Review

HIF1, HSF1, and NRF2: Oxidant-Responsive Trio Raising Cellular Defenses and Engaging Immune System

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ABSTRACT: Cellular homeostasis is continuously challenged by damage from reactive oxygen species (ROS) and numerous reactive electrophiles. Human cells contain various protective systems that are upregulated in response to protein damage by electrophilic or oxidative stress. In addition to the NRF2-mediated antioxidant response, ROS and reactive electrophiles also activate HSF1 and HIF1 that control heat shock response and hypoxia response, respectively. Here, we review chemical and biological mechanisms of activation of these three transcription factors by ROS/reactive toxicants and the roles of their gene expression programs in antioxidant protection. We also discuss how NRF2, HSF1, and HIF1 responses establish multilayered cellular defenses consisting of largely nonoverlapping programs, which mitigates limitations of each response. Some innate immunity links in these stress responses help eliminate damaged cells, whereas others suppress deleterious inflammation in normal tissues but inhibit immunosurveillance of cancer cells in tumors.
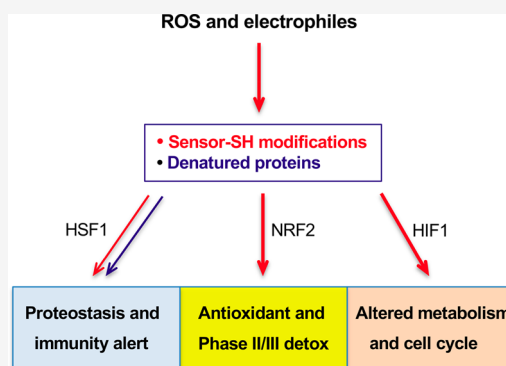
INTRODUCTION

Biological injury by oxidants and reactive electrophiles is causally linked to the development of major human pathologies such as neurodegeneration, atherosclerosis, diabetes, and cancer.[1−5] Chronic production of these toxic molecules also contributes to declining tissue functioning in aging. Endogenous reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive carbonyls, and other electrophiles produced during inflammation, normal metabolism, and lipid peroxidation are usually the major endogenous causes of chemical damage to proteins and DNA. Ambient pollution and the presence of various undesired xenobiotics in food and drinking water also lead to human exposure to a large number of exogenous toxicants. The most frequently modified/oxidized chemical group in proteins is Cys-SH, especially when cysteines are positioned next to positively charged amino acids.[6,7] Other targets for electrophile adduction in proteins include side chains of histidine, lysine, and arginine. DNA is also readily oxidized and chemically modified at various sites, especially in nucleobases. Protein damage induces a range of physiological dysfunctions, whereas DNA damage leads to mutations and, ultimately, to cancerous transformation of cells. There is a growing body of evidence that stable chemical modifications in histones by electrophilic substances are also capable of causing abnormalities in the epigenome.[8] Methylglyoxal, a reactive byproduct of glycolysis, was recently found to produce adducts at histone Lys and Arg side chains, which affected global transcriptome and histone acetylation.[9] The threat to cellular fitness posed by biological oxidants and reactive electrophiles is so great that all organisms contain specific protein sensors and protection responses against these toxic substances.

The NRF2-induced transcription program has long been viewed as a central cellular response to oxidative stress and the overabundance of reactive electrophiles. However, this response is limited in its ability to address several important vulnerabilities in stressed cells, such as accumulation of toxic damaged proteins and generation of oxidants by damaged mitochondria. Other conservative stress-sensitive transcription factors are also induced by the same types of reactive toxicants as NRF2, indicating that the full cellular response to oxidants includes much more than the NRF2 pathway. Here, we review mechanisms of cytosolic stress sensing and complementary defense activities governed by three oxidant-activated transcription factors HSF1, HIF1, and NRF2 that control the heat shock (unfolded proteins) response, hypoxia response, and antioxidant response, respectively. Although HSF1, HIF1, and NRF2 pathways have been extensively reviewed individually, analyses of their collaborative activities have been limited to the tumor state/microenvironment such as between HIF1 and NRF2 in tumor hypoxia.[10] The focus of this review is to provide an integrated view of major functions of HSF1, HIF1, and NRF2

as components of a global cellular defense against protein-reactive oxidants and electrophiles. HSF1 is an archaic stress-responsive factor with well-conserved mechanisms of activation and protective properties of its effectors between yeast to humans.[11,12] HIF1 and NRF2 are evolutionarily younger systems that are best characterized in mammals, but orthologue regulatory pathways are also known to exist in lower animals. The closest homologue of NRF2 in yeast is the transcriptional factor Yap1 which directly senses oxidants via the disulfide formation blocking its nuclear export and permitting trans-activation of its targets.[13] The human KEAP1-NRF2 pathway is a more versatile system that senses a much greater range of Cys-reactive toxicants, including those that form adducts. HIF1 is a metazoan transcription factor.[14]

