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Transcription Factor NRF2 as a Therapeutic Target for Chronic Diseases: A Systems Medicine Approach

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Abstract——**Systems medicine has a mechanism-based rather than a symptom- or organ-based approach to disease and identifies therapeutic targets in a nonhypothesis-driven manner. In this work, we apply this to transcription factor nuclear factor (erythroid-derived 2)–like 2 (NRF2) by cross-validating its position in a protein–protein interaction network (the NRF2 interactome) functionally linked to cytoprotection in low-grade stress, chronic inflammation, metabolic alterations, and reactive oxygen species formation. Multiscale network analysis of these molecular profiles suggests alterations of NRF2 expression and activity as a common mechanism in a subnetwork of diseases (the NRF2 diseasome). This network joins apparently heterogeneous phenotypes such as autoimmune, respiratory, digestive, cardiovascular, metabolic, and neurodegenerative diseases, along with cancer. Importantly, this approach matches and confirms in silico several applications for NRF2-modulating drugs validated in vivo at different phases of clinical development. Pharmacologically, their profile is as diverse as electrophilic dimethyl fumarate, synthetic triterpenoids like bardoxolone methyl and sulforaphane, protein–protein or DNA–protein interaction inhibitors, and even registered drugs such as metformin and statins, which activate NRF2 and may be repurposed for indications within the NRF2 cluster of disease phenotypes. Thus, NRF2 represents one of the first targets fully embraced by classic and systems medicine approaches to facilitate both drug development and drug repurposing by focusing on a set of disease phenotypes that appear to be mechanistically linked. The resulting NRF2 drugome may therefore rapidly advance several surprising clinical options for this subset of chronic diseases.**

I. Introduction

Life span has almost doubled in the last century, and aging-specific diseases are now becoming prevalent. However, the pathologic mechanisms underlying most of them are poorly understood and treated rather by correcting symptoms or risk factors. Moreover, contrary to a hitherto linear approach that considered one disease, one medicine, chronic diseases demonstrate a high degree of connectedness and a need for more precise, mechanism-based disease definitions rather than the current organ- and symptom-based. After the human genome sequencing and the development of molecular networks, a new concept of disease is thus emerging, in which diseases are diagnosed not only by clinical symptoms, but mainly by the underlying molecular signatures (Goh et al., 2007). The fact that different pathophenotypes have a shared molecular mechanism provides also a rationale toward a new concept of therapy summarized as "several diseases, one medicine" and drug repurposing. Network medicine, i.e., the application of network concepts to the analysis of dynamic connections among diseases and drugs, provides a new opportunity to develop this new approach. Chronic diseases in the elderly are most likely characterized by the loss of homeostasis during aging or as a result of environmental factors, all of them leading to low-grade stress by pathologic formation of reactive oxygen species (ROS), chronic inflammation, and metabolic unbalance. Based on a network medicine approach, in this review we will present extensive evidence indicating that the nuclear factor (erythroid-derived 2)–like

ABBREVIATIONS: AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; AMPK, AMP-activated protein kinase; ARE, antioxidant response element; β-TrCP, β-transducin repeat containing E3 ubiquitin protein ligase; bZip, basic region-leucine zipper; CDDO, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oate; CDDO-Me, CDDO-methyl ester; COPD, chronic obstructive pulmonary disease; CUL3, Cullin 3; DC, dendritic cell; DMF, dimethyl fumarate; EAE, experimental autoimmune encephalomyelitis; ECH, erythroid cell–derived protein with Cap'n'collar homology; GCLC, γ-glutamyl cysteine ligase catalytic subunit; GCLM, γ-glutamyl cysteine ligase modulator subunit; GI, gastrointestinal tract; GSH, glutathione; GSK-3, glycogen synthase kinase 3; HFD, high-fat diet; HNSCC, head and neck squamous cell carcinoma; HO-1, heme oxygenase-1; IBD, inflammatory bowel disease; IκB, κ-B inhibitor; IKK, IκB kinase; IL, interleukin; KEAP1, kelch-like ECH-associated protein 1; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAF, musculoaponeurotic fibrosarcoma protein; MMF, monomethyl fumarate; MS, multiple sclerosis; NASH, nonalcoholic steatohepatitis; NF-κB, p65 subunit of nuclear factor κ-light-chain enhancer of activated B cells; NQO1, NADPH:quinone oxidoreductase; NRF2, nuclear factor (erythroid-derived 2)–like 2; PD, Parkinson disease; PI3K, phosphatidylinositol 3-kinase; PPI, protein–protein interaction; PTEN, phosphatase and tensin homolog; RA, rheumatoid arthritis; RBX1, RING-box protein 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SFN, sulforaphane; SLE, systemic lupus erythematous; SNP, single-nucleotide polymorphism; SQSTM1, sequestosome 1; STAT, signal transducer and activator of transcription; T2DM, type 2 diabetes mellitus; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; Treg, T regulatory.

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2 (NRF2), as the master regulator of multiple cytoprotective responses and a key molecular node within a particular cluster of diseases, provides a new strategy for drug development and repurposing.

II. From Nuclear Factor (Erythroid-Derived 2)–Like 2 Interactome to Nuclear Factor (Erythroid-Derived 2)–Like 2 Diseasome

A. Nuclear Factor (Erythroid-Derived 2)–Like 2 as a Master Regulator of Cellular Homeostasis

NRF2 is a basic region-leucine zipper (bZip) transcription factor (Fig. 1) that forms heterodimers with small musculoaponeurotic fibrosarcoma protein (MAF) K, G, and F in the nucleus. The heterodimer recognizes an enhancer sequence termed antioxidant response element (ARE) that is present in the regulatory regions of over 250 genes (ARE genes) (Ma, 2013; Hayes and Dinkova-Kostova, 2014). These ARE genes encode a network of cooperating enzymes involved in phase I, II, and III biotransformation reactions and antioxidant mechanisms that generate NADPH, glutathione (GSH), and thioredoxin reactions; lipid and iron catabolism; and interaction with other transcription factors, etc. (Hayes and Dinkova-Kostova, 2014). Recently, NRF2 was also found to regulate the expression of several proteasome subunits and autophagy genes, providing additional interest for its control of proteostasis (Pajares et al., 2015, 2016, 2017; de la Vega et al., 2016).

The great significance of NRF2 from a clinical perspective is that it might be targeted pharmacologically with patient benefit. The main mechanism regulating the transcriptional activity of NRF2 is the control of protein stabilization by the E3 ligase adapter Kelch-like erythroid cell–derived protein with Cap'n'collar homology (ECH)-associated protein 1 (KEAP1) (Fig. 2). KEAP1 is a homodimeric protein that bridges NRF2 with the E3 ligase complex formed by Cullin 3 and RING-box protein 1 (CUL3/RBX1). Under homeostatic conditions, the N-terminal domain of the KEAP1 homodimer binds one molecule of NRF2 at two amino acid sequences with low (aspartate, leucine, and glycine; DLG) and high (glutamate, threonine, glycine, and glutamate; ETGE) affinity, and hence presents NRF2 to ubiquitination by CUL3/RBX1 (Tong et al., 2007) and subsequent degradation by the proteasome. KEAP1 is a redox and electrophile sensor that upon modification of critical cysteines loses its ability to repress NRF2 (Fig. 2; *Biomarkers as Nuclear Factor (Erythroid-Derived 2)–Like 2 Signature and for Monitoring Target Engagement*). An alternative mechanism of regulation of NRF2 stability is the phosphorylation mediated by glycogen synthase kinase 3 (GSK-3) (Fig. 2). This kinase phosphorylates a domain of NRF2 (aspartate, serine, glycine, isoleucine, serine; DSGIS) and hence creates a recognition motif for the E3 ligase adapter β-transducin

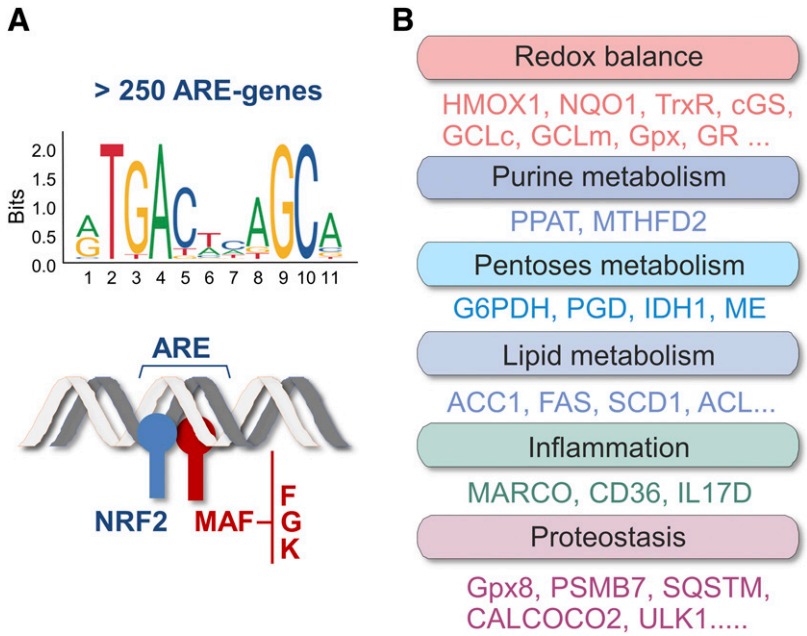

Fig. 1. NRF2 as a master regulator of cytoprotective responses. (A) NRF2 heterodimerizes with the members of MAF family through their bZip domain. The heterodimer binds to an enhancer sequence termed ARE that is present in the regulatory regions of over 250 genes (ARE genes). (B) These genes participate in the control of redox metabolism, inflammation, and proteostasis balance, as indicated. The existence of susceptibility SNPs in *NFE2L2*, elevated levels of its target genes in brain necropsies, and positive data from preclinical studies suggests that the imbalance in proteostasis, redox, and inflammatory control may be counterbalanced by NRF2 activation. ACl, ATP citrate lyase; ACC1, acetyl-coenzyme A carboxylase 1; CALCOCO2, calcium binding and coiled-coil domain 2; cGS, c-glutamate cysteine synthetase; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; Gpx, glutathione peroxidase; Gpx8, gluthation peroxidase 8; GR, glutathione reductase; HMOX1, heme oxygenase-1; IDH1, isocitrate dehydrogenase 1; ME, malic enzyme; MTHFD2, methylenetetrahydrofolate dehydrogenase 2; PGD, phosphogluconate dehydrogenase; PPAT, phosphoribosyl pyrophosphate amidotransferase; PSMB7, proteasome subunit β type-7; SCD1, stearoyl-CoA desaturase; TrxR, thioredoxin reductase; ULK1, unc-51 like autophagy activating kinase 1.

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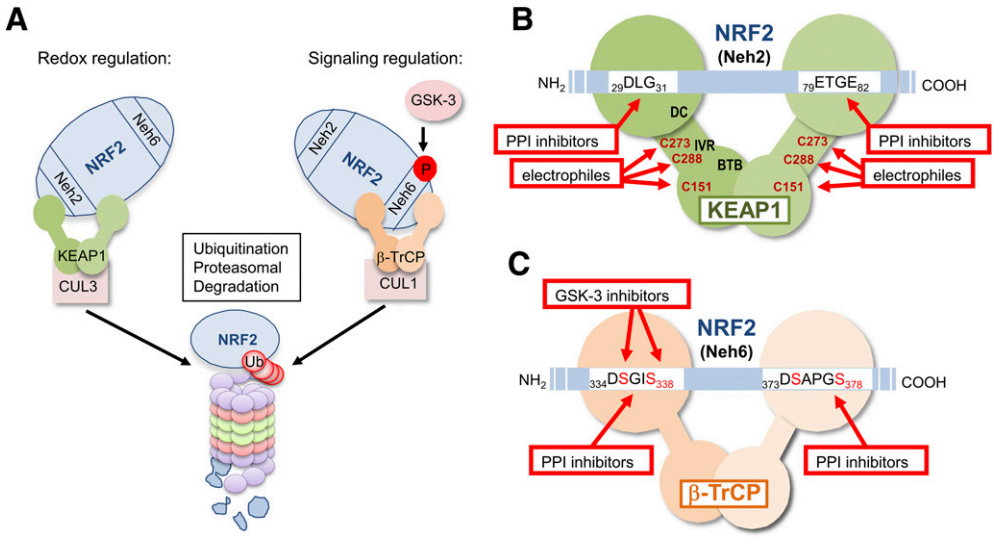

Fig. 2. Regulation of NRF2 stability by KEAP1 and β-TrCP and its pharmacological targeting. (A) According to the dual regulation model (Rada et al., 2011), two domains of NRF2, termed Neh2 and Neh6, participate in NRF2 degradation in response to redox and electrophile changes (KEAP1) and to signaling kinases (β-TrCP), respectively. The Neh2 domain binds the E3 ligase adapter KEAP1 that presents NRF2 for ubiquitination to a CUL3/RBX1 complex. The Neh6 domain requires previous phosphorylation by GSK-3 to bind the E3 ligase adapter β-TrCP and subsequent ubiquitination by a CUL1/RBX1 complex (see text for details). (B) Detail of the binding between NRF2 and KEAP1 and current strategies to target this interaction. The KEAP1 homodimer binds NRF2 at two motifs of the Neh2 domain: the low-affinity (29-DLG-31) and the high-affinity (79-ETGE-82) binding sites. Current strategies to disrupt this interaction include the following: electrophiles that alter sulfhydryl groups of cysteines C151, 273, and 288; PPI inhibitors that alter the docking of NRF2 to the DC domain of KEAP1. (C) Hypothetical binding of NRF2 and β-TrCP and suggested strategies to target this interaction. The β-TrCP homodimer binds to the Nhe6 domain at the phospho-motif 334-DSGIS-338 when it is phosphorylated by GSK-3 (Rada et al., 2011, 2012) and at the phospho-motif 373-DSAPGS-378 independently of GSK-3 (Chowdhry et al., 2013). In this figure we postulate that, by analogy with KEAP1, one β-TrCP homodimer interacts with one molecule of NRF2 at the two phospho-motifs, but experimental evidence is still lacking. Two possible strategies to disrupt this interaction include the use of GSK-3 inhibitors and PPI inhibitors.

repeat containing E3 ubiquitin protein ligase (β-TrCP) that presents NRF2 to a CUL1/RBX1 complex, leading to an alternative pathway for ubiquitin-dependent proteasome degradation of NRF2. Therefore, KEAP1 and GSK-3/β-TrCP tightly control NRF2 protein levels in the context of redox homeostasis and cell signaling, respectively (Cuadrado, 2015). Other mechanisms of NRF2 regulation at protein, mRNA, or gene level have been reported (Hayes and Dinkova-Kostova, 2014), but at least these two are amenable to pharmacological regulation.

B. Positioning Nuclear Factor (Erythroid-Derived 2)–Like 2 and Its Regulatory Pathway in the Human Interactome and Diseasome

The gene encoding NRF2, termed *NFE2L2*, is highly polymorphic and presents a mutagenic frequency of 1 per every 72 bp. An excellent review on this topic reported in 2015 up to 18 single-nucleotide polymorphisms (SNPs), most of them in the 5′ regulatory region and in intron 1 (Cho et al., 2015). Several of these SNPs might constitute functional haplotypes that are associated with risk at onset or progression of chronic diseases. Variations in functional haplotypes may have a subtle impact on a proportion of individuals who exhibit clinical symptoms of specific diseases, yet they may have a profound effect at the population level and may define specific strategies to target this gene in precision medicine.

Recent advances in network medicine have provided quantitative tools to characterize how the interplay between genes and their interactions (interactome) is related to pathology (Barabasi et al., 2011; Vidal et al., 2011; Guney et al., 2016), how dysregulated molecular networks are common to various diseases (Menche et al., 2015), and how diseases manifest in particular tissues (Kitsak et al., 2016). To understand the relevance of NRF2 in pathology from the systems medicine perspective, first we have generated the human interactome map. We have integrated and curated information on physical interactions among proteins involved in the NRF2-regulating pathway (Hayes and Dinkova-Kostova, 2014; Cuadrado, 2015). The interaction data have been taken from the recently published human interactome that compiles data across several protein–protein interaction (PPI) resources (Turei et al., 2013; Menche et al., 2015). However, the currently available information for development of the NRF2 interactome is limited by the fact that, because NRF2 is a very short half-life protein, some meaningful interactions may be undetected. Nevertheless, some well-known proteins that physically interact with NRF2 are found in the interactome, including KEAP1, β-TrCP, and MAFs. Another group of NRF2-interacting proteins corresponds to nuclear proteins with functions in regulation of gene expression. These include proteins related with bZip transcription factors, nuclear receptors or coactivators, or proteins involved in histone acetylation. Therefore, the NRF2 interactome evidences additional mechanisms of gene regulation beyond those directly connected to this

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transcription factor. NRF2 is phosphorylated at several residues, and therefore it is expected to interact with several kinases that include GSK-3 and several protein kinase C isoforms. These kinases are downstream of some membrane receptors and adapter/scaffold proteins. In addition to this physical interaction, we have identified several biologic functions that are enriched in the NRF2 neighborhood, including metabolic processes, such as biosynthesis of pentose, tetrapyrrole, heme, glucose 6-phospate, cysteine, GSH, glyceraldehyde-3-phosphate, and NADPH. Most of these regulatory proteins do not interact directly with each other but are connected through proteins acting as mediators (Fig. 3; a more detailed description of specific interacting molecules can be found at http://sbi.imim.es/data/nrf2/).

