions. It has been reported that KEAP1 is a downstream target of NRF2, with an ARE located in the proximal promoter of the KEAP1 gene [134]. In Hepa-1 and HepG2 hepatocytes, t-BHQ or DEM treatment can activate NRF2 and induce NRF2 binding to the promoter, resulting in transcriptional upregulation of KEAP1 [134, 135]. This forms a negative feedback loop between NRF2 and KEAP1. In Hepa-1 and HepG2 cells, tBHQ treatment first induced fast nuclear BACH1 exportation, but it was followed by delayed BACH1 nuclear re-accumulation [90,97]. This accumulation requires *de novo* protein synthesis with concomitant upregulation of BACH1 mRNA expression [97]. This regulated induction of KEAP1 and BACH1 may facilitate deactivation of NRF2 as the oxidative stress recedes.

3.3. Significance and implications

While cellular toxic, ROS especially H_2O_2 are also highly important second messengers mediating a variety of physiological signaling events [136–138]. Therefore, the cellular ROS and redox states have to be at a trade-off balance to prevent either an over-oxidized or over-reduced state [4]. Treating many disease conditions associated with oxidative stress or H_2O_2 signaling disruption requires fine-tuning of the cellular antioxidant capacity with high precision. A quantitative grasp of the KEAP1-NRF2-ARE signaling pathway, its URM modules, and the larger feedback and feedforward circuitry will help identify sensitive molecular entities and processes as therapeutic targets beyond the realm of direct or single-target NRF2 activators or inhibitors. As an example, synthetic triterpenoids CDDO-TFEA and CDDO-Me can have dual actions – they promote nuclear exclusion of BACH1 in addition to activating NRF2 through inhibiting KEAP1 [105]. Pharmacologically manipulating these targets to either enhance or suppress the cellular antioxidant capacity, aided with computational models, will lead the way toward precision medicine for redox-related diseases. Mathematical modeling has played an essential role in understanding the KEAP1-NRF2-ARE antioxidant response pathway and its responses to a variety of chemical stressors [21,22,115,135,139–146]. However, only a limited scope of interactions among the pathway components was explored in these earlier efforts. The URM modules and their integration as we laid out here will provide an important framework for future quantitative exploration of the antioxidant response pathway and its utility in systems pharmacology. The KEAP1-NRF2-ARE axis is also an important toxicity pathway, which if sufficiently perturbed, can lead to adverse health outcomes. The interactions between KEAP1, NRF2, sMaf, p62, and BACH1 represent key events (KEs) in the adverse outcome pathways (AOPs) that can be perturbed by many environmental oxidative chemicals. Monitoring the NRF2 dynamics in cells [147] and characterizing the KE relationship (KER) with mathematically modeling [135] will be crucial to developing quantitative AOPs for next-generation health risk assessment of a variety of environmental chemicals that perturb this important stress pathway [148,149].

Declaration of competing interest

The authors declare no conflict of interest.

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