NRF2 ANTIOXIDANT RESPONSE AND ITS LIMITATIONS

NRF2 is a constitutively expressed transcription factor, commonly known as a master regulator of the oxidative/electrophilic stress-induced response in cells. Despite its ongoing production, protein levels of NRF2 in normal cells are low due to its rapid destruction by 26S proteasomes.[15,16] The stability of NRF2 is primarily controlled by KEAP1, which is a substrate-binding component of the CUL3-RBX1 E3 ubiquitin ligase complex. KEAP1 also serves as a sensor of the cellular abundance of electrophiles in the activation of the NRF2 response. This electrophile-sensing potential of KEAP1 is created by the presence of numerous functionally important Cys-SH groups, which can be oxidized or adducted with the ensuing loss of the ubiquitin ligase activity toward NRF2 (Figure 1). In addition to its overall large content of Cys-SH (27 in humans, 25 in mice), KEAP1 sensitivity toward reactive toxicants is further enhanced by embedding its critical cysteines next to positively charged amino acids which lower the pK_a of the SH group.[15] Deprotonation of the SH group enhances its reactivity with carbonyls and the most common cellular ROS and RNS (H_2O_2, $O_2^{\bullet-}$, $CO_3^{\bullet-}$, $^{\bullet}NO_2$, $ONOO^-$, $HOCl$), which have higher reaction rates with thiolate (RS^-) than with RSH.[17] KEAP1 inactivation by damage to its sensory Cys-SH groups results in the accumulation of NRF2, its nuclear translocation, heterodimerization with bZip domain-containing proteins such as small MAFs, and transactivation of genes whose promoters contain the antioxidant response element sequence. In addition to its canonical regulation by inactivation of KEAP1 during electrophilic stress, NRF2 stability can be affected by other mechanisms which either diminish NRF2-KEAP1 interactions or engage an alternative degradation pathway of NRF2 involving its phosphorylation by GSK3β and then ubiquitination by the β-TrCP E3 ligase.[15,16] NRF2-induced genes are primarily involved in ROS detoxification (glutathione reductase, glutathione peroxidase, glutaredoxin, peroxiredoxin, thioredoxin), Phase II xenobiotics inactivation (NQO1, GSTA1, GSTA3, carbonyl reductase), glutathione biosynthesis (GCLC, GCLM), and regulation of heme and Fe metabolism (HMOX1, ferritin chains).[15,18,19] NRF2 also regulates expression of a Phase III detoxification protein MRP2 which excretes conjugates of electrophilic compounds with glutathione, glucuronate, or sulfate.[20]

NRF2 plays a dual role in carcinogenesis by inhibiting its initiation but promoting the progression of already transformed cells.[21,22] Consistent with its control of cellular defenses against reactive electrophiles, abrogation of NRF2 increased the susceptibility of animals to oncogene-, chemical-, and
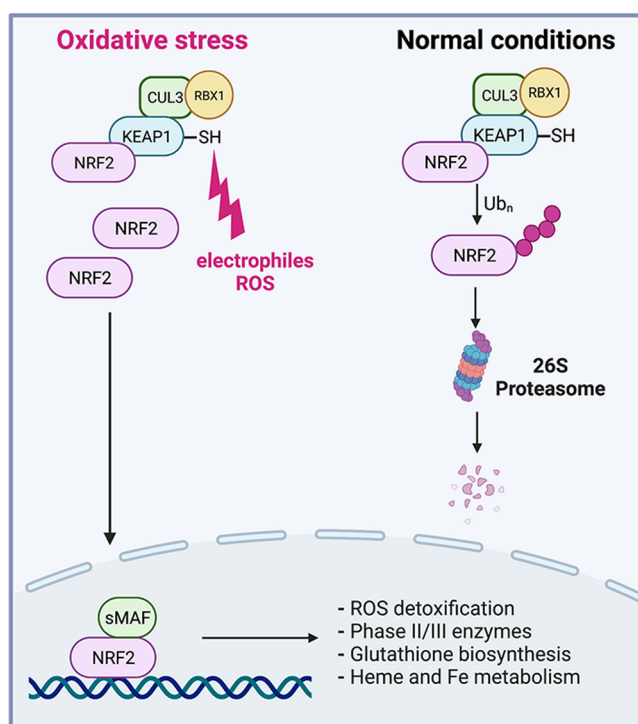

Figure 1. NRF2 regulation under normal and stress conditions. The transcription factor NRF2 is constitutively expressed, but its protein levels are low in normal cells due to its rapid destruction by 26S proteasomes. Constitutive polyubiquitination of NRF2 is performed by an E3 ubiquitin ligase complex containing SH-rich KEAP1 which serves as a sensor of cellular ROS and electrophiles in the activation of the NRF2 response. Damage to sentinel SH groups in KEAP1 causes its inactivation leading to the stabilization and accumulation of NRF2, its nuclear translocation, heterodimerization with bZip domain-containing proteins such as small MAFs, and transactivation of genes involved in many direct antioxidant activities such as ROS detoxification, Phase II/III detoxification reactions, glutathione biosynthesis, and iron/heme metabolism.

inflammation-induced cancers, as evident by the incidence and size of tumors.[22−27] However, once tumors were formed, NRF2 inactivation was detrimental to tumor growth.[26,27] In many human cancers, NRF2 is constitutively elevated due to its higher stability and mRNA expression.[21,22] Approximately 20% of lung cancers contain mutationally inactivated KEAP1, which eliminates constitutive ubiquitination and degradation of NRF2, and another 10% have activating mutations in this transcription factor. Another, more unexpected mechanism for persistent NRF2 activation in a large subset of human tumors is a recently discovered inactivation of KEAP1 via its neomorph binding by the oncogenic mutant BRAF[V600E].[28] BRAF mutations are particularly common in human epidermal melanoma, colon, and thyroid cancers. An analysis of TCGA data revealed that somatic gain-of-function mutations in NRF2 are common and correlate with poor prognosis.[29] Thus, cancers benefit from elevated protection against electrophile stress, which allows them to thrive in challenging growth conditions.