Perturbations of the NRF2 interactome have been reported in several diseases. We have developed a diseasome map by curating a list of 37 NRF2-related diseases based on DisGeNET (Pinero et al., 2017) and GeneCards (Stelzer et al., 2016) databases, as well as knowledge from some studies in animal models. We have also retrieved disease–gene associations for these pathologies using DisGeNET, OMIM, and GWAS databases (Menche et al., 2015) (Table 1). The interactome-based proximity (Guney and Oliva, 2014) of NRF2 to known disease genes for each of the NRF2-related disease phenotypes is shown in Fig. 4. NRF2 is significantly closer to the known disease genes of the digestive system and cancers, such as prostate, liver, and lung neoplasms, compared with randomly selected proteins, highlighting the key functional role of NRF2 in these pathologies. Moreover, NRF2 was found to be proximal to various proteins related to metabolic and cardiovascular diseases, such as diabetes, hyperglycemia, ischemia, middle cerebral artery infarction, and atherosclerosis. Protein interactions of NRF2 also connect it to genes associated to respiratory disorders such as asthma, pulmonary fibrosis, and pulmonary emphysema, as well as to neurodegenerative conditions such as Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS).

The interactome-based proximity therefore offers a perspective on how NRF2 can be linked to various pathologic conditions. The relationships among diseases have been previously summarized as a network, termed diseasome, that is connecting them based on genetic (Goh et al., 2007) and clinical (Hidalgo et al., 2009; Zhou et al., 2014) commonalities. In Fig. 5, we have defined a network of diseases, the NRF2 diseasome, based on shared genes, symptom similarity, and comorbidities. NRF2 appears to connect diseases governed substantially by inflammatory processes, such as acute kidney injury, liver cirrhosis, and atherosclerosis. Furthermore, neurodegenerative diseases such as AD, PD, Huntington's disease, and ALS constitute a cluster consistent with recent studies implicating also NRF2 in neuroinflammatory processes (Rojo et al., 2010; Lastres-Becker et al., 2012, 2014; Jung et al., 2017; Wang et al., 2017b). The regulation of NRF2 by kinase-signaling cascades (Jung et al., 2017) could explain the well-connected cluster of cancers, particularly supporting the involvement of NRF2 in pathologic ROS

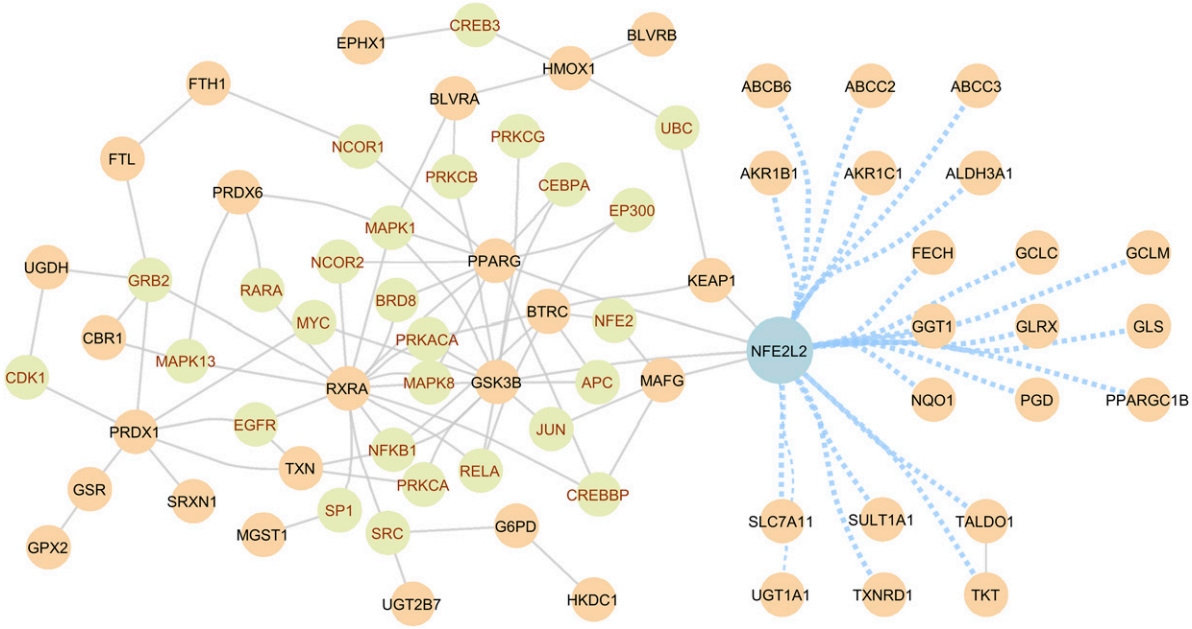

Fig. 3. Locating the NRF2 regulatory pathway in the human interactome. NRF2 plays a key role in pathologic ROS formation as well as inflammatory and metabolic responses by coordinating the activity of various proteins. These interactions constitute a molecular interaction network, the NRF2 interactome. The known physical interactions between proteins involved in the NRF2 regulatory pathway (i.e., regulator proteins, brown circles) and the proteins through which they are connected (i.e., mediator proteins, green circles) are shown with a gray link. The regulator proteins that involve more than one mediator protein to connect to NRF2 are shown with a blue link. The regulator proteins are curated from literature, and their interactions are retrieved from various resources, including IntAct, MINT, BioGRID, HPRD, KEGG, and PhosphoSite. The list of all the proteins that interact with NRF2 in the human interactome is available online at http://sbi.imim.es/data/nrf2/.

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TABLE 1
Cluster of diseases with evidence of NRF2 association

Disease phenotypes with evidence of genetic association with NRF2 were selected from the DisGeNet database. DisGeNet integrates disease–gene association information from various resources, such as UniProt, ClinVar, GWAS Catalog, and Comparative Toxicogenomics Database and scores disease–gene associations according to the number of resources and publications supporting these associations. The curation of the list was based on the following criteria: 1) only pathophenotypes with more than one citation in Pubmed were selected; 2) the score of reliability was set to a threshold of 0.001; and 3) disease entries with very similar names or overlapping terms were simplified to one single entry.

Pathophenotype	Reliability Score	Pathophenotype	Reliability Score
Diabetic nephropathy	0.2016	Diabetic cardiomyopathy	0.0803
Liver cirrhosis	0.2005	Middle cerebral artery infraction	0.0800
Nonalcoholic steatohepatitis	0.2005	Breast neoplasms	0.0087
Acute kidney injury	0.2000	Vitiligo	0.0076
Pulmonary fibrosis	0.2000	Atherosclerosis	0.0067
Nonsmall cell lung carcinoma	0.1252	Asthma	0.0043
Squamous cell carcinoma	0.1243	Leukemia	0.0038
Liver neoplasms	0.1238	Colon neoplasm	0.0038
Hyperglycemia	0.1208	Gastrointestinal diseases	0.0029
Drug-induced liver injury	0.1200	Parkinson disease	0.0026
Prostatic neoplasms	0.1200	Systemic lupus erythematous nephritis	0.0026
Chronic obstructive pulmonary disease	0.0899	Glioma	0.0024
Colorectal neoplasms	0.0847	Amyotrophic lateral sclerosis	0.0022
Alzheimer disease	0.0837	Ischemia	0.0016
Type 2 diabetes mellitus	0.0814	Pulmonary emphysema	0.0013
Chronic kidney disease	0.0808	Pancreatic neoplasms	0.0013
Diabetic retinopathy	0.0805	Vascular diseases	0.0013
Huntington's disease	0.0805	Sepsis	0.0013

formation underlining colon (Gonzalez-Donquiles et al., 2017) and breast (Lu et al., 2017) tumors.

III. Target Validation of Nuclear Factor (Erythroid-Derived 2)–Like 2 in Human Disease States

A. Key Role of Nuclear Factor (Erythroid-Derived 2)–Like 2 in Resolution of Inflammation

Persistent inflammation is a hallmark of all pathophenotypes found in the NRF2 diseasome. This is most likely because inflammation is associated with increased local and systemic pathologic formation of reactive oxygen species (ROS). In fact, ROS and reactive nitrogen species (RNS) stimulate and aggravate inflammatory responses that are mechanistically related to the activation of transcription factor, p65 subunit of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) (Wenzel et al., 2017). Very simplified, in resting immune cells, NF-κB is retained in the cytosol through interaction with the nuclear κ-B inhibitor (IκBα). Pathogen-associated molecular pattern molecules derived from microorganisms as well as damage-associated molecular pattern molecules released in

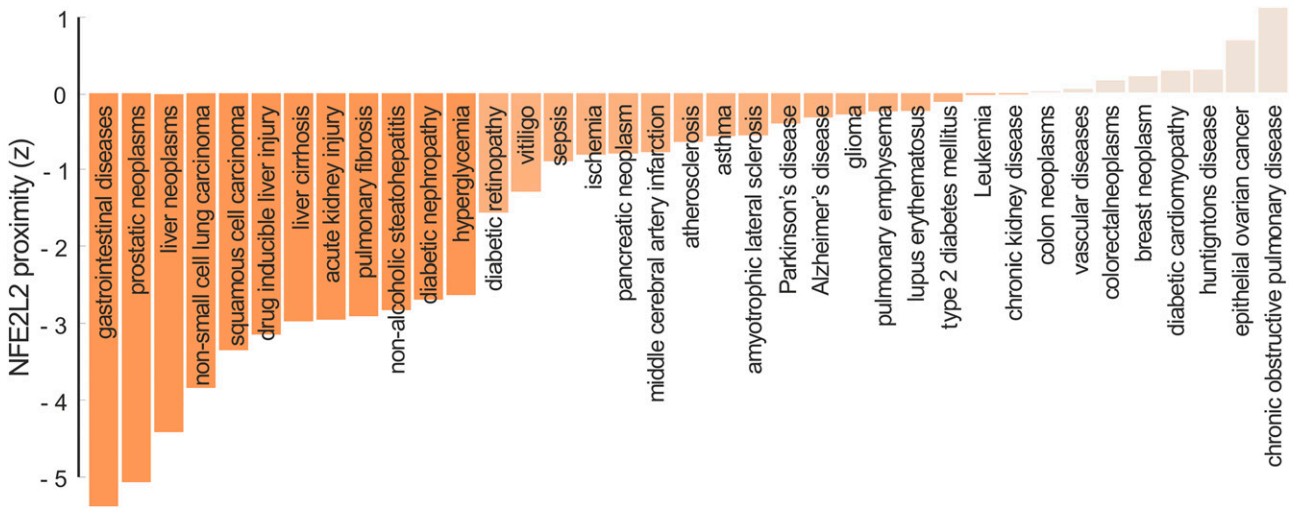

Fig. 4. Systems medicine view of NRF2 from the perspective of the human interactome. Diseases are triggered by mutations that perturb proteins and their interactions, affecting a certain neighborhood in the NRF2 interactome. Interactome-based proximity measures the distance of genes to those disease neighborhoods. The bars show the proximity of NRF2 gene (*NFE2L2*) to known disease genes that participate in NRF2-related pathophenotypes. These bars highlight the role of NRF2 in digestive system diseases and cancers. For a given disease, the proximity first calculates the distance from NRF2 to the closest known disease gene and then compares that distance to a random expectation that is estimated using average distances between randomly selected proteins in the interactome. The *z*-score reported by interactome-based proximity corresponds to the significance of the observed distance between NRF2 and the disease genes. A negative value indicates that the observed distance is lower than what would be expected by chance. The bars are colored in varying tones of orange based on the *z*-score: significantly proximal (dark orange), proximal (orange), not proximal (light orange), respectively. The known disease genes are taken from DisGeNet, OMIM, and GWAS Catalog.

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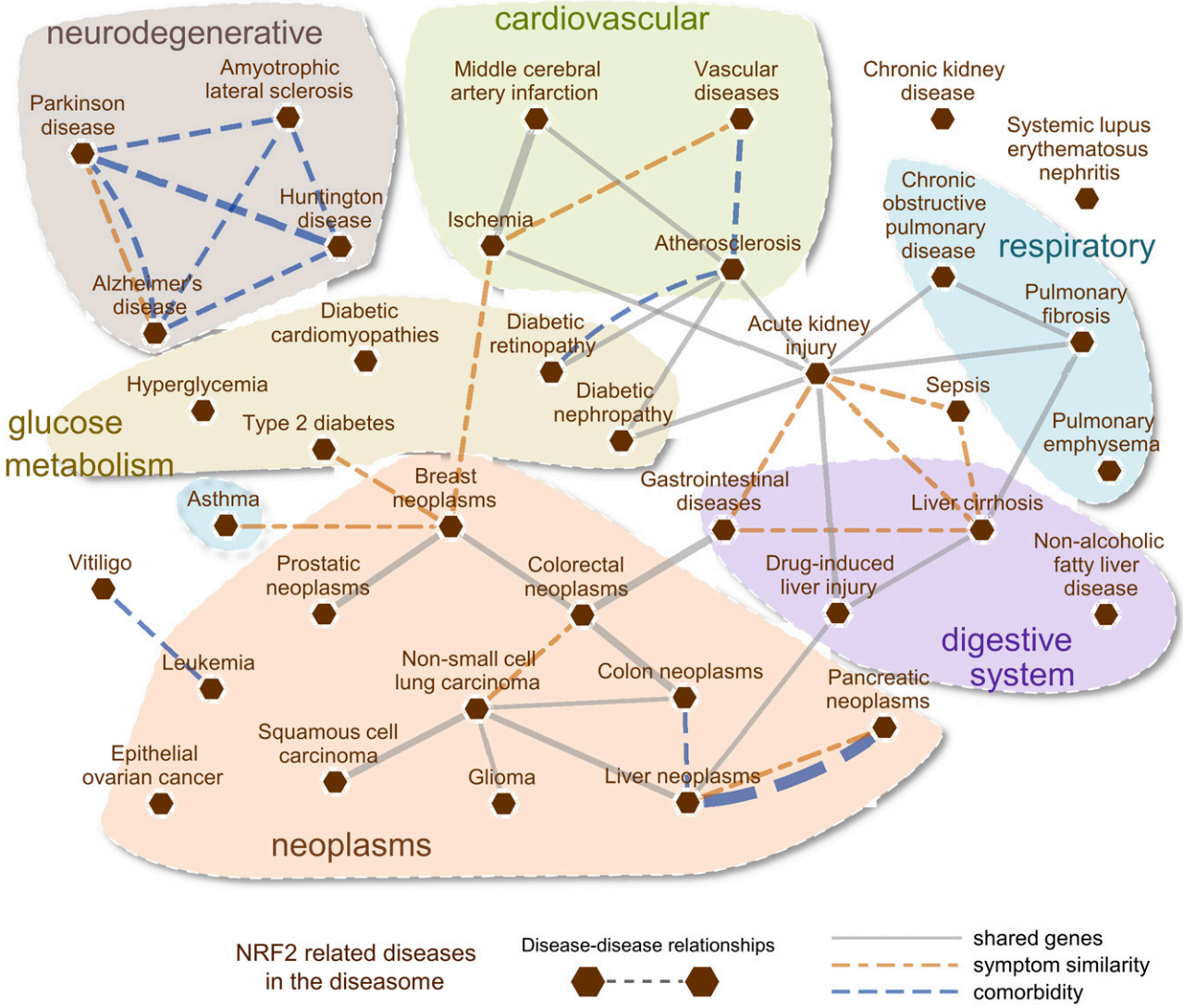

Fig. 5. Current status of the NRF2 diseasome. The relationships between diseases are represented as a network in which pathophenotypes are linked by common genetic and clinical descriptors. In the figure, nodes (red hexagons) represent diseases, and the edges are similarities among them based on shared genes, common symptoms, and comorbidities (gray, orange, and blue lines, respectively). The genes and symptoms associated with the diseases are used to identify disease pairs that have a significant genetic and symptomatic overlap calculated using the Jaccard index. Among significant disease–disease connections ($P < 0.05$, assessed by Fisher's exact test based on the observed gene or symptom overlap), only the links that have an elevated overlap and comorbidity are shown to eliminate potentially spurious connections. Accordingly, the diseases that share at least 10% of the disease-associated genes and more than half of the associated symptoms are included in the figure. The comorbidity information is extracted from medical insurance claims, representing disease pairs that tend to occur together in the population (relative risk >2).

response to tissue damage stimulate cognate receptors expressed by immune cells that lead to activation of IκB kinase (IKK)β. This kinase phosphorylates IκBα, targeting it for degradation and allowing nuclear translocation and activation of NF-κB (Napetschnig and Wu, 2013). These events are submitted to redox control through several modes of regulation of IκBα (Bowie and O'Neill, 2000; Morgan and Liu, 2011; Siomek, 2012), but one that has been described recently involves the regulation of IKKβ stability by KEAP1. Just like NRF2, IKKβ possesses an ETGE motif that enables its binding to KEAP for ubiquitination and proteasomal degradation. Therefore, under basal redox conditions, active KEAP1 targets IKKβ for degradation and then IκBα inhibits NF-κB. By contrast, in the presence of ROS, KEAP1 is inhibited and IKKβ is stabilized, phosphorylating IκBα and leading to its degradation and therefore to upregulation of NF-κB (Lee et al., 2009).

Because NRF2 is a master regulator of redox homeostasis, it exerts an indirect control on NF-κB activity. Lipopolysaccharide (LPS) activates simultaneously a fast, proinflammatory NF-κB response and a slow NRF2 response. The NF-κB response is subsequently inhibited when NRF2 is maximally active (Cuadrado et al., 2014). For instance, Ras-related C3 botulinum toxin substrate 1, a small G protein of the Rho family, activated the NF-κB pathway and NRF2 overexpression blocked, whereas NRF2 knockdown enhanced NF-κB–dependent transcription (Cuadrado et al.,

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2014). Consistently, in NRF2-deficient (*Nrf2*$^{-/-}$) mice challenged with LPS or tumor necrosis factor (TNF)-α, the activity of IKK was exacerbated and led to increased phosphorylation and degradation of IκB (Thimmulappa et al., 2006a).