Activation of the NRF2 pathway does not appear to involve significant adjustments to cell cycle progression or respiratory metabolism in order to minimize consequences of the oxidant-induced damage to the genome or diminish the production and/or leakage of ROS by mitochondria, respectively. The NRF2 response also does not directly regulate the cellular capacity to stabilize unfolded damaged proteins or target them for

proteasomal degradation, although it can promote another protein quality control mechanism − autophagy.[30] The impact of the KEAP1-NRF2 pathway on the elimination of DNA double-stranded breaks (DSBs), the most toxic form of DNA damage by oxidants, is directed toward their repair via nonhomologous end-joining (NHEJ). NHEJ is error-prone but faster than the accurate repair of DSBs by homologous recombination, which requires a long-range resection of DSB ends and fully functional BRCA1 and BRCA2 proteins. KEAP1 inactivation leads to a stimulation of NHEJ through (i) the NRF2-mediated induction of 53BP1,[31] which acts as an antagonist of the homologous recombination factor BRCA1,[32] and (ii) stabilization of the second KEAP1 target EMSY, which suppresses BRCA2 functions.[33] For the vast majority of cells *in vivo*, which are either nondividing or rarely dividing, NHEJ activation is likely beneficial as homology-based repair can operate only in S and G2 phases, and a long-range resection of DSBs in G1 phase is genetically deleterious.[34] In actively proliferating cancer cells, however, mutational inactivation of KEAP1 results in the genomic instability and presents as a BRCA deficiency phenotype.[33]

■ HIF1 RESPONSE

HIF1 is the primary transcriptional mediator of the hypoxia response activated in all human cells by low O_2 or chemical agents acting as hypoxia mimetics.[35,36] HIF1 is a heterodimer-containing stable HIF1β and O_2-regulated unstable HIF1α. Similar to NRF2, HIF1α is a constitutively expressed protein that is present in normoxic cells at very low levels due to its continuous degradation by 26S proteasomes (Figure 2). This process is initiated by hydroxylation of Pro402 and Pro564 in HIF1α that are recognized by VHL, which acts as a substrate adaptor in the Cullin 2-elongin B/C-Rbx1 E3 ubiquitin ligase. HIF1α-targeting hydroxylases PHD1-3 are Fe(II)-dependent enzymes that split O_2 to hydroxylate Pro and the cosubstrate 2-oxoglutarate. In hypoxia, this Pro hydroxylation reaction is suppressed due to the lack of O_2. Stabilization of HIF1α by hypoxia or by drug-induced inhibition of its Pro hydroxylases activate a large transcription program that includes upregulation of several hundreds of genes.[37,38] This hypoxia adaptation response promotes cell survival by rewiring cellular energy metabolism, which includes shifting to O_2-independent, glycolysis-based production of ATP and decreasing nonessential metabolic activities. The metabolic switch that directs the glucose metabolite pyruvate away from mitochondria results from the inactivation of pyruvate dehydrogenase by the HIF1 target PDK1.[39,40] Other effects of HIF1 on mitochondrial metabolism include the substitution of the cytochrome c oxidase subunit COX4-1 with COX4-2 to improve efficiency of electron transfer to O_2 and lower ROS production.[41] HIF1 also upregulates expression of the NADH dehydrogenase NDU-FA4L2 that inhibits mitochondrial Complex I activity and thereby diminishes O_2 consumption and ROS formation.[42] In moderate hypoxia, however, ATP production by the mitochondrial respiratory chain is not yet significantly diminished, and the primary purpose of the switch to glycolysis in these conditions is to prevent excessive formation and leakage of mitochondrial ROS.[35] Consistent with this role, mitochondrial ROS are also important for the stabilization of HIF1α in hypoxia.[43−46]

Similar to the oxidant-sensing function of cysteines in KEAP1, several Cys-SH in the catalytic domain in PHD2 (the main HIF1α prolyl hydroxylase, also known as EGLN1) act as targets for inactivation by various ROS, RNS and toxic metals.[47−50]
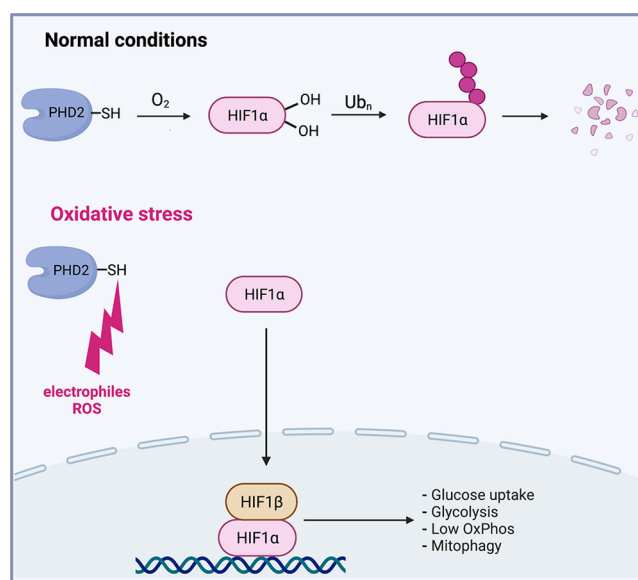

Figure 2. Regulation of HIF1 by O_2 and oxidants. The hypoxia-inducible transcription factor HIF1 is a heterodimer composed of stable HIF1β and O_2-regulated unstable HIF1α subunits. HIF1α is constitutively expressed, but it is present in normoxic cells at very low levels due to its degradation by 26S proteasomes. Hydroxylation of two Pro residues in HIF1α by the O_2-dependent hydroxylases PHD1-3 (primarily PHD2) acts as a trigger for its proteolytic ubiquitination. In hypoxia, Pro hydroxylation of HIF1α is suppressed due to the lack of O_2. Several Cys-SH in the catalytic domain of PHD2 (also known as EGLN1) are sensitive to oxidation or adduction by various ROS, RNS, and toxic metals, causing the loss of its Pro hydroxylation activity. Stabilization of HIF1α activates a specialized transcription program known as hypoxia response which stimulates glucose uptake, upregulates glycolysis, downregulates oxidative phosphorylation in mitochondria, and promotes mitophagy.