NRF2 also induces an anti-inflammatory phenotype that modulates the functions of CD8$^+$ T cells (Sha et al., 2015) as well as in macrophages and microglia (Rojo et al., 2010, 2014a; Brune et al., 2013). This is because NRF2 increases cysteine and GSH levels in macrophages through regulation of the cystine/glutamate transporter and the GSH-synthesizing enzyme γ-glutamyl cysteine ligase modulator and catalytic subunits [γ-glutamyl cysteine ligase modulator subunit (GCLM) and γ-glutamyl cysteine ligase catalytic subunit (GCLC)]. Conversely, GSH depletion sensitizes macrophages to NRF2 activation by LPS (Diotallevi et al., 2017). All of these studies point to NRF2 as an anti-inflammatory factor, crucial in controlling the intensity and duration of inflammatory responses (Fig. 6).

NRF2 and NF-κB crosstalk through feed forward and feedback mechanisms (Fig. 7). At the transcriptional level, NF-κB activates NRF2 expression due to the existence of several functional binding sites in the promoter region of the *NFE2L2* gene, thus inducing a negative feedback loop (Rushworth et al., 2012). Moreover, both NF-κB and NRF2 transcription factors require the coactivator CBP/p300, which is a histone acetyltransferase that acetylates and increases the DNA-binding capacity. As such, NF-κB overexpression hampers the availability of CBP/p300 for NRF2, hence reducing its transcriptional capacity, whereas NF-κB knockdown shows the opposite effect (Liu et al., 2008). Additionally, NF-κB may promote interaction of histone deacetylase-3 with MAF proteins, therefore preventing their dimerization with NRF2 (Liu et al., 2008). NF-κB binds and translocates KEAP1 to the nucleus, thus favoring NRF2 ubiquitination and degradation in this cellular compartment (Yu et al., 2011). The E3 ligase adapter β-TrCP tags both IκBα (Winston et al., 1999) and NRF2 (Rada et al., 2011, 2012; Cuadrado, 2015) for proteasomal degradation, and therefore it can lead to increased NF-κB activity.

The anti-inflammatory activity of NRF2 was thought to rely only on modulation of redox metabolism or crosstalk with NF-κB. However, NRF2 can also directly block the transcription of the proinflammatory genes interleukin (IL)-6 and IL-1β in macrophages upon exposure to LPS (Kobayashi et al., 2016). LPS exposure or pharmacological activation of NRF2 leads to its binding to the proximal promoters of these proinflammatory genes and blocks the recruitment of RNA pol II. The mechanism appears to be independent of the binding of NRF2 to its well-established ARE enhancer. In other studies, NRF2 could directly regulate the expression of several other macrophage-specific genes, such as macrophage receptor with collagenous structure, a receptor required for bacterial phagocytosis, or CD36, a scavenger receptor for oxidized low-density

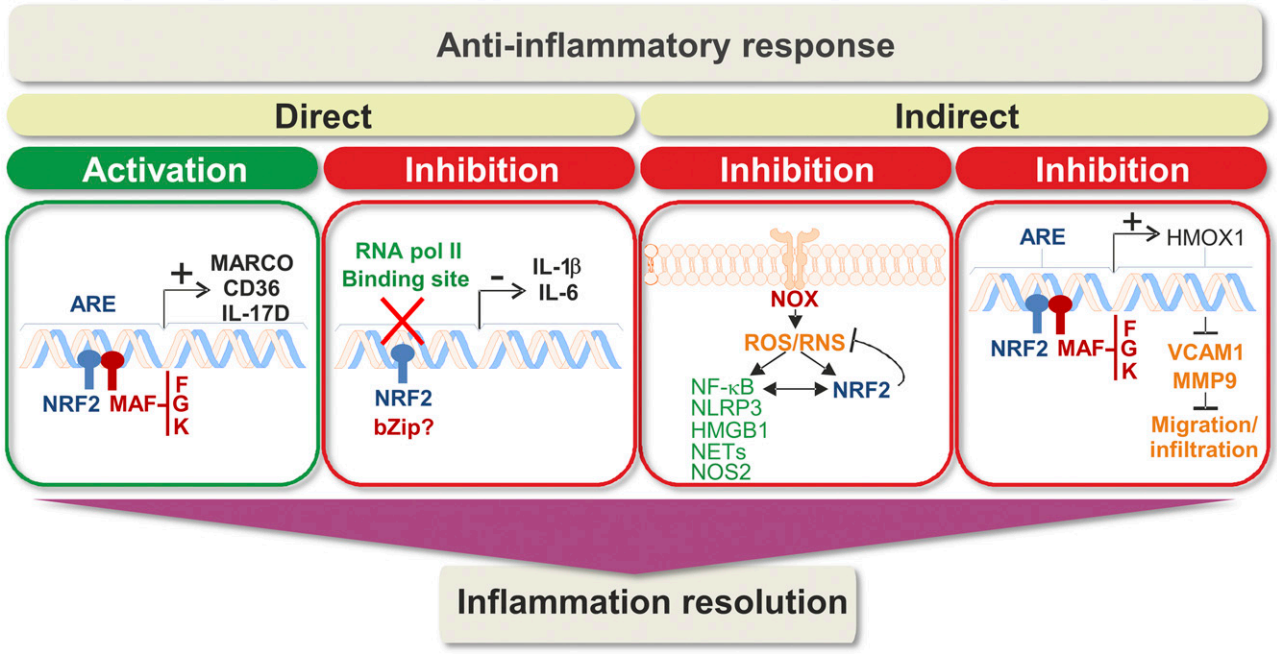

Fig. 6. Direct and indirect regulation of inflammation by NRF2. Direct mechanisms of action include transcriptional induction of anti-inflammatory genes as well as transcriptional repression of proinflammatory genes. In the second case, the quotation mark indicates that further work is required to identify the bZip partner of NRF2 in this function, if any. Indirect mechanisms to counteract inflammation involve ROS/RNS modulation and inhibition of migration/infiltration of immune cells. Overall, these pathways lead to an anti-inflammatory response that helps to properly resolve inflammation. The existence of polymorphisms in *NFE2L2* associated with reduced transcriptional activity, the altered levels of target genes in patients, and promising data from preclinical studies support a relevant role of NRF2 in inflammation resolution.

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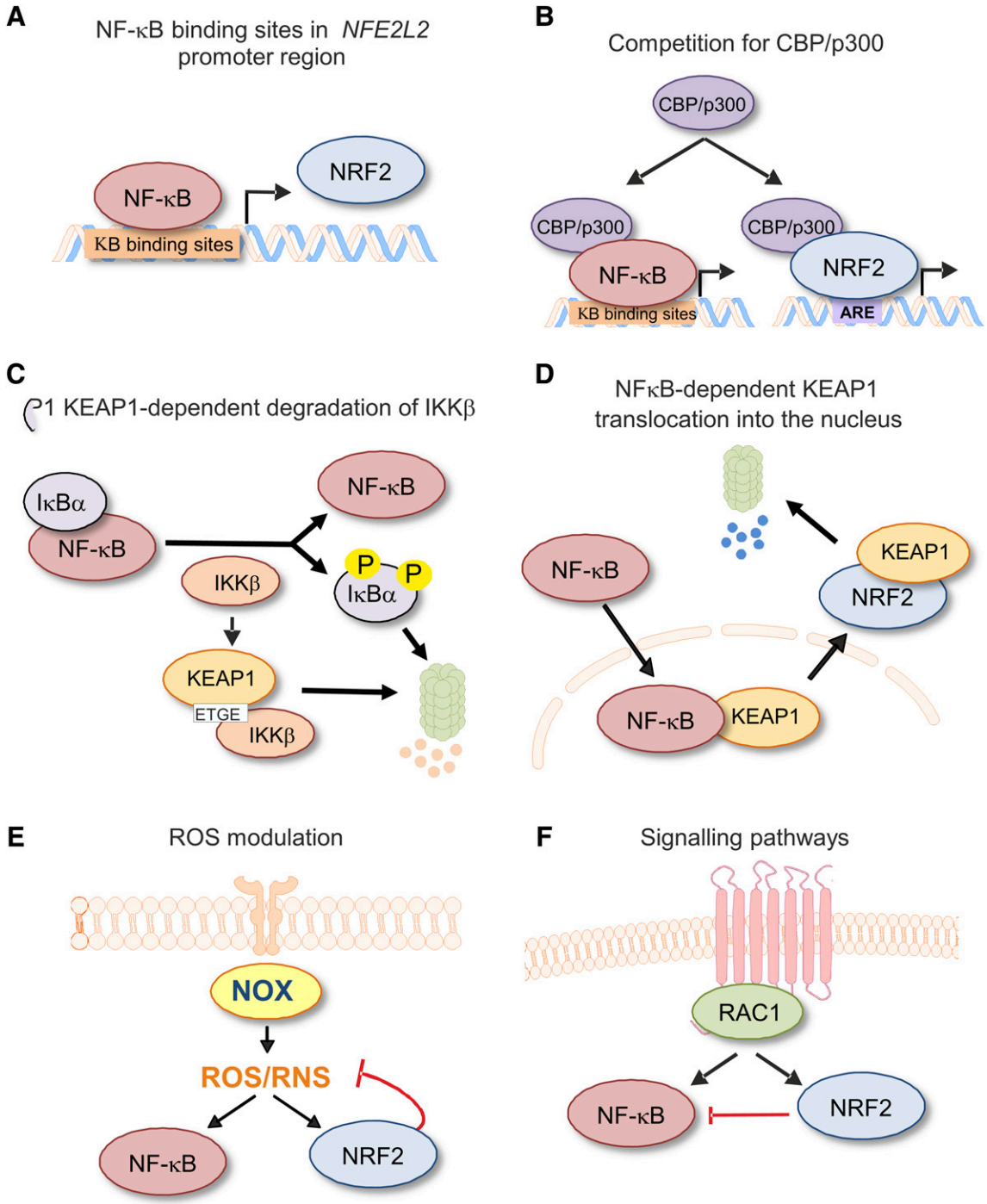

Fig. 7. Crosstalk between NF-κB and NRF2 occurs at different levels. (A) Responsive elements have been identified in the promoter region of *NFE2L2*. (B) Both NRF2 and NF-κB transcription factors compete for binding to the transcriptional coactivator CREB-binding protein (CBP/p300). (C) The NF-κB activating kinase IKKβ contains an ETGE motif allowing KEAP1 binding and subsequent ubiquitin–proteasome degradation. (D) NF-κB was reported to bind and translocate KEAP1 to the nucleus, thereby promoting NRF2 degradation. (E) ROS produced during inflammation activate NF-κB and NRF2; finally, NRF2 attenuates ROS and consequently NF-κB activity. (F) Different proinflammatory signals activate the Rho GTPase RAC1, which leads to NF-κB and NRF2 activation. Then NRF2 inhibits RAC1-mediated activation of NF-κB.

lipoprotein (Harvey et al., 2011; Ishii and Mann, 2014). Similarly, the gene encoding the proinflammatory cytokine, IL-17D, contains AREs, and this NRF2–T helper (Th)17 axis seems to confer protection against tumorigenesis and viral infections (Saddawi-Konefka et al., 2016).

Chronic inflammatory processes involve adhesion of leukocytes to the vascular endothelium and infiltration into the damaged tissue. Both processes appear to be modulated by NRF2 together with at least one of its target genes *HMOX1*, coding heme oxygenase-1 (HO-1). The NRF2/HO-1 axis inhibits adhesion of inflammatory

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cells to the endothelium by modulating the expression of several cell adhesion molecules such as vascular cell adhesion molecule 1 (Banning and Brigelius-Flohe, 2005; Wenzel et al., 2015). Additionally, NRF2/HO-1 inhibits metalloproteinase-9 in macrophages that are necessary for migration of immune cells within tissues (Bourdonnay et al., 2009).

Numerous preclinical studies have reported that activation of NRF2 by natural compounds (Satoh et al., 2013) or by disrupting its negative regulator KEAP1 leads to potent anti-inflammatory effects in myeloid leukocytes (Kong et al., 2011) and macrophages (Lin et al., 2008). In observational studies, a polymorphism in *NFE2L2* was associated with reduced transcriptional activity correlating with increased risk of inflammatory bowel disease (Arisawa et al., 2008b) and chronic gastritis (Arisawa et al., 2007). One example of the immune modulatory action of NRF2 is provided in the central nervous system. Injured neurons release fractalkine, a chemokine that specifically activates the phosphatidylinositol 3 kinase/AKT (PI3K/AKT) pathway in microglia, resulting in inhibition of GSK-3β and upregulation of NRF2 (Lastres-Becker et al., 2014). In this study, necropsies from AD and progressive supranuclear palsy patients exhibited a compensatory increase in fractalkine levels together with upregulated NRF2 protein, suggesting that this pathway contributes to limiting the inflammatory response in the diseased brain.

B. Nuclear Factor (Erythroid-Derived 2)–Like 2 in Autoimmune Diseases

At the periphery of the NRF2 diseasome cluster, we found several autoimmune disease phenotypes, such as vitiligo, asthma, multiple sclerosis (MS), and systemic lupus erythematosus (SLE). Indeed, extensive work in animal models of experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (RA), as well as clinical evidence in MS and psoriasis further points to a role of NRF2 in autoimmune diseases. Oxidative tissue damage and apoptosis may increase the generation of autoantigens, leading to activation of T cells and production of autoantibodies by B cells, e.g., as observed for 3-nitrotyrosine–positive proteins (Thomson et al., 2012). In addition, loss of phase II detoxification enzymes, many of which are transcriptionally regulated by NRF2, results in increased production of reactive intermediates that contribute to formation of haptens or damaged macromolecules that sometimes become immunogenic, increasing hence the pool of autoantigens that trigger autoimmune reactions. Because NRF2-regulated enzymes play a critical role in the detoxification of many chemicals, it is conceivable that NRF2 may be a protective mechanism against the environmental contribution to autoimmune pathogenesis (Ma, 2013). Potential mechanisms for NRF2-mediated regulation of autoimmunity also involve suppression of proinflammatory Th1 and Th17 responses and activation of immunosuppressive T regulatory (Treg) and Th2 ones. There is also increasing evidence that NRF2 may control the differentiation and function of dendritic cells (DCs) and macrophages involved in antigen presentation and regulation of adaptive immune responses. In fact, NRF2 deficiency was shown to alter the function and phenotype of DCs by increasing the expression of costimulatory molecules and consequently the antigen-specific T cell reactivity (Al-Huseini et al., 2013).

MS is a chronic inflammatory disease characterized by infiltration of autoreactive immune cells into the central nervous system. The absence of NRF2 exacerbates the development of EAE, which is a mouse model of MS (Johnson et al., 2010). Part of the effects associated with NRF2 deficiency may be related to the reduced levels of HO-1. Thus, mice with a myeloid-specific HO-1 deficiency exhibited higher incidence of lesions, accompanied by activation of antigen-presenting cells and infiltration of inflammatory Th17 and myelin-specific T cells (Tzima et al., 2009). Knockdown of KEAP1 (Kobayashi et al., 2016) or treatment with a wide range of small molecules that activate NRF2 (Buendia et al., 2016) inhibited the development and severity of disease. NRF2 is strongly upregulated in active MS lesions, and the expression of NRF2-responsive genes is predominantly found in areas of initial myelin destruction (Licht-Mayer et al., 2015). In MS brains, NRF2 and its targets NADPH:quinone oxidoreductase (NQO1) and HO-1 are mainly expressed in infiltrating macrophages and to a lesser extent in astrocytes, most likely as a compensatory response against pathologic ROS formation. In contrast, there is a lack of NRF2 and antioxidant gene expression in oligodendrocytes, and this may underlie their damage and loss in MS (van Horssen et al., 2010). As a consequence of the altered immune and redox homeostasis, HO-1 expression is reduced in peripheral blood mononuclear cells of MS patients and is downregulated during exacerbation of the disease (Fagone et al., 2013). Of note, gene expression profiling in interferon-β–treated patients identified NRF2 as a potential mediator of long-term antioxidant response and neuronal preservation (Croze et al., 2013).

SLE is underlined by high oxidative environment, deregulated cell death, and defects in removal of dead cells, which leads to cell necrosis as source of autoantigens. Female mice deficient in NRF2 develop with age a multiorgan autoimmune disorder similar to SLE, characterized by increased DNA oxidation, lipid peroxidation, splenocyte apoptosis, presence of antibodies against double-strand DNA and the Smith antigen, along with important tissue damage (vasculitis, glomerulonephritis, hepatitis, and myocarditis) (Li et al., 2004). The fact that only female mice show progression to SLE suggests that female-specific factors may contribute to break immune tolerance to self-antigens

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(Li et al., 2004). NRF2 deficiency also results in enhanced proliferative responses of $CD4^+$ T cells, altered $CD4^+/CD8^+$ ratio, and promotion of proinflammatory Th17 in SLE (Ma et al., 2006; Zhao et al., 2016). In fact, NRF2 depletion has been associated with Th17 differentiation and function during development of lupus nephritis, which seems to be mediated by regulation of the suppressor of cytokine signaling 3/phosphorylated signal transducer and activator of transcription (STAT)3 pathway and IL-1β signaling (Zhao et al., 2016). In addition, the salivary glands of *Nrf2*$^{-/-}$ mice show intense lymphocyte infiltration, reminiscent of the Sjögren syndrome, which is often associated with SLE (Ma et al., 2006). SLE patients exhibit alterations in repair mechanisms of oxidative DNA damage (Evans et al., 2000), high serum levels of oxidized proteins, apolipoprotein C3 (Morgan et al., 2007), oxidized phospholipids, and autoantibodies against oxidatively modified lipoproteins (Frostegard et al., 2005). NRF2 polymorphisms have not been associated with SLE susceptibility, although the SNP rs35652124 was related to increased risk of nephritis in childhood onset of Mexican females (Cordova et al., 2010).