PHDs also sense the depletion of cellular antioxidants such as ascorbate,[51] which is important for the reduction of Fe(III) to Fe(II) in the catalytic center of these enzymes.[52] In addition to its accumulation due to inactivation of PHDs, human HIF1α is also stabilized by oxidants-induced covalent modifications at its Cys520 residue (Cys533 in mouse HIFα) such as glutathionylation (protein-glutathione disulfide) and S-nitrosylation which inhibit ubiquitination by VHL at the nearby Lys532 in the oxygen degradation domain.[53,54] Mitochondria can be damaged by a large number of endogenous and exogenous toxic agents, and the resulting distortions in the respiratory metabolism produce excessive amounts of ROS.[55,56] Thus, it is reasonable to view HIF1-driven suppression of the entry of pyruvate into the Krebs cycle[35] and, consequently, a decreased flow of electrons through the respiratory chain as a general protective response from ROS overproduction by diseased mitochondria, including in normoxic conditions. Upregulation of BNIP3 and BNIP3L by HIF1 also stimulates mitophagy which eliminates severely damaged mitochondria that generate especially large amounts of ROS.[35] In addition to ROS, mitochondrial stress also leads to cytosolic release of the Krebs cycle intermediate fumarate which activates HIF1 through inhibition of PHD2[57] and NRF2 via inactivation of KEAP1.[58,59] Fumarate is an α,β-unsaturated carbonyl that nonenzymatically reacts with proteins generating cysteine-S-succination adducts.[60] High levels of fumarate are also present in individuals with inactivating mutations in fumarate hydratase, where it acts as an oncometabolite causing leiomyomatosis and renal cell carcinoma.[61]

Increased expression of glycolytic factors by activated HIF1 is also important to counteract losses of the essential glycolytic enzyme GAPDH which is susceptible to oxidative inactivation, resulting in the collapse of ATP production during strong oxidative stress.[62] HIF1-induced routing of pyruvate into the production of lactic acid results in the increased acidification of cells, which together with increased utilization of NAD^+ leads to the global slowdown of cell cycle,[63] a beneficial response, preserving energy and genome integrity in stressed cells. Lowering cellular pH is also likely protective by globally decreasing oxidation of protein-SH groups due to their increased protonation. As discussed above, the protonated SH group is much less reactive with various oxidants and electrophiles than the thiolate anion (RS^-). Consistent with its importance in antioxidant protection, HIF1 depletion led to hyperactivation of NRF2 in cells experiencing moderate oxidative stress.[47] HIF1 is known to promote malignant characteristics in established cancers,[64] which already acquired metabolic adaptation neutralizing growth inhibitory effects of high HIF1.[63] In contrast, HIF1 activity in normal tissues is anticarcinogenic as evidenced by its role as a tumor suppressor gene in the development of human kidney cancer[65] and animal models of leukemia and squamous cell carcinoma.[66,67]

■ HSF1 RESPONSE

HSF1 is the primary transcriptional mediator of a rapid cellular response to proteotoxic stress known as the heat shock response. The main gene targets and effectors of HSF1 are heat shock proteins which are molecular chaperones acting to prevent aggregation of misfolded proteins, to promote their refolding or when proteins are damaged beyond repair, and to stimulate their degradation by proteasomes and via autophagy.[11,12,68] HSF1 also contributes to cytoprotection against toxic xenobiotics by promoting their efflux by MDR1 (ABCB1). MDR1 is a plasma membrane-based pump removing a range of toxic substances from cells. The promoter of the MDR1 gene is stress responsive and contains several HSF1-binding heat shock elements.[69] The introduction of a constitutively active HSF1 into cells increased MDR1 expression, which was dependent on HSF1 binding to the heat shock elements in the MDR1 promoter.[70]

In unstressed cells, HSF1 is an inactive monomer localized in the cytoplasm and bound by HSP70 and HSP90 protein chaperones (Figure 3). In stress conditions, HSF1 undergoes a multistep activation process which involves dissociation of both HSPs, trimerization, translocation to the nucleus and binding to the target promoters.[11,12] HSPs expression and HSF1 activity are higher in cancers due to dysregulated metabolism producing proteotoxic conditions in malignant cells.[71] Although HSF1 was initially characterized as a regulator of cellular responses to hyperthermia, its activation is also well-established for many other protein-damaging agents, including cellular oxidants and electrophilic lipid peroxidation products.[72−74] For 4-hydroxynonenal, one of the most toxic products of lipid oxidation, HSF1 was more important for cytoprotection than NRF2.[75] Sulforaphane, a chemopreventive isothiocyanate compound from cruciferous vegetables and one of the most widely studied NRF2 activators, also acts as a strong inducer of the heat shock response.[76] Stimulation of anti-inflammatory and antiapoptotic responses by electrophilic nitro-fatty acids is mediated by both NRF2 and, even more significantly, HSF1.[77] A side-by-side comparison of several structurally distinct chemical NRF2 activators, all of which react with Cys-SH groups in KEAP1, found a strong upregulation of the canonical HSF1 target gene
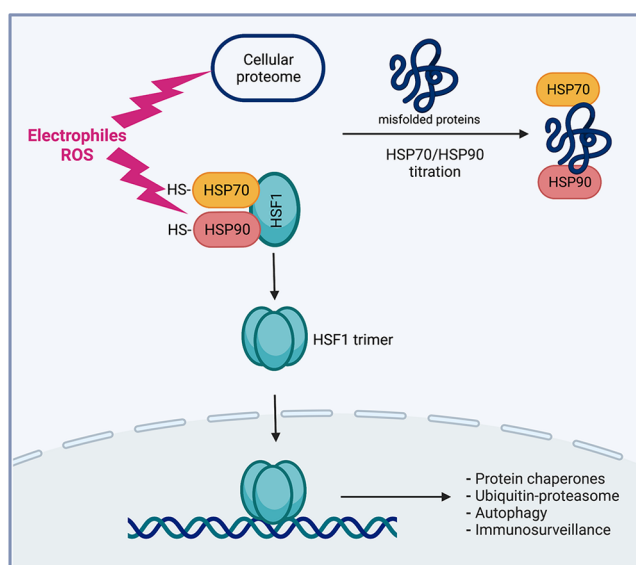