Rheumatoid arthritis (RA) is a systemic inflammatory disease with a complex but still elusive autoimmune profile, in which neutrophils, macrophages, and lymphocytes are actively recruited and activated in the inflamed joints. This results in increased secretion of proinflammatory mediators such as ROS/RNS, eicosanoids, cytokines (IL-17, TNF-α, interferon-γ, IL-6, and IL-1β), and catabolic enzymes that trigger hyperproliferation of synovial fibroblasts, joint swelling, and progressive destruction of cartilage and bone (Roberts et al., 2015). Deletion of the NRF2 gene increases vulnerability to joint alterations in experimental RA models. For instance, in mice expressing the T cell receptor KRN and the major histocompatibility complex class II molecule A(g7) (the K/B$\times$N arthritis model), and in antibody-induced arthritis, NRF2 deficiency accelerates the incidence and aggravates the disease course (Maicas et al., 2011; Wu et al., 2016b). NRF2 deficiency dramatically upregulates migration of inflammatory cells, expression of cyclooxygenase-2 and inducible nitric oxide synthase, production of ROS and RNS, and release of proinflammatory cytokines and chemokines. Moreover, NRF2 may be a protective factor for bone metabolism in arthritis (Maicas et al., 2011), and NRF2/HO-1 activation exerts anti-inflammatory and antioxidant effects in animal models of RA and in human synovial fibroblasts (Wu et al., 2016b). It is interesting that antirheumatic gold(I)-containing compounds that stimulate the antioxidant response through activation of NRF2 and upregulation of HO-1 and GCLC proved clinical efficacy in RA (Kobayashi et al., 2016). Moreover, NRF2/HO-1 activation mediates the induction of synovial cell apoptosis and inhibition of proinflammatory cytokine production by cilostazol (Park et al., 2010) as well as the anti-inflammatory effects of H_2S and related compounds, which are able to modify by sulfhydration the cysteine residues of KEAP1 (Wu et al., 2016b). Other drugs that induce NRF2 and HO-1 signaling, such as rebamipide, can divert the differentiation of human and murine $CD4^+$ T cells toward an immunosuppressive Treg phenotype and inhibit the differentiation of $TCD4^+$ cells toward inflammatory Th17 cells through specific inhibition of STAT3 (Moon et al., 2014). Excessive ROS generation within the inflamed synovium appears to contribute to the pathogenesis of RA because patients show a marked increase in ROS formation, lipid peroxidation, protein oxidation, DNA damage, and decrease in the activity of the antioxidant defense mechanisms, all of these contributing to tissue damage and disease progression (Datta et al., 2014). In response to pathologic ROS formation, the NRF2 pathway is activated in synovial cells of RA patients and in joints of antibody-induced arthritic mice, but this response is apparently insufficient to counteract the disease progression (Wu et al., 2016b).

Vitiligo is a skin inflammatory disorder characterized by the accumulation of ROS in the epidermis, which participates in the death of melanocytes. These molecules modify DNA and melanosomal proteins with formation of autoantigens and activation of an autoimmune response against melanocytes (Xie et al., 2016). Genetic studies have revealed associations of NRF2 promoter SNPs with susceptibility to develop vitiligo, such as the SNP at -650 position (Guan et al., 2008), whereas the C allele of rs35652124 was shown to be associated with protective effects in a Han Chinese population (Song et al., 2016). NRF2 and its downstream detoxification target genes NQO1, GCLC, and GCLM are upregulated in the epidermis of vitiligo patients, suggesting insufficient activation of this defensive mechanism (Natarajan et al., 2010).

C. Nuclear Factor (Erythroid-Derived 2)–Like 2 in Chronic Respiratory Diseases

The relevance of NRF2 in respiratory diseases was reviewed in 2010 (Cho and Kleeberger, 2010), and in this work we will highlight only the most relevant findings (Fig. 8). Cigarette smoke is a main risk factor for chronic obstructive pulmonary disease (COPD). COPD patients have dysfunctional alveolar macrophages that lead to uncontrolled ROS production, proinflammatory mediators, defective phagocytosis, and an array of metalloproteinases that participate in tissue damage. In fact, the emphysematous lung tissue shows a direct correlation between alveolar macrophage density in the parenchyma and severity of lung destruction (Finkelstein et al., 1995). Impaired phagocytic activity of alveolar macrophages is a major cause of recurrent bacterial and viral infections that cause acute

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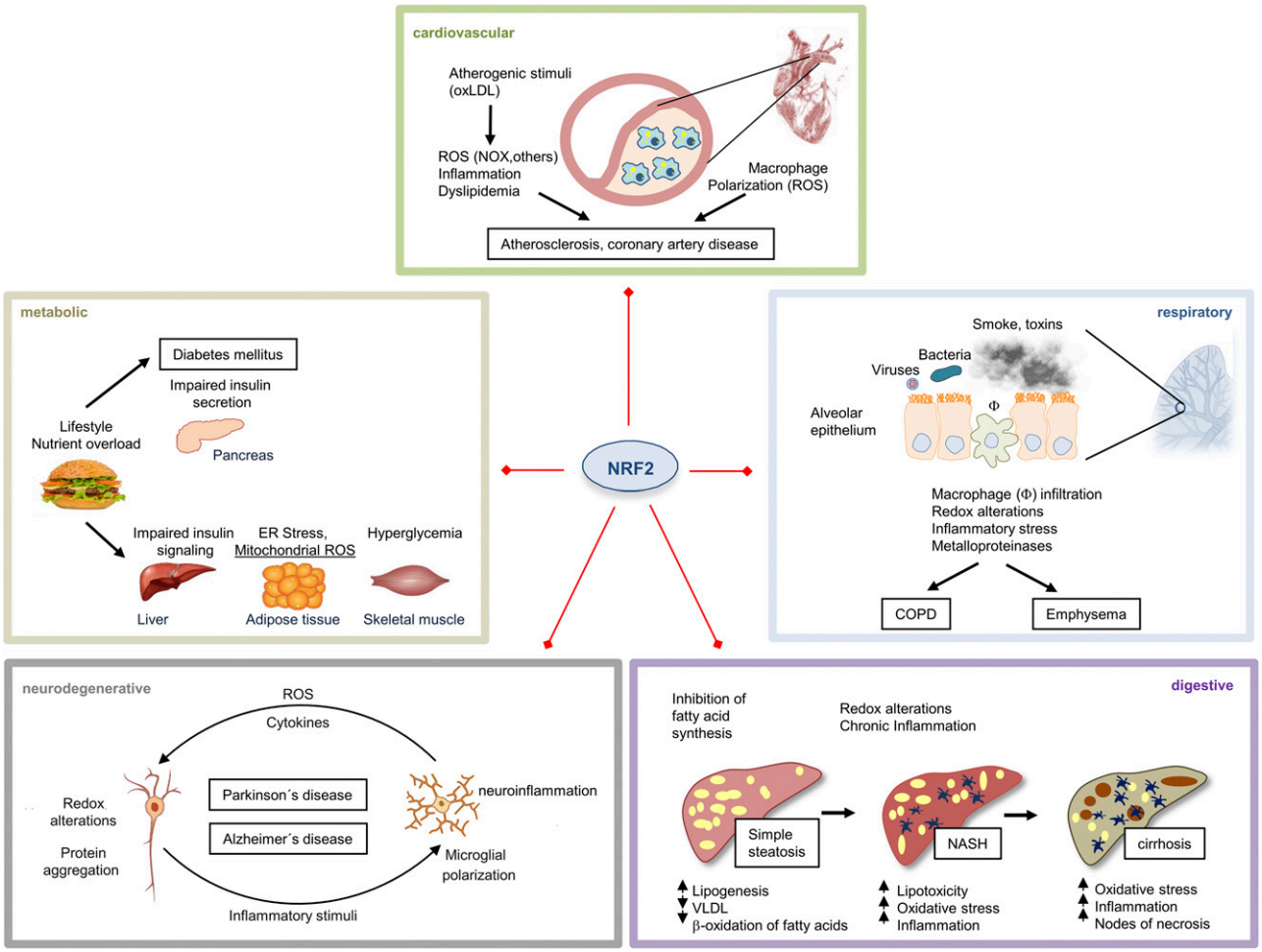

Fig. 8. Role of NRF2 in common mechanisms and pathophenotypes of chronic diseases. The picture provides some examples extracted from the NRF2 diseasome cluster of Fig. 5. Common pathomechanisms of these diseases include abnormally high ROS levels and low-grade chronic inflammation that participate in tissue damage. NRF2 provides a cytoprotective signature against these and other tissue-specific alterations by regulating the expression of numerous cytoprotective genes.

exacerbations of COPD and are a major source of morbidity and mortality. *Nrf2*$^{-/-}$ mice exhibit enhanced susceptibility to cigarette smoke-induced emphysema (Rangasamy et al., 2004). Importantly, activation of NRF2 with the isothiocyanate sulforaphane (SFN) restores bacteria recognition and phagocytosis, enhances pulmonary bacterial clearance by alveolar macrophages, and reduces inflammation in wild-type mice, but not in *Nrf2*$^{-/-}$ mice exposed to cigarette smoke (Harvey et al., 2011). In humans, the transcriptional signature of NRF2 is decreased in alveolar macrophages from patients with smoking-related lung emphysema as compared with smoking and nonsmoking patients without emphysema (Goven et al., 2008). Decreased NRF2 expression is associated with increased macrophage expression of the lipid peroxidation product 4-hydroxynonenal. In the *NFE2L2* promoter, a functional haplotype constituted by three SNPs and one triplet repeat, polymorphism that produces low to medium NRF2 expression (Yamamoto et al., 2004), was associated with increased risk to develop COPD (Hua et al., 2010). The low-expressing haplotypes were significant predictors for developing respiratory failure. Thus, the −617A allele of SNP rs6721961 had a significantly higher risk for developing acute lung injury (Marzec et al., 2007).

Pathologic ROS formation may play a role in the pathogenesis of chronic lung fibrosis. An early study demonstrated that pulmonary fibrosis induced by bleomycin is more severe in *Nrf2*$^{-/-}$ than in wild-type mice (Cho et al., 2004). In fact, wild-type mice induced an antioxidant and anti-inflammatory response by upregulating NRF2, and this could not be achieved in the *Nrf2*$^{-/-}$ mice. Later, it was verified that patients with idiopathic pulmonary fibrosis or chronic sarcoidosis/hypersensitivity pneumonitis exhibit increased expression of NRF2 and augmented levels of low-mol. wt. antioxidants in bronchoalveolar lavage fluids, such as uric acid, ascorbic acid, retinol, and α-tocopherol, suggesting an unsuccessful adaptive response to the ROS challenge (Markart et al., 2009). Mechanistically, NRF2 deficiency increases myofibroblast differentiation, whereas pharmacological induction of NRF2 with SFN results in lower number of myofibroblasts and

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attenuation of the profibrotic effects of transforming growth factor-β (TGF-β) (Artaud-Macari et al., 2013).

D. Nuclear Factor (Erythroid-Derived 2)–Like 2 in the Digestive System

The prominent position of pathophenotypes of the digestive system in the NRF2 diseasome highlights the relevance of the transcriptional signature of NRF2 as a potent adaptive mechanism to chronic oxidative damage and inflammatory stress triggered by xenobiotics. In the gastrointestinal tract (GI), chronic exposure to xenobiotics triggers dysfunctional interactions between the microbiota of the intestinal lumen and the immune system (Aviello and Knaus, 2017). This can lead to chronic diseases of the GI tract, like the inflammatory bowel disease (IBD) phenotypes comprising Crohn's disease and ulcerative colitis, in which there is evidence of activation of a protective NRF2 response. For instance, in colonic tissues from IBD patients, colon epithelial cells responded to inflammatory signals through a NRF2-dependent adaptation that was associated with increased proteasome protein expression (Kruse et al., 2016). Monocyte-derived macrophages from IBD patients evidenced a specific NRF2-dependent gene expression profile that was exacerbated in response to LPS, further suggesting an attempt to attenuate the inflammatory challenge (Baillie et al., 2017). At the genetic level, a particular genotype of the *NFE2L2* gene (−686−684) was associated with the development of ulcerative colitis, especially in females, in a Japanese cohort (Arisawa et al., 2008a). In fact, pathologic processes in the GI are highly dependent on the genetic background of the host in relation with dysfunctional interaction between the microbiota of the intestinal lumen and the immune system (Aviello and Knaus, 2017).

One of the symbiotic effects of the microbiota of the GI is to release moderate amounts of ROS that elicit a cytoprotective response mediated by NRF2 in epithelial colonocytes and infiltrating immune cells (Jones et al., 2015). Moreover, cytoprotective molecules that are under the transcriptional control of NRF2 in eukaryotes can also be produced by commensal bacteria. For instance, the HO-1 homologs in the microbiota may greatly contribute to GI homeostasis, and this can be therapeutically exploited for local delivery of carbon monoxide to the intestine (Onyiah et al., 2014).

The proven involvement of NRF2 in maintaining GI homeostasis makes this transcription factor a promising therapeutic target in IBD. Thus, several chemical compounds and dietary supplements might exhibit beneficial effects, like melatonin, 3-(3-pyridylmethylidene)-2-indolinone, butyrate, *Lactobacillus casei*, L-carnitine, 4-vinyl-2,6-dimethoxyphenol (canolol), lacto-wolfberry (formulated product of wolfberries in skimmed milk), etc. (Orena et al., 2015). Therefore, it is of utmost importance to define the involvement of NRF2 in chronic and acute diseases of the GI tract for better guidance on the therapeutic approach for modulating the NRF2 pathway.

The liver is also a first line of defense against food xenobiotics. Therefore, it is not surprising that the NRF2 diseasome highlights the relevance of this transcription factor in pathophenotypes associated with liver damage. Early work with the *Nrf2*$^{-/-}$ mouse model demonstrated its protective effect against acetaminophen-induced hepatocellular injury, benzo[a]pyrene-induced tumor formation, and Fas- and TNF-α–mediated hepatocellular apoptosis (Aleksunes and Manautou, 2007). The higher sensitivity of *Nrf2*$^{-/-}$ mice to chemical toxicity correlated with reduced basal and inducible expression of detoxification enzymes. In humans, the functional haplotype of three NRF2 promoter SNPs that result in reduced NRF2 expression was significantly associated with development of gastric mucosal inflammation, either independently or by interacting with *Helicobacter pylori* infection (Arisawa et al., 2007). Analysis of the transcriptional signature of NRF2 in patients with primary biliary cholangitis indicated that these patients exhibit reduced NRF2 expression together with low levels of HO-1 and GCLC proteins, and these impairments are more advanced in patients with cirrhosis (Wasik et al., 2017).

Pathologic ROS formation is a key mechanism of hepatocellular injury and disease progression in patients with nonalcoholic steatohepatitis (NASH) (Fig. 8). This disease evolves in two phases, one of progressive accumulation of fatty acids in hepatocytes and a second that involves liver injury and inflammatory pathologic ROS formation (Wang et al., 2018). Accordingly, mice fed with a high-fat diet (HFD) developed a simple steatosis, characterized by increased hepatic fat deposition without inflammation or fibrosis, but *Nrf2*$^{-/-}$ mice presented exacerbated hepatic steatosis and substantial inflammation, consistent with NASH (Reccia et al., 2017). It is interesting, however, that the hepatocyte-specific *KEAP1* deletion, while reducing liver steatosis, did not alter inflammation during development of NASH, suggesting a compensatory mechanism (Ramadori et al., 2016). At least in the rat model of NASH, dietary NRF2 activators attenuate the progression of liver fibrosis (Shimozono et al., 2013). Markers of pathologic ROS formation were increased in liver biopsies of NASH patients, and the NRF2 signature was increased, suggesting an attempt to reduce the oxidant and inflammatory burden (Takahashi et al., 2014).

E. Nuclear Factor (Erythroid-Derived 2)–Like 2 in the Cardiovascular System

The NRF2 diseasome cluster points to the high susceptibility of the cardiovascular system to changes in the cellular redox balance and the development of well-known comorbidities like atherosclerosis, hypertension, and diabetes (Griendling and FitzGerald, 2003a,b; Harrison et al., 2003; Jay et al., 2006)

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(Fig. 8). A role of NRF2 in preventing these pathophenotypes has been demonstrated in *Nrf2*$^{-/-}$ mice, which exhibit impaired cardiac structure (more remodeling events) and function (less fractional shortening) in response to chronic endurance exercise (Shanmugam et al., 2017a). They are also more susceptible to develop heart failure after myocardial infarction (Strom and Chen, 2017). In contrast, constitutive activation of NRF2 creates a reductive state, characterized by increased cardiac GSH/glutathione disulfide ratio and decreased ROS formation and malondialdehyde levels (Shanmugam et al., 2017b). In humans, microarray analysis in Tako–Tsubo cardiomyopathy indicated an increase in pathologic ROS levels and a compensatory upregulation of NRF2 during the acute phase of this contractile dysfunction (Nef et al., 2008). Recently, systemic inflammation and pathologic ROS formation in hemodialysis patients were associated with downregulation of NRF2 (Pedruzzi et al., 2015), and two promoter polymorphisms (rs35652124 and rs6721961) were associated with increased risk of mortality in these patients (Shimoyama et al., 2014).