Figure 3. HSF1 activation by oxidants and other protein-damaging stressors. In unstressed cells, HSF1 is present in the cytoplasm as an inactive monomer that is repressed by HSP70 and HSP90 chaperones. In proteotoxic conditions causing dissociation of both HSPs, HSF1 forms a trimer which translocates to the nucleus and binds to the target promoters. HSF1 activation by oxidants and other reactive electrophiles results from dissociation of inhibitory HSP70 and HSP90 by direct damage to their SH groups and titration away of these chaperones by unfolded protein substrates. The HSF1-driven transcription program upregulates protein quality mechanisms (through increased abundance of protein chaperones, components of ubiquitin-proteasome systems, and autophagy factors) and enhances immunosurveillance of damaged cells (through expression of stress markers recruiting innate immune cells).

HSP70.[78] HSP70 induction was HSF1-dependent but NRF2-independent. Analysis of the concentration dependence showed that activation of HSF1 closely followed that of NRF2, which is remarkable considering the evolutionarily selected structure of KEAP1 designed to act as a sensor of SH-reactive chemicals. The basal expression of NRF2 targets was higher in HSF1-knockout MEFs,[78] suggesting that the diminished expression of molecular chaperones in these cells increased the abundance of reactive species sensed by the KEAP1-NRF2 pathway.

Activation of HSF1 by heat shock and by oxidants such as H_2O_2 was initially linked to the disulfide cross-linking of HSF1 molecules to form active trimers.[79,80] Two out of five cysteines in human HSF1 were found to mediate intermolecular cross-linking that was needed for HSF1 trimerization and DNA binding. The disulfide bridging of HSF1 into trimers by heat stress and proteasome inactivation has been recently attributed to the protein disulfide isomerase activity of transglutaminase 2.[81] This study found that heat shock and proteasome inhibition induced the trimerization of HSF1 only in $Tg2^{+/+}$ MEFs and these multimeric products were present exclusively in the nuclear pool of HSF1. However, the multimers constituted only a small fraction of the total nuclear HSF1, and structural studies of DNA-bound HSF1 at a single site in the heat shock element[82] do not support the importance of the earlier reported Cys36-Cys103 cross-linking.[79] However, cross-linking between HSF1 trimers bound to the heat shock element containing multiple binding sites could not be excluded. Irrespective of its significance for H_2O_2, it seems unlikely that Cys-Cys cross-linking or other forms of Cys damage in HSF1 could represent a

general route to its activation by numerous other electrophilic chemicals. Chemically diverse forms of Cys-SH modifications can serve as a sensitive and effective mechanism for the inactivation of proteins (KEAP1 as an example) but not for the formation of a specific active conformation.

A generally accepted mechanism for activation of HSF1 by the overabundance of misfolded proteins in the absence of chemical protein damage (for example, after heat shock or proteasome inhibition) involves dissociation of HSP70 and HSP90 from monomeric HSF1, permitting the formation of active trimers and gene expression.[11,12] This process is fine-tuned by numerous posttranslational modifications, especially by phosphorylation.[83,84] The loss of HSF1-bound inhibitory HSP70 and HSP90 is believed to result from their titration by unfolded proteins (higher affinity targets) that compete for a limited pool of these chaperones in unstressed cells. Recent studies provided strong evidence that the main source of these unfolded proteins triggering HSF1 activation during moderate proteotoxic stress are nascent polypeptides that are more vulnerable to heat-induced unfolding and aggregation due to their immature, unstable structures.[85,86] In comparison to mature proteins, the unfolded structure of newly translated proteins is also expected to make them more vulnerable to chemical damage due to greater steric accessibility of reactive amino acids and higher abundance of Cys-SH groups prior to the formation of physiological disulfide bridges stabilizing specific protein folds. Therefore, it seems likely that a major source of unfolded proteins induced by oxidants and other electrophiles and sensed by HSF1 also originates from nascent polypeptides. Consistent with this notion, newly synthesized proteins were the major pools of degraded proteins after both heat shock and oxidative damage.[87] There is also strong evidence that HSP70 and HSP90 can act as direct sensors of electrophilic stressors, leading to the dissociation of these chaperones from HSF1 and allowing its activation. HSP70 has been found to undergo different modifications of Cys-SH groups, such as conjugation with 4-hydroxynonenal,[75,88] glutathione,[89] and other compounds.[73,90,91] HSP90 was also chemically modified by reactive electrophiles, predominantly targeting Cys-SH groups but also histidine residues.[73,78,92] Overall, it is likely that HSF1 activation by oxidants and other reactive electrophiles reflects a combined effect of derepression from HSP70 and HSP90 by direct damage to these chaperones and their titration away from HSF1 by the buildup of unfolded substrates, predominantly originating from nascent polypeptides and intrinsically unstable proteins (Figure 3).