One of the most relevant targets of NRF2 in endothelial homeostasis is HO-1, which is usually paralleled by upregulation of ferritin, hence decreasing free iron levels and preventing Fenton-type reactions. Bilirubin, which is generated from the combined activity of HO-1 and biliverdin reductase, is one of the most powerful endogenous antioxidants that scavenges ROS/RNS (Jansen et al., 2010), and is highly efficient in preventing lipid peroxidation in vitro (Stocker et al., 1987). *Hmox1*$^{-/-}$ mice show increased pulmonary hypertension in response to chronic hypoxia (Christou et al., 2000), and pharmacological HO-1 induction improves diabetic complications (Kruger et al., 2006), as well as nitroglycerin-induced vascular dysfunction (nitrate tolerance) (Wenzel et al., 2007). Recently, *Hmox1*$^{-/-}$ mice were shown to display upregulated NADPH oxidase-2, vascular pathologic ROS formation, markers of inflammation, endothelial dysfunction, and higher blood pressure in response to angiotensin-II (Wenzel et al., 2015). In fact, high serum levels of bilirubin are inversely correlated with the incidence of coronary artery disease (Hopkins et al., 1996). Bilirubin prevents the activation of the vascular NADPH oxidase (Kwak et al., 1991), involved in the development of cardiovascular diseases (Griendling and FitzGerald, 2003a,b; Harrison et al., 2003; Jay et al., 2006). Patients suffering from peripheral artery disease, which is a common manifestation of atherosclerosis, present reduced levels of HO-1 (Signorelli et al., 2016).

F. Nuclear Factor (Erythroid-Derived 2)–Like 2 in Metabolic Diseases

Type 2 diabetes mellitus (T2DM) is one of the most common chronic metabolic diseases and is highly underlined in the NRF2 diseasome. Pathologic ROS formation in insulin-sensitive tissues, as well as in pancreas, has been found in T2DM patients, resulting in severe impairment of both insulin secretion by pancreatic β cells and insulin action in peripheral tissues (Uruno et al., 2015) (Fig. 8). Likewise, pathologic ROS levels also contribute to the pathogenesis of diabetic complications due to nonenzymatic glycation of proteins. This has been evidenced in diabetic nephropathy, in which the glomeruli exhibit pathologic ROS levels and a compensatory elevation of NRF2 (Jiang et al., 2010). Since 2007, several studies have addressed the role of NRF2 in T2DM and its complications using animal models and cell lines. In vitro studies in human cells reported that NRF2 activation is achieved with acute exposure to high glucose, whereas longer incubation times or oscillating glucose concentration failed to activate NRF2 (Ungvari et al., 2011; Liu et al., 2014). Accordingly, these studies pointed out that NRF2 activation is dependent on glucose concentration and dynamics. In contrast, NRF2 is downregulated in peripheral blood mononuclear cells of prediabetic and diabetic patients, suggesting that NRF2 could be an important therapeutic target (Jimenez-Osorio et al., 2014).

The impact of NRF2 deficiency on hyperglycemia was first shown in *Nrf2*$^{-/-}$ mice, where oxidative and nitrosative alterations were enhanced and led to early-stage renal injury (Yoh et al., 2008). In a subsequent study, streptozotocin-induced diabetic *Nrf2*$^{-/-}$ mice exhibited exacerbated glomerular injury, together with high ROS production and increased expression of the profibrotic markers TGF-β and fibronectin (Jiang et al., 2010). In this diabetic model, NRF2 protected against dysfunction of the blood–retina barrier and the progression of diabetic retinopathy (Xu et al., 2014). Likewise, HFD-induced increase in vascular ROS levels was significantly exacerbated in *Nrf2*$^{-/-}$ mice and was accompanied by a severe endothelial dysfunction, as shown by diminished acetylcholine-induced relaxation of aorta and increased expression of intercellular adhesion molecule-1 and TNF-α (Ungvari et al., 2011).

NRF2 plays a complex role in tissue-specific insulin resistance. Thus, HFD-fed *Nrf2*$^{-/-}$ mice displayed better insulin sensitivity due to enhanced insulin signaling in liver and skeletal muscle than their wild-type counterparts, but conversely, these mice developed a severe NASH due to excessive hepatic lipotoxicity linked to pathologic ROS formation (Meakin et al., 2014). Accordingly, this study dissociated hepatic insulin resistance from the development of NASH. In light of these data, a subsequent study demonstrated that the livers of HFD-fed *Nrf2*$^{-/-}$ mice exhibited higher pathologic ROS formation by a significant depletion of GSH due to attenuated expression of the CYP2A5 enzyme (Cui et al., 2013). The knockdown of NRF2 in hepatocytes enhanced the apoptosis induced by palmitate, a fatty acid that is highly elevated in insulin-resistant

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obese patients. This effect was correlated with increased production of pathologic ROS, again reinforcing the key role of NRF2 in the progression of NASH (Pilar Valdecantos et al., 2015).

To further examine the role of NRF2 in the metabolic syndrome, NRF2 was ablated in leptin-deficient (ob/ob) mice, a model with an extremely positive energy balance (Xue et al., 2013). Interestingly, global ob/ob/*Nrf2*$^{-/-}$ mice or adipocyte-specific ob/ob/*Nrf2*$^{-/-}$ mice displayed reduced white fat mass, revealing NRF2 as a key player in adipogenesis. These mice had an even more severe metabolic syndrome that was characterized by hyperlipidemia, aggravated insulin resistance, and hyperglycemia, suggesting a mechanistic linkage between the metabolic syndrome and pathologic ROS formation.

Another subset of studies has evaluated the effects of persistent induction of NRF2 in glucose metabolism. Genetic NRF2 induction making use of a hypomorphic allele of *Keap1* (*Keap1*flox/- mutant) decreased blood glucose in the obese diabetic db/db mice by suppressing hepatic glucose 6 phosphatase through the repression of cAMP-CREB signaling in hepatocytes, as well as other gluconeogenic genes, such as peroxisome proliferator-activated receptor coactivator-1α (Uruno et al., 2013). Additionally, enhancement of NRF2 activity in *Keap11*-knocked down mice increased the phosphorylation of AMP-activated protein kinase (AMPK) in the liver, as well as insulin signaling in skeletal muscle, resulting in a substantial improvement of glucose tolerance (Xu et al., 2013). Due to the pleiotropic activities of NRF2 in the context of T2DM, the results of all these and other studies have evidenced the need to design multiple genetic and pharmacological strategies to elucidate the full array of NRF2 functions in tissues involved in the control of whole-body glucose homeostasis.

In addition to diabetic factors like age, body weight, and blood glucose, genetic factors that are linked to NRF2 have been poorly studied in humans. In a Chinese population, the SNP rs6721961 has been associated with pathologic ROS formation and risk of newly diagnosed T2DM and may also contribute to impaired insulin secretory capacity and increased insulin resistance (Wang et al., 2015). The same SNP was associated with diabetes in Mexican mestizo men (Jimenez-Osorio et al., 2017). In a case-control study performed with Han volunteers, a significant difference in genotypic and allelic frequencies of four SNPs of the *NFE2L2* gene was found between T2DM patients with and without complications, including peripheral neuropathy, nephropathy, retinopathy, foot ulcers, and microangiopathy (Xu et al., 2016b).

G. Nuclear Factor (Erythroid-Derived 2)–Like 2 in Neurodegenerative Diseases

The NRF2 diseasome provides evidence of NRF2 involvement in several neurodegenerative diseases, including AD and PD, which represent the most prevalent cognitive and motor disorders of the elderly. In neurodegenerative diseases, the connection between low-grade pathologic ROS formation and proteostasis is particularly relevant because most of these pathophenotypes are characterized by abnormal aggregation of specific proteins (Fig. 8). Evidence pointing to pathologic ROS formation in proteinopathy, as well as NRF2 as regulator of proteasome and autophagy was provided in cellular and animal models (Pajares et al., 2017). Initially, it was reported that the autophagy cargo protein sequestosome 1 (SQSTM1) competes with NRF2 for binding to KEAP1. SQSTM1 takes KEAP1 to the autophagosome degradative pathway, therefore upregulating NRF2 (Komatsu et al., 2010). More recently, it was found that NRF2 regulates the expression of autophagy genes involved in autophagy initiation, cargo recognition, elongation, and autolysosome clearance (Pajares et al., 2016). In this study, amyloidopathy and tauopathy induced by transgene overexpression of human mutant amyloid precursor protein and tau were aggravated in *Nrf2*$^{-/-}$ mice. A connection between NRF2 deficiency and neurodegeneration is supported by a growing body of evidence in animal models (Johnson and Johnson, 2015). The general point of view is that damaged neurons try to activate NRF2-dependent transcription, presumably to increase their own survival. Additionally, upregulation of NRF2 in astrocytes participates in metabolic compensations, including increased supply of GSH for augmenting their proliferative capacity (Bolanos, 2016), whereas NRF2 upregulation in microglia returns this immune cell to a resting state (Rojo et al., 2014a).

Ramsey et al. (2007) evidenced the nuclear localization of NRF2 in dopaminergic neurons of patients with PD. Other studies found that amyloid precursor protein– and tau-injured neurons expressed increased levels of NRF2 and its target SQSTM1, probably as compensatory mechanism to clear these toxic proteins through autophagy (Lastres-Becker et al., 2014; Pajares et al., 2016). In agreement with these results, the levels of HO-1, NQO1, GCLM, and SQSTM1 are increased in AD and PD brains (van Muiswinkel et al., 2004; Cuadrado et al., 2009; Schipper et al., 2009; Lastres-Becker et al., 2016). However, there is some controversy in the field, as Ramsey's study described the accumulation of NRF2 in the cytosol of AD-injured neurons, suggesting an impaired capacity of these neurons to upregulate NRF2 transcriptional activity. In addition, the cytoprotective proteins associated with NRF2 expression, such as NQO1 and SQSTM1, were partly sequestered in Lewy bodies, suggesting impaired neuroprotective capacity of the NRF2 signature in PD patients (Lastres-Becker et al., 2016). One possible explanation for this discrepancy could be that the levels of NRF2 and its target genes might change during ageing and disease progression.

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Some SNP haplotypes of *NFE2L2* were associated with decreased risk or delayed onset of ALS, AD, or PD. The onset of ALS was analyzed in two studies regarding three functional promoter SNPs that were previously linked to high gene expression. Interestingly, this haplotype was associated with a 4-year delay in onset of ALS (Bergstrom et al., 2014), but another study did not find a clear association (LoGerfo et al., 2014). Regarding AD, one haplotype allele was associated with 2-year earlier age onset of AD, suggesting that variants of the *NFE2L2* gene may affect AD progression (von Otter et al., 2010b). Genetic association of *NFE2L2* with PD has been analyzed in more detail. Three SNPs in the *NFE2L2* promoter (rs6721961, rs6706649, and rs35652124) were evidenced as protective haplotype in a case-control study (von Otter et al., 2010a). Such haplotype delayed the onset of disease in a Swedish cohort or even reduced the risk of PD in a Polish cohort. These results were supported by four new independent European case-control studies (von Otter et al., 2014), but were not replicated in a Taiwanese population (Chen et al., 2013), suggesting disparity in ethnicities and environmental factors. As an alternative approach, PD cells derived from olfactory mucosa were exposed to smoke extract or pesticide to assess gene–environment interaction, and several SNPs were identified that affect the susceptibility to these toxins (Todorovic et al., 2015). Altogether, it is possible that a slight activation of NRF2, such as that found for some functional haplotypes of the *NFE2L2* gene, should be enough to trigger protective mechanisms in the brain.

IV. The Kelch-Like ECH-Associated Protein 1 Paradox in Cancer

An apparent dichotomy appears to exist in the role of NRF2 in tumorigenesis and further tumor progression. On one hand, by activating biotransformation reactions, NRF2 protects against chemically induced carcinogenesis. Preclinical studies have demonstrated complete protection against aflatoxin B(1)-induced liver cancer after pharmacological activation of NRF2 in rats (Johnson et al., 2014). In contrast, the protective responses elicited by NRF2 provide a growth advantage in established cancers, and this will be the focus of this section.

Constantly increased levels of ROS can sustain tumorigenesis through alteration of genomic stability, along with activation of specific redox signaling circuits and inflammatory processes that favor survival and proliferation of tumor cells (Reuter et al., 2010). Therefore, upregulation of NRF2 represents a mechanism of adaptation of cancer cells to tolerate high ROS levels that propel tumor progression (Schumacker, 2006) as well as to maintain cancer stem cells that are responsible for tumor relapse and formation of distant metastases (Ryoo et al., 2016). For instance, the NRF2 signature in cancer stem cells from human colorectal tumors pointed out protective mechanisms mediated by high levels of GCLC, glutathione peroxidase, and thioredoxin reductase-1 that underlie the ability of these cells to counteract stressors and chemotherapeutics (Emmink et al., 2013). From this perspective, NRF2 behaves in cancer cells like an oncogene that by inducing chronic activation of ARE-mediated cytoprotective responses affords adaptation to their oxidative environment (Panieri and Santoro, 2016). Several mechanisms of malignant activation of NRF2 have been reported, including somatic mutations, epigenetics, and oncogenic signaling alterations.

Close to 600 somatic mutations have been reported in cancer along the coding sequence of *NFE2L2* (Gao et al., 2017). In Fig. 9, we show the results from a dataset of 10,000 cancer patients (Zehir et al., 2017). In most cases, these mutations alter the interaction of the DLG and ETGE motifs of NRF2 with KEAP1, hence inducing hyperactivation of NRF2 in several solid tumors, including esophagus, skin, lung, and larynx carcinomas (Kim et al., 2010b; Taguchi and Yamamoto, 2017). For instance, in advanced esophageal squamous cancer, gain-of-function mutations of NRF2 were associated with tumor recurrence and poor prognosis due to increased proliferation, attachment-independent survival, and resistance to chemo- and radiotherapy (Shibata et al., 2011). Loss-of-function mutations in the *KEAP1* gene are also frequent in some solid tumors such as lung cancer (Singh et al., 2006). Based on the strong evidence that this pathway regulates β-TrCP/NRF2, it is strange that disrupting somatic mutations have not been found at the interface between NRF2 and β-TrCP. This fact suggests that such mutations are not viable for unknown reasons or that the increase in NRF2 levels that would result from the escape of β-TrCP is not sufficient to drive oncogenicity.

Nonetheless, somatic mutations account for chronic NRF2 activation only in a fraction of cancer patients. At the level of gene expression, it is interesting that an allele of the SNP rs6721961 ($-617C > A$) located at the ARE enhancer of the human NRF2 gene abolished self-induction of NRF2, and this correlated with remarkable survival of these cancer patients (Okano et al., 2013). Epigenetic changes due to promoter hypermethylation of three CpG sites of *KEAP1* have been described in lung tumors, resulting in consequent NRF2 activation that could be reversed by 5-aza-2′-deoxycitydine treatment (Wang et al., 2008). The role of miRNAs in the post-transcriptional regulation of NRF2 levels has been reviewed recently (Kurinna and Werner, 2015). Briefly, miR200a targets the KEAP1 mRNA in human breast cancer cells, leading to its degradation and consequent activation of NRF2 (Eades et al., 2011). In turn, miR28 facilitates the degradation of the NRF2 mRNA (Yang et al., 2011).

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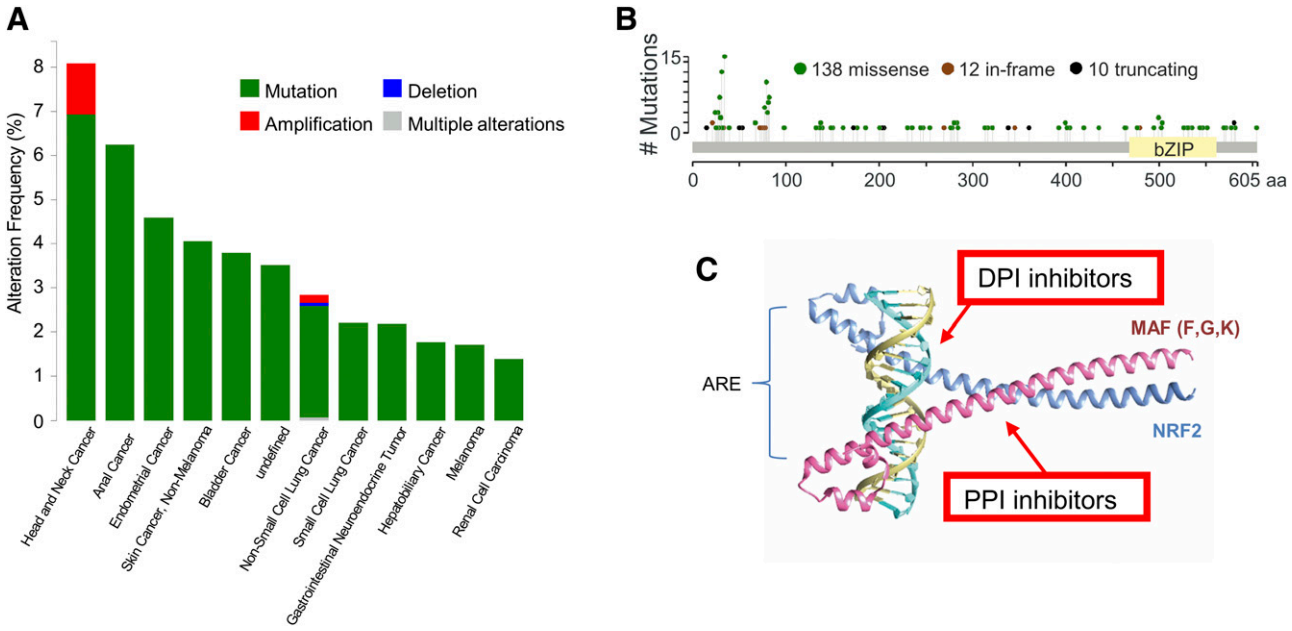

Fig. 9. Somatic mutations found in tumors of the MSK-IMPACT Clinical Sequencing Cohort (MSKCC) study (Zehir et al., 2017) and pharmacologic strategies to inhibit NRF2. (A) Percentage of tumors with NRF2 mutations. (B) Distribution of mutations along the NRF2 polypeptide. (C) PyMOL representation of the interaction between the NRF2/MAFF heterodimer and the ARE element. Blue, NRF2; pink, MAFF. The red arrows indicate possible mechanisms of inhibition of NRF2 by small molecules that could target the bZip domains of interaction between NRF2 and MAF proteins (PPI inhibitors) or the interface of interaction of the NRF2-MAF heterodimer with the ARE [DNA–protein interaction (DPI)].