■ STRESS-REGULATED IMMUNOSURVEILLANCE OF DAMAGED CELLS

Recent successes in the treatment of human malignancies by derepression of anticancer immune responses provided clear evidence for the central role of the immune system in the maintenance and growth of highly immunogenic tumors.[93] However, the functions of the immune system, especially components of its innate immunity branch, are also critical for the prevention of cancer. Mouse models with systemic losses of NK cells, NK/T cell perforin deletion, or ablation of epidermal γ/δ T cells all showed increased susceptibility to carcinogen-induced tumorigenesis.[94−96] In humans, drug-induced immunosuppression for the prevention of organ transplant rejection is associated with several-fold higher risks of melanoma and other skin cancers.[96] For the innate immune system to identify and eliminate damaged cells, its targets need to emit activation/

recruitment signals and express distress markers. Two key groups of immunostimulatory danger markers expressed by damaged cells are the MIC and RAET1 families. These proteins serve as ligands for NKG2D receptors expressed by NK cells, subpopulations of γ/δ T cells, and by all human CD8^{+} T cells.[97] Promoter regions of NKG2D receptors-engaging MICA and MICB contain heat shock elements that are bound by activated HSF1 to upregulate transcription.[98] Promoters of the ULBP1-6 members of the RAET1 family of NKG2D ligands also contain heat shock elements and are likely to be upregulated by HSF1 during proteotoxic stress.[71]

Another cellular response stimulating the innate immune system is the production of interferon type I (IFN-I).[99] IFN-I operates in a paracrine manner to stimulate macrophages and NK cells and in an autocrine manner to activate cellular production of a number of immunostimulatory cytokines. The binding of IFN-I to the plasma membrane receptors triggers activation of STAT1 by JAK kinase. Tyrosine-phosphorylated STAT1 dimerizes and translocates to the nucleus, leading to the induction of IFN-responsive genes. Although it has been initially characterized in pathogen-infected cells, activation of IFN-I is now a well-established cellular response to damage-associated molecular patterns, which are abnormal molecules in the cytosolic compartment. These abnormal molecules include leaked fragments of nuclear DNA and mitochondrial DNA and dsRNA.[100,101] A recent report by Marzio et al. (2022)[33] provided strong evidence from experimental models and human clinical studies that INF-I response is regulated by KEAP1 as a separate function from its control of NRF2. Similar to its regulation of NRF2 in unstressed cells, KEAP1 was found to target a proto-oncogene EMSY for ubiquitin-mediated proteasomal degradation. EMSY acts as a negative regulator of IFN-stimulated genes, and consequently, its build-up as a result of KEAP1 inactivation limits the production of proinflammatory cytokines under the conditions of oxidative/electrophilic stress in cells, which restricts recruitment of inflammatory immune cells and their release of oxidants in pathogen-free tissue. Prolonged or overly robust inflammation in the absence of pathogen infections is undesirable and associated with higher cancer risks.[102,103] In tumors, proinflammatory cytokines are necessary for efficient recruitment of immune cells and cancer immunosurveillance, and consequently, KEAP1 inactivation/EMSY upregulation is protumorigenic due to suppression of IFN-I response.[33] Inflammatory responses in sterile tissues are also regulated by the transcription factor NF-κB which modulates the strength and duration of inflammation.[104,105] It was reported that IKKβ, a positive regulator of NF-κB, was a ubiquitination target of KEAP1 and its stabilization upon KEAP1 inactivation led to NF-κB activation.[106] However, this function of KEAP1 was not confirmed in a more recent study[28] and is inconsistent with suppression of proinflammatory cytokines production in cells with inactive KEAP1.[33] Although hypoxic tumor microenvironment is known to be immunosuppressive, this state is induced by low O_2 and not by HIF1/HIF2,[107] indicating that the oxidant-activated hypoxia response in normoxic cells would not interfere with immunomodulatory actions of HSF1 or KEAP1.

■ OXIDANT-INDUCED HSF1, HIF1, AND NRF2 RESPONSES: INTEGRATIVE VIEW

Proteotoxicity and the resulting cell injury by oxidants and other reactive electrophiles arise from damage to different amino acids, but Cys-SH is by far the most vulnerable group. Protein-

Cys is approximately 1000-times more nucleophilic than the next two most reactive amino acids in proteins: histidine and lysine.[108] This sensitivity of Cys-SH was evolutionarily incorporated into the design of specific protein sensors of electrophilic toxicants using Cys-SH as thiol-based switches for the inactivation of negative regulators of cellular stress responses. Operating on this principle are KEAP1 (sensor of oxidants/electrophiles in the NRF2 pathway), PHD2 (oxidant-sensitive regulator of HIF1), and to some extent, HSP70 and HSP90 (negative regulators of HSF1). Another trigger of the heat shock response is the accumulation of oxidant/chemically damaged proteins, which activate HSF1 in a manner similar to that by other protein-denaturing stressors such as hyperthermia. Cys-SH is susceptible to oxidation by all common ROS and RNS (H_2O_2, $O_2^{\bullet-}$, $CO_3^{\bullet-}$, $^\bullet NO_2$, $ONOO^-$, $HOCl$),[17,108] which can originate from both normal metabolism and redox cycling and other prooxidant activity of numerous xenobiotics in their parental or metabolically activated forms. The SH group is a soft Lewis nucleophile (base), making it highly reactive with soft Lewis electrophiles (acids) such as several toxic metals. Numerous organic electrophiles of endogenous and exogenous origin with high Cys-SH reactivity include quinones, epoxides, aldehydes, and other reactive carbonyls, especially α,β-unsaturated aldehydes. Reactivity of these carbonyl compounds with the protein-SH groups involves a rapid Michael addition at the highly polarized β-carbon of the double bond.[17]