Oncogenes or mutated tumor suppressors may enhance the activation of NRF2 in cancer. Endogenous oncogenic alleles of KRAS, BRAF, or c-MYC upregulate NRF2, presumably through oncogene-mediated ROS generation and consequent chronic inactivation of KEAP1 (DeNicola et al., 2011). The mutated form of the tumor suppressor p53, which sustains the growth of cancer cells by enhancing nutrient uptake and synthesis of building blocks, can also upregulate NRF2, possibly through the crosstalk with the Sp1 transcription factor that binds to the NRF2 promoter (Tung et al., 2015).

The phosphorylation of NRF2 (phospho-NRF2) by various protein kinases is a potential mechanism of activation in 107 hepatocellular carcinomas. Increased levels of phospho-NRF2 were associated with reduced KEAP1 expression and poor 5-year overall survival of patients exhibiting this distinctive phenotype (Chen et al., 2016). Additionally, mutations in the phosphatase and tensin homolog (PTEN) tumor suppressor sustain hyperactive and oncogenic phosphatidylinositol-3-kinase (PI3K)–AKT signaling and consequent increase in NRF2 activity due to the downregulation of the PTEN/GSK-3/β-TrCP pathway for the proteasomal degradation of NRF2 (Rada et al., 2011, 2012; Cuadrado, 2015). Therapeutic interventions targeting the PTEN/GSK-3/β-TrCP pathway should take into consideration that GSK-3 may act both as tumor suppressor and tumor promoter, and is also implicated in the generation of cancer stem cells (McCubrey et al., 2014).

Some stress-induced proteins interact with KEAP1 and thus compete with its NRF2 binding in cancer cells. Consequently, NRF2 escapes KEAP1-mediated degradation. One of the NRF2 competitors for KEAP1 binding is the phosphorylated form of the autophagy-adaptor protein SQSTM1 that occurs during selective autophagy used by cancer cells for sustaining their own growth (Shimizu et al., 2016). The cyclin-dependent kinase inhibitor p21 that promotes cell cycle arrest in cancer stem cells was also demonstrated to inhibit the association of NRF2 with KEAP1 through the interaction of its KRR motif with DLG and ETGE motifs in NRF2 (Chen et al., 2009). Recently, it was shown that dipeptidyl-peptidase 3, which bears an ETGE motif, may compete with NRF2 for binding to KEAP1 (Hast et al., 2013). Overexpression of dipeptidyl-peptidase 3, possibly induced by chronic alteration of the redox status, correlates with increased expression of ARE genes and poor prognosis, particularly in estrogen receptor–positive breast cancer (Lu et al., 2017).

NRF2 induces metabolic changes that contribute to cancer progression. For instance, a multiplatform nontargeted metabolomics study identified patterns of metabolite changes in breast tumor samples (Tang et al., 2014). They found that GSH and 3-(4-hydroxyphenyl)lactate were positively correlated with the involvement of BRCA1 in redox homeostasis through interaction with NRF2. Metabolomics studies also indicate that NRF2 can increase aerobic glycolysis in cancer cells to support their high-energy requirements. This occurs through NRF2-mediated induction of Mn-superoxide dismutase expression, leading to elevated mitochondrial production of hydrogen peroxide

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and to activation of AMPK. The process is regulated by caveolin-1, which binds directly to both NRF2 and KEAP1, and impedes on NRF2 activation and hence on the glycolytic shift. This is apparently one explanation why glycolytic tumors, which are generally more aggressive, have a caveolin-1^{low}/Mn-superoxide dismutase^{high} phenotype (Hart et al., 2016). NRF2 can also drive glucose and glutamine toward anabolic pathways required for tumor cell proliferation (Mitsuishi et al., 2012). In the presence of active PI3K–AKT signaling and loss of KEAP1 activity, NRF2 was shown to induce the shift of glucose metabolism from glycolysis toward anabolic pathways (purine synthesis) in cancer (Mitsuishi et al., 2012; Xu et al., 2016a). This is underlined by NRF2-mediated transcription of genes involved in the pentose phosphate pathway and generation of NADPH (glucose 6 phosphate dehydrogenase; phosphogluconate dehydrogenase, malic enzyme 1, isocitrate dehydrogenase 1, transketolase, and transaldolase), along with genes involved in purine nucleotide synthesis (phosphoribosyl pyrophosphate amidotransferase, methylenetetrahydrofolate dehydrogenase 2).

The fact that activation of NRF2 confers a growth advantage to cancer cells might argue that its pharmacologic activation in chronic diseases presented in this work might imply a high risk of developing cancer. However, it must be considered that the oncogenic activity of NRF2 requires mutations in its gene or in *KEAP1*, which results in very high and persistent induction of NRF2 signaling. This is not the case in pharmacological therapy, in which it is possible to modulate drug dosing and NRF2 activity. Moreover, empirical evidence indicates that subjects enrolled in clinical trials with NRF2 activators do not exhibit increased cancer risk. This is best exemplified in the case of patients with MS, who have been taking the NRF2 activator dimethyl fumarate for several years since it was approved by the regulatory agencies in 2013. Conversely, the use of NRF2 inhibitors in cancer patients might lead to manifestation of the pathophenotypes described in the NRF2 diseasome. This is a possibility that will need further investigation when NRF2 inhibitors reach the clinic.

V. Nuclear Factor (Erythroid-Derived 2)–Like 2 Drugome

This section attempts to develop a NRF2 drugome that might be useful for future clinical directions to target therapeutically NRF2 centered on the pathophenotypes of the NRF2 diseasome. As stated in Fig. 2B, the pharmacological activation of NRF2 is being pursued for increasing its stability by targeting KEAP1. These strategies are based on the discovery of either electrophile compounds that alter the KEAP1 structure or small molecules that prevent the docking of NRF2 to KEAP1. Although not yet demonstrated empirically, the GSK-3 inhibitors should prevent the recognition of NRF2 by β-TrCP and some compounds could be discovered to prevent binding of NRF2 to β-TrCP. The inhibition of NRF2 is being analyzed with compounds that target the bZip dimerization domain to prevent formation of the active NRF2/MAF heterodimer. By comparison with other transcription factors, it might be possible to find small molecules that impede binding of the NRF2/MAF heterodimer to the ARE (Fig. 9C). This section summarizes the most important findings from a translational point of view in both de novo drug discovery and repurposing.

A. Electrophilic Nuclear Factor (Erythroid-Derived 2)–Like 2 Inducers

The majority of known physiologic or pharmacological NRF2 inducers are electrophilic molecules that covalently modify, by oxidation or alkylation, cysteine residues present in the thiol-rich KEAP1 protein (Hur and Gray, 2011; Satoh et al., 2013). KEAP1 is one of the best-suited proteins to act as electrophilic or redox sensor, as it contains 27 cysteine residues in humans and functions as an electrophile trap. The cysteines C151, C273, and C288 of KEAP1 appear to be the most prone to electrophile reaction (Fig. 2B), although there are some specificities (Yamamoto et al., 2008; Saito et al., 2015). Electrophile adducts inhibit KEAP1 in two different ways. One is induction of a conformational change in KEAP1 that will result in loss of its binding capacity to NRF2. The other is the blockade of the interaction between KEAP1 and CUL3/RBX1, resulting in sequestration of KEAP1 with NRF2 and further stabilization of newly synthetized NRF2 (Rachakonda et al., 2008; Baird and Dinkova-Kostova, 2013; Cleasby et al., 2014; Saito et al., 2015).

At least 30 recent patents for NRF2 modulators are indexed in the World International Property Organization. These patents are protecting chalcone derivatives, novel amide triterpenoid derivatives, deuterium-substituted fumarate derivatives, 3-alkylamino-1H-indolyl acrylate derivatives, withanolide, a benzyl derivative containing an activated vinyl group, andrographolide or $[S]^{+}$apomorphine, and sesquiterpene lactone derivative (Sun et al., 2017). Although most of these compounds proved to be useful to some degree from a preclinical proof-of-concept perspective, their clinical value is to date generally very limited. Only a few of them have entered clinical trials, and regulatory bodies, such as Food and Drug Administration or European Medicines Agency, have approved even fewer. We in this study discuss the most developed NRF2 activators along the translational pipeline.

Fumaric acid esters are the most prominent example of a KEAP1 modifier, and dimethyl fumarate (DMF) is to date the only Food and Drug Administration– and European Medicines Agency–approved drug registered as NRF2 activator. The monoester form of DMF,

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monomethyl fumarate (MMF), was described as its active metabolite. DMF and MMF are Michael acceptors that directly react with cysteine residues present in KEAP1 (Lin et al., 2011).

DMF and other fumaric acid esters have been used for treating psoriasis for over 50 years, starting at a time when the function of NRF2 was still unknown. This compound was licensed in Europe under the commercial name of Fumaderm. Clinical trials showed a decrease in the psoriasis area and severity index to 50%–80% after 12–16 weeks of DMF therapy (Altmeyer et al., 1994; Mrowietz et al., 1998). More recently, DMF has demonstrated its efficacy in the treatment of adults with moderate to severe chronic plaque psoriasis in a phase III trial (BRIDGE) (Mrowietz et al., 2017). The mechanism of action underlying fumarates in remission of psoriatic lesions includes the decrease in the number of peripheral T cells along with a shift from a Th1 toward a Th2 immune response (Ghoreschi et al., 2011; Tahvili et al., 2015). In another autoimmune disease, SLE, fumaric acid esters have been used as systemic combination therapy in the treatment of severe, extensive, and refractory cutaneous manifestations (Saracino and Orteu, 2017).

DMF was approved in 2013 for the treatment of MS under the commercial name Tecfidera (Xu et al., 2015). The use of DMF in MS patients was propelled by positive results obtained in the MS mouse model of EAE. Significant therapeutic effects on the disease course and histology were associated with a markedly reduced macrophage-mediated inflammation in the spinal cord. Multiplex cytokine analysis in blood evidenced an increase of the anti-inflammatory cytokine IL-10 in DMF-treated animals (Schilling et al., 2006). Moreover, DMF also improved preservation of myelin, axons, and neurons in wild-type, but not in *Nrf2*$^{-/-}$ mice (Ellrichmann et al., 2011). In humans, DMF demonstrated a significant reduction of lesions and annualized relapse rate in MS (Schimrigk et al., 2006). Two phase III clinical trials, DEFINE and CONFIRM, substantiated these results (Fox et al., 2012; Gold et al., 2012). Therefore, DMF is currently used as the first line of treatment of relapsing-remitting MS that cannot be treated by traditional therapies. New formulations of DMF are being tested and patented to improve drug bioavailability and efficacy (Sun et al., 2017). For instance, MMF has been used to develop a second generation of NRF2 inducers as prodrugs (Zeidan et al., 2014). The lead compound ALKS-8700, a 2-(2,5-dioxo-1-pyrrolidinyl)ethyl ester derivative of MMF, is rapidly converted into MMF in the body, hence increasing its bioavailability and reducing gastrointestinal side effects. ALKS-8700 is currently under phase III clinical trial (EVOLVE MS).

DMF and MMF modulate the immune response. For example, they inhibit the maturation of DCs by reducing the release of inflammatory cytokines and hence the ability of DCs to process antigens. Moreover, DMF and MMF activate natural killer cells to lyse DCs and enhance apoptosis of both DCs and T cells (Ghoreschi et al., 2011; Al-Jaderi and Maghazachi, 2015). As such, DMF and MMF impede T cell–mediated autoreactivity. Some studies indicate that DMF also induces type II DCs by triggering GSH depletion, which results in enhanced HO-1 activity and suppression of STAT1 phosphorylation. These classic type II DCs suppress Th1- and Th17-mediated responses in favor of Th2 ones. Furthermore, the increased production of IL-10 by DCs favors the differentiation of $CD4^+$ T cells toward a suppressive Treg phenotype (Ockenfels et al., 1998; Ghoreschi et al., 2011). DMF also inhibits the nuclear translocation of NF-κB (Peng et al., 2012) and consequently the production of inflammatory mediators, such as TNF-α, IL-1β, IL-6, chemokines, adhesion molecules, and nitric oxide in microglia and astrocytes (Brennan et al., 2017), as well as in peripheral blood mononuclear cells (Eminel et al., 2017). In addition, DMF exerts antiangiogenic effects that are dependent on the downregulation of vascular endothelial growth factor receptor-2 expression in endothelial cells (Meissner et al., 2011). Recent findings indicated that DMF reduced the number of $CD4^+$, $CD8^+$, Th1, and Th17 cells, whereas the $CD4^+/CD8^+$ ratio and the Th2 subset were increased in the blood of these patients. Interestingly, the inhibitory effects of DMF/MMF on T cell activation were confined mainly to memory T cells (Wu et al., 2017). These immunomodulatory activities of DMF or MMF are important for the protection of oligodendrocytes against ROS-induced cytotoxicity (Scannevin et al., 2012).

Additional mechanisms might explain the inhibition of NF-κB independently of NRF2 activation. Thus, DMF may interact with cysteine residues in several proteins that regulate NF-κB signaling (Blewett et al., 2016). In addition, DMF inhibits ubiquitin-conjugating enzymes and thus prevents the degradation of the IκB repressor of NF-κB in response to IL-1β or Toll-like receptor agonists (McGuire et al., 2016). Moreover, DMF binds directly to specific cysteine residues in protein kinase C-θ, a key kinase involved in signaling by the T cell receptor (Blewett et al., 2016). In addition, MMF and DMF activate the hydroxycarboxylic acid receptor-2, resulting in inhibition of NF-κB and downregulation of proinflammatory cytokines and adhesion molecules (Chen et al., 2014; Gillard et al., 2015) and leading to decreased neutrophil infiltration (Chen et al., 2014). Although these NRF2-independent effects would be relevant in the acute inflammatory phase of EAE, the neuroprotective efficacy of DMF during chronic autoimmune demyelination depends on NRF2 activation (Linker et al., 2011). The clinical benefit of DMF treatment in both *Nrf2*$^{-/-}$ and wild-type mice was associated with a reduction of inflammatory Th1 and Th17 cells, as well as with induction of

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anti-inflammatory M2 monocytes. At the same time, decreased expression of CD80 and CD86 costimulatory molecules was observed in wild-type, but not in *Nrf2*$^{-/-}$ mice, indicating that at least these effects were NRF2-dependent (Schulze-Topphoff et al., 2016).

The success of DMF for the treatment of autoimmune diseases indicates that other diseases that share common pathomechanisms underlined by chronic, low-grade inflammation and pathologic ROS formation might benefit from the repositioning of this drug. In a mouse model of Huntington's disease, the survival rate, muscle function, and body weight were preserved with DMF treatment, and this was associated with an increased number of intact neurons (Ellrichmann et al., 2011). Also, in a recent preclinical study of PD, using the α-synucleinopathy model of this disease, DMF was neuroprotective in wild-type, but not in *Nrf2*$^{-/-}$ mice due to impaired autophagy induction (Lastres-Becker et al., 2016).

DMF was shown to prevent endothelial dysfunction and cardiovascular pathologic ROS formation and inflammation in diabetic mice (Sharma et al., 2017), and decreased atherosclerosis, kidney dysfunction, and other diabetic complications were reported in apolipoprotein E–deficient mice after streptozotocin injection (Tan et al., 2014). Additionally, several studies indicated that DMF might exert antitumor activity by inhibiting the NF-κB pathway, hence adding therapeutic value in the treatment of aggressive cancers (Kastrati et al., 2016). DMF is a relevant example of the drug-repurposing concept within the network pharmacology approach.

Synthetic triterpenoids are derivatives of 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oate (CDDO; bardoxolone, RTA401) that resemble the natural product oleanolic acid. They exhibit Michael acceptor activity through its α-β unsaturated scaffold and represent the most potent inducers of NRF2 (Sun et al., 2017). They interact with C151 of KEAP1 and impede its interaction with CUL3, hence leading to NRF2 activation (Cleasby et al., 2014). Proof-of-principle studies strongly support the use of synthetic triterpenoids for degenerative diseases and are being the focus of intensive research as antioxidant modulators of inflammation by Reata/Abbott. For instance, CDDO-imidazole (CDDO-Im RTA403) induced in peritoneal neutrophils of wild-type but not *Nrf2*$^{-/-}$ mice the expression of various antioxidant genes (*Hmox1*, *Gclc*, *Gclm*, and *Nqo1*) and attenuated LPS-induced ROS generation and production of proinflammatory cytokines, consequently decreasing mortality (Thimmulappa et al., 2006b). CDDO-ethyl amide (RTA405) and CDDO-CDDO-trifluoethyl amide (RTA 404) had significant effects across all endpoints measured in a toxin-induced PD model (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Kaidery et al., 2013). In the EAE model of MS, CDDO-CDDO-trifluoethyl amide suppressed inflammation, pathologic ROS formation, and myelin degeneration (Pareek et al., 2011).

CDDO-methyl ester (CDDO-Me, RTA 402) was the first CDDO that reached in clinical trials for the treatment of diabetic nephropathy (Pergola et al., 2011). Although the results of the phase II were very encouraging, CDDO-Me was later withdrawn at phase III (BEACON trial) due to cardiovascular safety issues (Zhang, 2013) that were not related to NRF2 but most likely to an off-target alteration of endothelin signaling (de Zeeuw et al., 2013; Chin et al., 2014). Currently, CDDO-Me is under clinical study as potential treatment of Alport syndrome and pulmonary hypertension (Table 2). In an effort to improve its safety profile, further studies have led to the development of CDDO-difluoropropionamide (RTA-408, omaveloxone), which is currently in phase II trial for the treatment of Friedreich's ataxia, ocular inflammation, and pain after ocular surgery.

Oltipraz is an organosulfur compound that is used as an antischistosomal agent and is currently in phase III trial for the treatment of nonalcoholic steatohepatitis. Advanced clinical trials for the treatment of Huntington's disease are under development with minocycline, an antibiotic that has demonstrated neuroprotective properties due to NRF2 activation (Kuang et al., 2009). Another NRF2 inducer in phase I clinical study for the treatment of acute kidney disease is CXA-10, a nitro fatty acid with anti-inflammatory properties through the activation of NRF2 (Batthyany and Lopez, 2015). Many other NRF2 inducers with the same mechanism of action have been described in the last years (Buendia et al., 2015a,b, 2016), and some are in preclinical studies, such as the compound VEDA-1209, a chalcone derivative with a good anti-inflammatory profile for the treatment of ulcerative colitis.

SFN is an isothiocyanate produced from enzymatic cleavage of the organosulfur compound glucoraphanin, which is present in sprouts of broccoli, cabbage, and other *Brassicacea* plants. The catalytic reaction is driven by the enzyme myrosinase that is found in plants and microbiota of the GI tract (Kensler et al., 2013). More recently, SFN has been obtained by chemical synthesis (Kim et al., 2015). Translation of SFN to the clinic has been achieved by administration of SFN-containing broccoli sprout powder to patients with T2DM (Bahadoran et al., 2012). Broccoli powder decreased plasma malondialdehyde and oxidized low-density lipoprotein (LDL) and increased the total antioxidant capacity. Cardiovascular risk factors such as serum triglycerides, oxidized LDL/LDL ratio, and atherogenic index of plasma (log of triglycerides/high-density lipoprotein ratio) were also reduced. Furthermore, proinflammatory markers such as C-reactive protein and IL-6 were decreased. In a more recent study, SFN administered as concentrated broccoli sprout extract suppressed glucose production from

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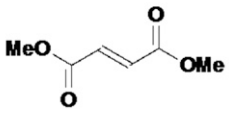
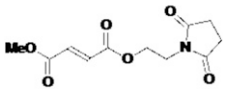
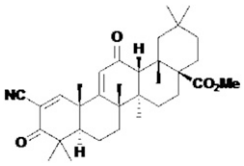
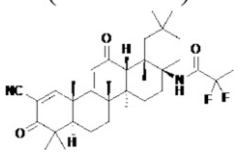
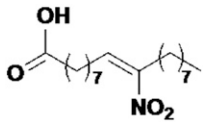
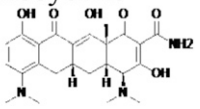
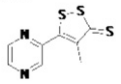
TABLE 2
Selected NRF2 inducers acting as electrophilic modifiers of KEAP1
The reference corresponds to the code in ClinicalTrials.gov.

Compound	Disease	Clinical Trial	Reference
Dimethyl fumarate 	Multiple sclerosis	Approved	
	Psoriasis	Approved	
	Rheumatoid arthritis	Phase II	NCT00810836
	Adult brain glioblastoma	Phase I	NCT02337426
	Cutaneous T cell lymphoma	Phase II	NCT02546440
	Obstructive sleep apnea	Phase II	NCT02438137
	Chronic lymphocytic leukemia small lymphocytic lymphoma	Phase I	NCT02784834
ALKS-8700 (MMF-derivate) 	Multiple sclerosis	Phase III	NCT02634307
CDDO-Me (RTA402) 	Diabetic nephropathy	Phase II	NCT00811889
	Chronic kidney disease, T2DM, diabetic nephropathy	Phase III	NCT01351675
	Liver disease	Phase I/II	NCT00550849
	Hepatic impairment	Phase I	NCT01563562
	Advanced solid tumors	Phase I	NCT00529438
	Lymphoid malignancies		NCT00508807
	Alport syndrome	PhaseII/III	NCT03019185
	Pulmonary hypertension	Phase III	NCT03068130
	Pulmonary arterial hypertension	Phase III	NCT02657356
	Renal insufficiency, T2DM	Phase II	NCT01053936
CDDO-DFPA (RTA-408) 	Mitochondrial myopathy	Phase II	NCT02255422
	Friedreich's ataxia	Phase II	NCT02255435
	Inflammation and pain following ocular surgery	Phase II	NCT02065375
	Corneal endothelial cell loss, ocular pain and inflammation, cataract surgery	Phase II	NCT02128113
	Melanoma	Phase I/II	NCT02259231
	Breast cancer	Phase II	NCT02142959
	Non-small cell lung cancer, melanoma	Phase I	NCT02029729
CXA-10 	Acute kidney injury	Phase I	NCT02248051
Minocycline 	HD	Phase II/III	NCT00277355
	Intracerebral hemorrhage	Phase I/II	NCT01805895
	Retinitis pigmentosa	Phase I/II	NCT02140164
	Intracerebral hemorrhage	Phase I/II	NCT03040128
Oltipraz 	NASH	Phase III	NCT02068339
	Schistosomiasis	Approved	
	Lung cancer	Phase I	NCT00006457

(*continued*)

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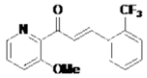
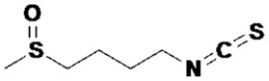
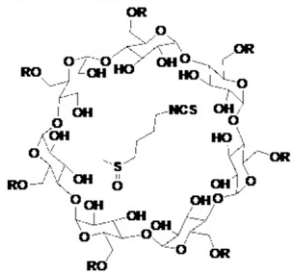
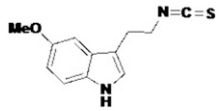
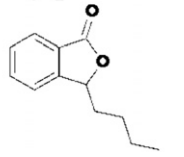
TABLE 2—*Continued*

Compound	Disease	Clinical Trial	Reference
VEDA-1209 	Ulcerative colitis	Preclinical PK	——
Sulforaphane 	Cystic fibrosis	Phase II	NCT01315665
	Schizophrenia	Phase II/III	NCT02880462
		Phase II	NCT02810964
		Phase II	NCT01716858
	COPD	Phase II	NCT01335971
	Atopic asthmatics	Phase I	NCT01845493
	Autism	Phase II	NCT01474993
		Phase II	NCT02909959
		Phase II	NCT02677051
		Phase II	NCT02654743
		Phase I/II	NCT02561481
	Healthy	Phase I	NCT01008826
		Phase I	NCT02023931
	Melanoma	Phase I	NCT01568996
	Asthma	Phase I	NCT01845493
		Phase I/II	NCT01183923
	Prostate cancer	Phase II	NCT01228084
	Breast cancer	Phase II	NCT00843167
	Lung cancer	Phase II	NCT03232138
	Environmental carcinogenesis	Phase II	NCT01437501
	Alcohol sensitivity	Phase II	NCT01845220
	Aging	Phase II	NCT03126539
	Allergic rhinitis	Phase II	NCT02885025
	Helicobacter pylori infection	Phase IV	NCT03220542
	T2DM	Phase II	NCT02801448
	Head and neck squamous cell carcinoma, head and neck cancer, tobacco-related carcinoma	Phase I	NCT03182959
Sulforadex 	Subarachnoid hemorrhage	Phase II	NCT02614742
	Breast neoplasm	Phase I/II	NCT02970682
	Prostate cancer	Phase I	NCT02055716
			NCT01948362
ITH12674 	Brain ischemia	Preclinical PK	—
DL-3-n-butylphthalide 	Alzheimer disease	Phase II	NCT02711683
	Acute ischemic stroke	Phase I/II	NCT02149875
	Vascular cognitive impairment	Phase II/III	NCT02993367
	Cerebrovascular occlusion, collateral blood circulation, anterior cerebral circulation infarction	Phase IV	NCT02594995

(*continued*)

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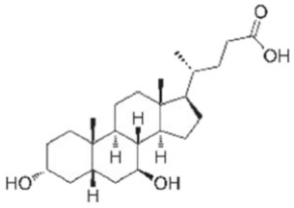
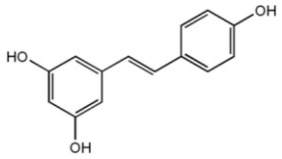
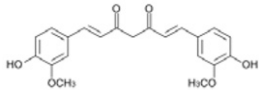
TABLE 2—*Continued*

Compound	Disease	Clinical Trial	Reference
Ursodiol 	Cholestasis	Phase II/III	NCT00846963
	HD	Phase I	NCT00514774
	Barrett esophagus, low-grade dysplasia	Phase II	NCT01097304
	Chronic hepatitis C	Phase III	NCT00200343
	T2DM	Phase II	NCT02033876
Resveratrol 	T2DM	Phase I	NCT01677611
	Colon cancer	Phase I	NCT00256334
	COPD	Phase III	NCT02245932
	Friedreich ataxia	Phase I/II	NCT01339884
	NASH	Phase II/III	NCT02030977
	Non-ischemic cardiomyopathy	Phase III	NCT01914081
	Endometriosis	Phase IV	NCT02475564
	Chronic renal insufficiency	Phase III	NCT02433925
	Metabolic syndrome X	Phase II	NCT02114892
	Chronic subclinic inflammation	Phase III	NCT01492114
	AD	Phase II	NCT01504854
		Phase III	NCT00743743
Curcumin 	Colorectal cancer	Phase I	NCT00433576
	HD	Phase III	NCT02336633
	T2DM, cardiovascular risk	Phase IV	NCT01052025
	Schizophrenia, cognition, psychosis	Phase I/II	NCT02104752
	Acute kidney injury, abdominal aortic aneurysm	Phase II/III	NCT01225094
	Chronic kidney diseases, T2DM	Phase II/III	NCT03262363
	AD	Phase I/II	NCT00164749
	Neoplasms	Phase II	NCT02944578
	Crohn's disease	Phase III	NCT02255370
	Chronic schizophrenia	Phase IV	NCT02298985
	Mild cognitive impairment	Phase II	NCT01811381
	Prostate cancer	Phase III	NCT02064673
	Major depression	Phase IV	NCT01750359

hepatocytes by nuclear translocation of NRF2 and decreased expression of key enzymes involved in gluconeogenesis. Moreover, SFN reduced fasting blood glucose and glycated hemoglobin in obese patients with T2DM (Axelsson et al., 2017). SFN-induced activation of NRF2 protected renal cells against lupus nephritis by reducing the ROS burden and by inhibiting the NF-κB and TGF-β1 signaling pathways (Jiang et al., 2014a).

In regard to neurodegenerative diseases, it has been shown that SFN crosses the blood brain barrier and provides sufficient cerebral bioavailability to activate the NRF2 signature and to reduce LPS-elicited neuroinflammation, as reflected in the reduction of proinflammatory markers (inducible nitric oxide synthase, IL-6, TNF-α) and microgliosis in the hippocampus (Innamorato et al., 2008). SFN also safeguarded dopaminergic neurons against the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and attenuated astrogliosis and microgliosis (Jazwa et al., 2011). In line with these findings, SFN reduced the levels of phosphorylated tau and increased Beclin-1 and LC3-II, suggesting that NRF2 activation might facilitate degradation of this toxic protein through autophagy in the brain (Jo et al., 2014). SFN-treated rats subjected to spinal cord injury had significantly decreased levels of inflammatory cytokines, reduced contusion volume, and improved coordination (Wang et al., 2012). This drug also ameliorated EAE by preserving the blood-brain barrier and by reducing pathologic ROS formation and the number of inflammatory cells (Li et al., 2013). SFN has been used to date in at least 32 clinical studies addressing chronic diseases such as cancer, asthma, chronic kidney disease, T2DM, cystic fibrosis, autism, and schizophrenia (Duran et al., 2016; Houghton et al., 2016) (Table 2).

Altogether, these observations paved the way for the development of other SFN-derived compounds exhibiting an improved pharmacokinetic profile. SFN is an oily substance with low stability in hydrophilic media. Its physicochemical profile prompted Evgen Pharma (Wilmslow, Cheshire, England) to develop a cyclodextrin complex formulation, Sulforadex, which is under phase II clinical trial for the treatment of subarachnoid hemorrhage. SFN was also hybridized with melatonin to generate the ITH12674, a compound that was designed to have a dual drug–prodrug mechanism of action for treatment of brain ischemia (Egea et al., 2015).

Curcumin is the main curcuminoid found in turmeric and has been used for the treatment of obesity, metabolic syndrome, and prediabetes. A nontargeted

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metabolomics study to investigate the effects of curcumin on rat liver was conducted by means of gas chromatography with electron impact mass spectrometry. The intermittent intake of curcumin upregulated NRF2 and displayed antioxidant and anti-inflammatory roles in the protection against liver damage (Qiu et al., 2016). Oral consumption of curcumin is effective in lowering serum triglycerides, IL-1β, IL-4, and vascular endothelial growth factor, and in increasing adiponectin levels in blood. In T2DM patients, curcumin decreases the levels of fasting blood glucose, glycated hemoglobin, serum free fatty acids, triglycerides, and uric acid, and increases the levels of lipoprotein lipase (Na et al., 2013; Chuengsamarn et al., 2014).

Resveratrol is a polyphenol that protects plants against fungal infection and is found in the skin of grapes, red wine, berries, and many other plants. Resveratrol exerts antioxidant properties through activation of NRF2 signaling by downregulating KEAP1 expression and by activating the protein deacetylase sirtuin-1 (Ungvari et al., 2010). In healthy subjects, the dietary administration of resveratrol prevented the elevation in plasma of cholesterol, endotoxins, prooxidants, and inflammatory markers (p47phox, KEAP1, IL-1β, and TNF-α). These events correlated with the elevation of NRF2 activity as determined by enhanced expression of its targets NQO1 and glutathione S-transferase (Ghanim et al., 2011). In T2DM patients, insulin sensitivity was improved after 4 weeks of treatment, as determined by enhanced insulin signaling via AKT, decreased pathologic ROS formation, and reduced levels of glycated hemoglobin (Brasnyo et al., 2011; Bhatt et al., 2012). Overall, resveratrol was reported to prevent major cardiovascular, inflammatory, oxidative, and metabolic complications in hypertension, hypercholesterolemia, atherosclerosis, ischemic heart disease, diabetes, and metabolic syndrome in animal models and patients (Xia et al., 2017).

A problem that is frequently overlooked is the lack of selectivity of electrophilic KEAP1 inhibitors. Electrophiles react with different nucleophiles present in the cell, thus exhibiting off-target and nondesired side effects. For instance, CDDO-Im can interact with more than 500 different targets (Yore et al., 2011). In general, several protein phosphatases contain redox-sensitive cysteines in their catalytic center, and some KEAP1 inhibitors may modify and inactivate these phosphatases, hence disturbing signaling networks. One of these phosphatases is PTEN (Lee et al., 2002; Kitagishi and Matsuda, 2013; Han et al., 2015). The catalytic C124 residue of PTEN can be modified through adduct formation with strong electrophiles such as CDDO-Im (Pitha-Rowe et al., 2009) and tert-butylhydroquinone (Rojo et al., 2014b). Then, the increased activation of the PI3K/AKT pathway involves inhibition of GSK-3 and subsequent stabilization of NRF2 (Fig. 2C) (Rada et al., 2011, 2012). Moreover, KEAP1 interacts with other proteins that also contain the high-affinity binding motif ETGE (Hast et al., 2013), such as Bcl-2 and IKKβ (Kim et al., 2010a; Cazanave et al., 2014). Therefore, some results obtained from KEAP1-deficient cells may not necessarily be related to NRF2 activation.

B. Protein–Protein Interaction Inhibitors for Nuclear Factor (Erythroid-Derived 2)–Like 2 Activation

To overcome the pitfall of selectivity, a new class of NRF2 inducers that prevent the docking of NRF2 to KEAP1 has emerged (Richardson et al., 2015). The use of PPI inhibitors has been achieved by the prior elucidation of the X-ray crystal structure of KEAP1 (Padmanabhan et al., 2006) bound to a peptide containing the high-affinity binding ETGE motif of NRF2 (Lo et al., 2006). KEAP1 contains a six-bladed β-propeller with specific hydrophobic and hydrophilic residues that participate in the docking of the ETGE motif that adopts a β-hairpin structure. Docking is mainly favored by electrostatic interactions between several arginines of KEAP1 and the two glutamates in the ETGE motif (Lo et al., 2006; Padmanabhan et al., 2006). The docking to KEAP1 of the low-affinity DLG motif of NRF2 has also been characterized (Tong et al., 2007). Based on these interactions, peptidomimetic compounds were the first example of PPI inhibitors with significantly improved selectivity over electrophiles (Hu et al., 2013; Marcotte et al., 2013; Winkel et al., 2015). These inhibitors show weak activity in cells, and a new provocative strategy has now been reported based on the use of cyclic peptides. One of these peptides exhibited high-binding affinity for KEAP1 and activation of NRF2 and elicited anti-inflammatory effects in mouse macrophages (Lu et al., 2018).