In light of its evolutionary conserved function as a defense response to the buildup of denatured proteins, activation of HSF1 by oxidants and reactive electrophiles (which all primarily damage proteins) is not surprising. As discussed above, induction of the HIF1 responses by oxidants is also well-established.[43,44,47−50] To estimate HIF1 responsiveness to protein-damaging agents more generally, we compared the lists of activators of HSF1 (a marker of proteotoxic stress) and HIF1 that were identified in the currently largest screen of chemicals (∼7.5K chemical library, Tox21 program). Almost 80% (77.5%) of HIF1-activating chemicals also tested positive as activators of HSF1, indicating that chemicals with protein-damaging properties constitute a clear majority of the inducers of HIF1 pathway (Figure 4, Supporting Data File S1). As the number of chemicals screened for HSF1 response was smaller, the overlap in activators of HIF1 and HSF1 in the entire library
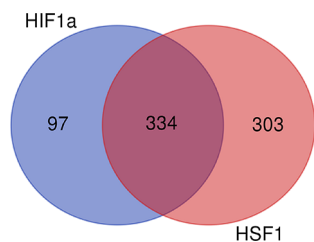

Figure 4. Venn diagram showing the number of chemicals activating HIF1, HSF1, or both transcription factors in a 7.5K chemical library. The diagram was generated using the results of high-throughput screens of HIF1 and HSF1 agonists (activators) performed as part of Tox21 program (https://comptox.epa.gov/dashboard/assay-endpoints; accessed on June 27, 2022). A DMSO-soluble library of stable, low-volatility chemicals was tested for activation of HIF1 (7871 chemicals) and HSF1 (7521 chemicals) in human cervical carcinoma cells. HSF1 agonists were screened in HeLa cells and HIF1 activators in ME-180 cells. HIF1 was activated by 431 and HSF1 by 637 chemicals. The same chemical library has not yet been screened for NRF2 activators.

was likely somewhat underestimated. The number of inducers of HSF1 was larger than that for HIF1, which could be due to the detection of chemicals that denature proteins and trigger the heat shock response in the absence of chemical damage. These nonreactive protein denaturants are not expected to act as selective inhibitors of negative regulators of the hypoxia response.

Activation of the NRF2 pathway represents the most direct protective response to oxidants/electrophiles due to the upregulation of enzymes with a variety of antioxidant activities, including detoxification of ROS, increased glutathione biosynthesis, inactivation of reactive toxicants by Phase II enzymes, and excretion of the resulting conjugates from cells by MRP2. Stimulation of the HIF1 pathway by oxidative inactivation of PHD2, evolutionarily selected primarily as part of cellular hypoxia sensing, suppresses the activity of the electron transport chain and the release of ROS by mitochondria. Mitochondria are frequently the main source of ROS in stressed cells due to their vulnerability to damage by various toxicants. The role of HIF1 as a guardian of mitochondrial health is further supported by its involvement in the elimination of severely diseased mitochondria through activation of mitophagy by two canonical hypoxia response-activated targets BNIP and BNIP3L.[35,109] Down-regulation of the mitochondrial respiratory chain is accompanied by increased uptake and flow of glucose through glycolysis, establishing O_2-independent generation of ATP and producing large amounts of lactic acid, which slows down cell cycle and promotes oxidant-protective protonation of protein-SH groups. Electrophile-induced upregulation of HSF1-inducible genes (such as protein chaperones and ubiquitin) acts to enhance repair and disposal of damaged/unfolded proteins. During chronic stress, all three oxidant-responsive pathways governed by NRF2, HSF1, and HIF1 are typically upregulated as observed in cancers.[21,22,35,71] In these conditions, the HSF1 pathway probably deals with unfolded proteins damaged by oxidants and electrophiles escaping detoxification activities of the NRF2-regulated processes and defensive metabolism promoted by HIF1. In the acute oxidative/electrophilic stress rapidly inducing large amounts of protein damage, HSF1 activation is probably the most immediate response due to the evolutionary selected processes for a rapid transcription and translation of HSF1 targets.[110] The unparalleled immediacy of the HSF1 response evolved to deal with a massive protein unfolding produced after even brief hyperthermia. Unlike gene expression and protein translation of HSF1 targets, cap-dependent mRNA translation (∼90% of total protein synthesis) and global transcription are actively repressed under severe stress conditions. Components of the cap-dependent translation machinery along with mRNA and damaged nascent polypeptides become insoluble and sequestered in the cytoplasmic granules.[110] Upregulation of chaperones and other protein quality control factors accelerate repair and removal of damaged proteins (mostly nascent proteins for both heat shock and oxidative damage),[87] allowing resumption of global transcription and translation, including those of NRF2 and HIF1 targets.

The above review of the major functional changes induced by NRF2, HSF1 and HIF1 indicated that their effects on cellular functions are largely nonoverlapping. To support this conclusion by quantitative analyses, we examined gene expression profiles for all three transcription factors to identify their unique and shared transcription targets. First, we compared HSF1 target genes from a comprehensive database described by Kovacs et

al.,[111] core transcription targets for NRF2 using high confidence NRF2-regulated genes from Ibrahim et al.,[112] and HIF1 targets using RNA-seq data[38] for the hypoxia-induced genes applying a 2-fold change as a cutoff. Analysis of these data sets showed that there were 0 genes induced by all three transcription factors and only 2 NRF2-activated genes (0.9% of NRF2 targets) were also upregulated by HIF1 (Figure 5A, Supporting Data File S2). The
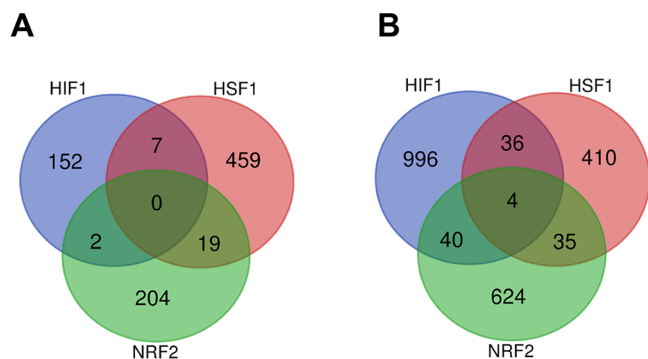

Figure 5. Venn diagrams depicting common and unique transcripts induced by HIF1, HSF1, and NRF2. (A) Venn diagram of overlapping and unique genes generated using a comprehensive HSF1 target genes data set,[111] a high confidence NRF2 genes data set (Table S5 in Ibrahim et al.[112]), and hypoxia-induced genes with 2 or higher fold change.[38] (B) Venn diagram of overlapping and unique genes from a comprehensive HSF1 target genes data set[111] and broader sets of target transcripts for NRF2 (Table S3 in Ibrahim et al.[112]) and HIF1 (all hypoxia-induced genes).[38]

percentage of NRF2 transcription targets that were also induced by HSF1 was higher but still small (8.4% of NRF2 genes). Next, we analyzed the same comprehensive data set for HSF1 target genes[111] together with all HIF1-upregulated genes,[38] which dramatically increased the number of transcripts from 161 to >1000, and a broader set of NRF2-regulated genes (Table S3 in Ibrahim et al.[112]). Even in these larger sets of genes, there were only 4 genes (0.6% of NRF2 targets) induced by all three transcription factors (Figure 5B, Supporting Data File S3). The percentages of NRF2-activated genes that were also upregulated by HIF1 and HSF1 also remained small: 5.7 and 4.9%, respectively. Although transcription programs driven by HSF1, HIF1, and NRF2 are clearly very distinct, these factors do not operate completely independently, as they can directly or indirectly affect each other's activity.[47,113,114]

Overall, NRF2, HIF1, and HSF1 pathways represent largely nonoverlapping transcription responses to oxidative/electrophilic stress, which collectively provide a comprehensive defense inside the cell and generate activation signals for surveillance of injured cells by innate immunity (Figure 6). The relative significance of these three responses probably varies dependent on the severity and complexity of the injury, abundance of unfolded proteins (more for bulky protein adducts than for simple SH oxidation), and the extent of mitochondrial damage. Some protein-reactive toxicants can inhibit a specific stress-responsive pathway, leading to the increased reliance of cells on the remaining protective processes. For example, HSF1 activators formaldehyde, acetaldehyde, and cadmium cause impairment of the hypoxia response by damaging HIFα subunits of HIF1 and the related transcription factor HIF2.[115] The importance of different oxidant-responsive pathways is also likely to vary among different types of cells. Epithelial cells in tissues that are most commonly exposed to xenobiotics (liver,
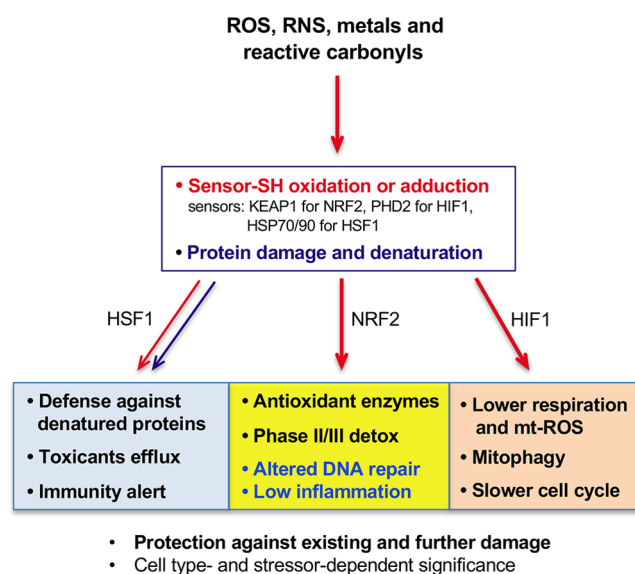

Figure 6. Flowchart depicting activation of HIF1, HSF1, and NRF2 and their main functions in protection against ROS and reactive electrophiles. All three factors are induced through inactivation of their negative regulators that act as sensors of ROS/reactive toxicants via modifications of their Cys-SH groups. HSF1 is also derepressed via titration of HSP70 and HSP90 by unfolded proteins which predominantly include damaged nascent polypeptides. KEAP1/NRF2-dependent promotion of NHEJ at the expense of homologous recombination represents a shift to a faster but more mutagenic repair of oxidants-induced DSBs. Suppression of sterile inflammation by KEAP1 inactivation during oxidative stress in normal tissue is beneficial by limiting a secondary damage. In tumor tissue, a constitutive loss of KEAP1 and the resulting ineffective production of proinflammatory cytokines inhibit cancer immunosurveillance.

intestine, lung) typically show the most robust upregulation of a broad set of defense genes by NRF2.[15] In lymphoid cells, activation of NRF2 induced a more limited transcription response with many canonical targets remaining unchanged.[19] Hematopoietic stem cells contain unusually high concentrations of the nonthiol antioxidant ascorbate,[52] and HIF1 plays a critical role in healthy functioning of these and other stem cells.[116]

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00131.

　　Supporting Data File S1: Raw data associated with Figure 4 (XLSX)

　　Supporting Data File S2: Raw data associated with Figure 5A (XLSX)

　　Supporting Data File S3: Raw data associated with Figure 5B (XLSX)

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■ ABBREVIATIONS

DSBs, DNA double-stranded breaks; IFN-I, interferon type I; NHEJ, nonhomologous end-joining; RNS, reactive nitrogen species; ROS, reactive oxygen species

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