The discovery of new peptides and small-molecule inhibitors of the KEAP1/NRF2 interaction has been reviewed recently (Abed et al., 2015; Jiang et al., 2016). Briefly, a series of truncated NRF2 peptides was initially evaluated as direct inhibitors of PPI using surface plasmon resonance and fluorescence polarization assays (Hu et al., 2013). The minimal peptide sequence with inhibitory capacity was the 9-mer sequence of LDE-ETGE-FL (Chen et al., 2011; Inoyama et al., 2012). In parallel, Wells and collaborators (Hancock et al., 2013) searched for new putative peptide ligands using a phage display library combined with high-throughput fluorescence polarization assay. They found that hybrid peptides based upon the ETGE motif of NRF2 and SQSTM1 have superior binding activity to KEAP1 compared with either native peptide alone. To facilitate cellular uptake, a peptide was designed with the ETGE motif fused to the cell transduction domain of the HIV-Tat protein and the cleavage sequence of calpain (DEETGE-Cal-Tat). This peptide showed neuroprotective and cognitive-preserving effects in a mouse model of cerebral ischemia (Tu et al., 2015).

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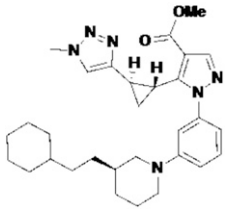
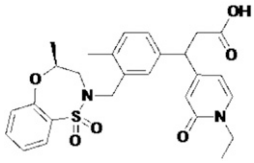
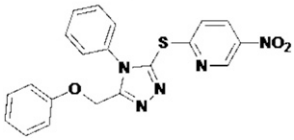
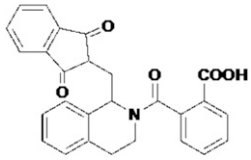
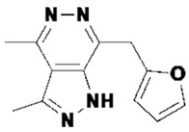
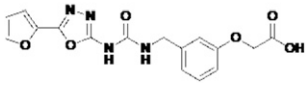
TABLE 3
Selected NRF2 inducers acting as NRF2–KEAP1 protein–protein interaction inhibitors

Compound	Patent	Title	Applicant
Compound 7 	WO2017060855	Arylcyclohexyl pyrazoles as NRF2 regulators	GlaxoSmithKline Astex Therapeutics
Compound 15 	WO2016/202253	NRF2 regulators	GlaxoSmithKline Astex Therapeutics, GlaxoSmithKline (China), R&D
BM19 	WO2014/197818	Small-molecule activators of NRF2 pathway	General Hospital, Regents of the University of California
LH601 	WO2013/067036	Direct inhibitors of KEAP1–NRF2 interaction as antioxidant inflammation modulators	Rutgers, The State University of New Jersey, Broad Institute
	WO2011/156889	Novel modulators of NRF2 and uses thereof	TRT Pharma Gerald Batist, Jian Hui Wu
AN-465/144580 	JP2011/0167537	KEAP1 protein-binding compound, crystal of complex between the same and KEAP1 protein, and method for producing the same	Toray Industries

(*continued*)

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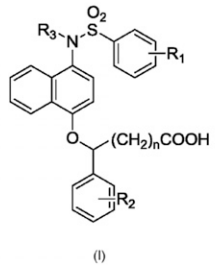
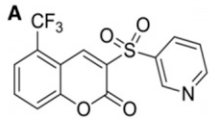
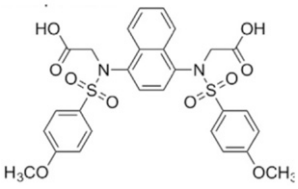
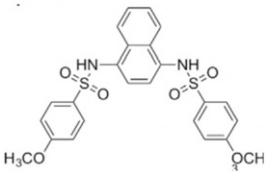
TABLE 3—*Continued*

Compound	Patent	Title	Applicant
3-(2-Oxo-2-phenylethylidene)-2,3,6,7-tetrahydro-1H-pyrazino[2,1-a]isoquinolin-4(11bH)-one (Compound (I))  (I)	WO2017124835	1-sulfonamido-4-aryloxy compound, and preparation method and medicinal application thereof	China Pharmaceutical University
3-(pyridin-3-ylsulfonyl)-5-(trifluoromethyl)-2H-chromen-2-one (PSTC) 	WO2015/092713	NRF2 regulators	GlaxoSmithKline Astex Therapeutics Limited
Compound 2 	WO2011156889A1	Novel regulators of NRF2 and uses thereof	TRT Pharma Gerald Batist, Jian Hui Wu
Compound 16 	WO2016/202253	NRF2 regulators	GlaxoSmithKline Astex Therapeutics, GlaxoSmithKline (China), R&D CO., LTD

Five families of PPI inhibitors have been described: tetrahydroisoquinoline (Jnoff et al., 2014; Richardson et al., 2015), thiopyrimidine (Marcotte et al., 2013), naphthalene (Jiang et al., 2014b), carbazone (Ranjan et al., 2014), and urea derivatives (Sato et al., 2013). Table 3 compiles recent patents addressing these small molecules. Although these compounds are very promising, it is still needed to demonstrate that they are selective for the KEAP1/NRF2 interaction, because KEAP1 also targets at least Bcl2 and IKK (Kim et al., 2010a; Hast et al., 2013; Cazanave et al., 2014).

From the large number of compounds indexed in the available libraries, the compounds LH601, benzenesulfonyl-pyrimidone 2, N-phenyl-benzenesulfonamide, and a series

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of 1,4-diphenyl-1,2,3-triazoles might be very well-suited candidates to inhibit the PPI with KEAP1 (Hu et al., 2013; Jnoff et al., 2014; Bertrand et al., 2015; Wen et al., 2015; Nasiri et al., 2016). These studies described in detail the atomic interaction with KEAP1, the affinity, and the thermodynamics parameters of binding. The therapeutic efficacy of these compounds is to be analyzed in future work in which safety, potency, and blood brain barrier permeability should be addressed.

C. Drug Targets Other Than Kelch-Like ECH-Associated Protein 1 for Nuclear Factor (Erythroid-Derived 2)–Like 2 Activation

Protein kinase GSK-3 phosphorylates the two serine residues in the DSGIS sequence of NRF2 to generate a phosphorylation-dependent degradation motif or phosphodegron (Fig. 2). This phosphodegron is recognized by the E3 ligase adapter β-TrCP, leading to ubiquitin-dependent proteasomal degradation of NRF2. Therefore, GSK-3 inhibitors should stop NRF2 degradation by preventing the generation of this phosphodegron. GSK-3 is an important kinase in AD and other pathophenotypes. It phosphorylates the cytoskeletal protein tau, facilitating the formation of neurofibrillary tangles, which are pathologic intracellular aggregates that disturb axonal transport and lead to neuronal death (Silva et al., 2014). Therefore, it has been speculated that GSK-3 inhibition might have the double benefit of preventing neurofibrillary tangle formation and NRF2 degradation. Unfortunately, most pipelines for the development of GSK-3 inhibitors have been discontinued due to futility, although in most cases there was not good evidence of target modulation (Palomo and Martinez, 2017).

Conceptually, inhibitors of the β-TrCP-phosphoNRF2 interaction should also lead to NRF2 activation as they should disrupt this branch of NRF2 degradation (Fig. 2). The molecular interactions between the β-propeller of β-TrCP and a peptide containing the NRF2 phosphodegron have been resolved by NMR (Rada et al., 2012). As it happens for KEAP1/EGTE, the most relevant amino acids appear to be several arginine residues of β-TrCP that interact with the two phosphoserines of the DpSGIpS motif. However, the discovery of small molecules that could inhibit the β-TrCP-phosphoNRF2 interaction is still to come.

Additional strategies have been developed to inhibit the NRF2 repressor BACH1, a bZip protein that makes heterodimers with MAF proteins and blocks expression of ARE genes. Efficient inhibition of BACH1 by the HPP-4382 compound has been described in vitro (Attucks et al., 2014), but, prior to a full clinical trial, the safety and efficacy profile of HPP-4382 will have to be demonstrated in vivo. Considering that other pathways may also influence NRF2 activity, it is reasonable to speculate that a combinatorial approach will be the best way to activate this transcription factor.

D. Nuclear Factor (Erythroid-Derived 2)–Like 2 Inhibitors

NRF2 has a "dark side" related to its oncogenic activity when constitutively and highly overexpressed. Therefore, NRF2 inhibition has been proposed as a mechanism to sensitize cancer cells to chemotherapeutic drugs or radiotherapy (Milkovic et al., 2017). Two strategies can be envisioned to inhibit NRF2 with small molecules: PPI inhibitors that disrupt the bZip interaction between NRF2 and MAFs, and DNA–protein interaction inhibitors that block binding of NRF2-MAF to the ARE (Fig. 9C). Both strategies are hampered by the need by such drugs to overcome the large free energy of association between protein–protein and, to a lesser extent, protein–DNA interfaces. Nevertheless, such drugs have been found for other bZip transcription factors such as STAT3-STAT3, MYC-MAX, and JUN-FOS (Yap et al., 2011), and new small molecules are being described for NRF2-MAF. For instance, malabaricone-A is a pro-oxidant compound that overcomes leukemia resistance by targeting NRF2 (Manna et al., 2015). Ascorbic acid (vitamin C), a well-known ROS scavenger, was found to sensitize imatinib-resistant cancer cells by decreasing the levels of the NRF2/ARE complex, reducing the expression of the *GCLC* gene and dropping GSH levels (Tarumoto et al., 2004). All-trans-retinoic acid is another example of NRF2 inhibitor that significantly decreases NRF2 activation by potent electrophilic NRF2 inducers in vitro and in vivo. It activates the retinoic acid receptor α, which forms a complex with NRF2, hence impeding the binding of the transcription factor to ARE genes (Wang et al., 2007).

Natural products such as brusatol (Ren et al., 2011; Olayanju et al., 2015), ochratoxin A (Tarumoto et al., 2004; Limonciel and Jennings, 2014), and trigonelline (Arlt et al., 2013) have also been found to inhibit NRF2. However, their mechanism of action is not fully understood. In fact, a significant issue related to currently available compounds is the profound off-target effect that they might have. For instance, promising results with brusatol were recently discouraged by the finding that this drug exerts a general and unspecific inhibition of protein synthesis, resulting in the drop of NRF2 levels, but also of many other proteins with rapid turnover (Harder et al., 2017). Similarly, the antiprotozoal agent halofuginone, used in veterinary practice, enhances the chemosensitivity of cancer cells by suppressing NRF2 accumulation, but this effect appears to be indirect by inhibiting prolyl–transfer RNA synthesis that is strongly required for ribosomal translation of NRF2 as well as many other proline-containing proteins (Tsuchida et al., 2017).

A novel approach to identify selective NRF2 inhibitors has been reported recently by the use of quantitative high-throughput screen of small-molecule inhibitors (Singh et al., 2016). The authors identified a first-in class compound, termed ML385, which most likely prevented

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the binding of NRF2 to other bZip coactivators. This compound blocked NRF2 transcriptional activity and sensitized KEAP1-deficient cells to carboplatin and other chemotherapeutic drugs. Additional studies are needed to confirm whether ML385 is selective for NRF2 or if it also inhibits other bZip transcription factors.

In light of the highly favorable systemic effects of NRF2 in various tumor pathophenotypes, a specific targeting of NRF2 with small-molecule inhibitors seems to provide an excellent clinical approach. However, it is necessary to determine whether cancer treatment with NRF2 inhibitors increases the risk of other pathophenotypes of the NRF2 diseasome.

E. Repurposing Instead of De Novo Drug Discovery and Development

As previously discussed, numerous compounds are under development to provide a benefit for the pathophenotypes associated with the NRF2 diseasome. An alternative approach is to give drugs that are already in clinical use for a certain pathomechanism a new use for the treatment of other pathomechanisms that are connected to NRF2. This section provides the basis for repositioning some commonly used drugs based on their role in NRF2 regulation.

Metformin is the first-line monotherapy for the T2DM. According to Fig. 6, it provides therapeutic benefit to the NRF2 subcluster of pathophenotypes related to glucose metabolism. In fact, SFN reduces hepatic glucose production and improves glucose control in patients with T2DM (Axelsson et al., 2017). Interestingly, some evidence suggests that metformin may be effective in preventing other nonglycemic pathophenotypes of the NRF2 diseasome, including cardiovascular (Nesti and Natali, 2017), respiratory (Sato et al., 2016), digestive (Bauer and Duca, 2016), neurodegenerative (Markowicz-Piasecka et al., 2017), autoimmune (Schuiveling et al., 2017), and neoplastic (Heckman-Stoddard et al., 2017) disorders. The mechanism of action of metformin is not completely clear, but it involves inhibition of mitochondrial complex I, thus increasing the AMP/ATP ratio (El-Mir et al., 2000; Owen et al., 2000) and leading to activation of the energy sensor AMPK (Hardie, 2004; Rena et al., 2017). Importantly, AMPK activates NRF2 (Wang et al., 2017a; Zhao et al., 2017), and pharmacological targeting of this axis attenuates inflammation after stroke (Wang et al., 2017c) or endotoxin exposure (Ci et al., 2017; Lv et al., 2017). Indeed, metformin activates NRF2 in an AMPK-dependent manner, resulting in inhibition of inflammatory responses in preclinical rodent models of transient global cerebral ischemia (Ashabi et al., 2015; Kaisar et al., 2017). Glucose metabolism and inflammation may not be the only pathomechanisms affected by metformin/NRF2. In fact, other salutary effects have been described for redox (Kocer et al., 2014; Kelleni et al., 2015) and protein homeostasis (Tsai et al., 2017).

Statins prevent and reduce cardiovascular pathophenotypes. In addition to a lipid-lowering effect, statins appear to protect against pathomechanisms associated with the NRF2 network such as inflammatory (Pantan et al., 2016; Wu et al., 2016a; Hwang et al., 2017) and pathologic ROS formation (Abdanipour et al., 2014). They are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, which catalyzes the rate-limiting reaction in cholesterol synthesis. Other pleiotropic effects include the upregulation of transcription factor Krüppel-like factor 2, which is induced early during progression of cirrhosis and lessens the development of hepatic vascular dysfunction (Marrone et al., 2015). Recent evidence indicates that at least some statins activate NRF2. In a proteomic study conducted in isolated hepatocytes, high concentrations of simvastatin activated NRF2, probably as a defensive mechanism (Cho et al., 2013). The pretreatment of neural stem cells with lovastatin activated the NRF2 pathway and elicited protection against hydrogen peroxide–induced cell death (Abdanipour et al., 2014). In liver cirrhosis, simvastatin activates an axis formed by Krüppel-like factor 2 and NRF2 to reduce the oxidative burden and inflammatory response of stellate cells, improving liver fibrosis, endothelial dysfunction, and portal hypertension. The mechanism of activation of NRF2 by simvastatin is not completely clear, but it appears to involve elements found in the NRF2 interactome, such as mitogen-activated protein kinase, PI3K/AKT pathways (Jang et al., 2016), and GSK-3 (Lin et al., 2016).

Other cases for drug repurposing can be inferred from the NRF2 interactome of Fig. 4, in particular with signaling kinases. As indicated in Fig. 2C, GSK-3 phosphorylates the Neh6 domain of NRF2, leading to the recognition by β-TrCP and further ubiquitin-dependent proteasomal degradation. GSK-3 is active in the absence of stimuli and inactive when signaling cascades that activate AKT and other kinases lead to phosphorylation of GSK-3 at its N-terminal pseudosubstrate domain. It follows that medications known to target signaling kinases may be used to upregulate (GSK-3 inhibitors) or downregulate (PI3K/AKT inhibitors) the NRF2 signature.

GSK-3 participates in at least some pathophenotypes found in the NRF2 diseasome such as diabetes and neurodegeneration (Beurel et al., 2015; Maqbool and Hoda, 2017). A broad spectrum of GSK-3 inhibitors has been discovered from natural and synthetic origins (Khan et al., 2017), but probably the best evidence for repurposing a GSK-3 inhibitor to increase NRF2 activity stems from the clinical use of lithium as mood stabilizer (Chiu et al., 2013). Although bipolar disorder and depression are not found at this time in the NRF2 diseasome, it is becoming evident that they exhibit neuroinflammatory and degenerative pathophenotypes that at least in mouse models imply deregulation of

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NRF2 (Martin-de-Saavedra et al., 2013; Freitas et al., 2016; Yao et al., 2016).

The NRF2 interactome also provides a justification for the inhibition of NRF2 by cancer drugs that block signaling kinases, thus activating GSK-3. For example, the epidermal growth factor receptor inhibitor erlotinib leads to NRF2 inhibition, participating in tumor cell sensation in nonsmall cell lung cancer (Xiaobo et al., 2016). The kinase cascade inhibitor sorafenib, used in therapy of hepatocellular carcinoma, also leads to inhibition of NRF2 and its downstream targets metallothionein-1 (Houessinon et al., 2016) and methylenetetrahydrofolate dehydrogenase 1 (Lee et al., 2017).

Finally, search for repurposing drugs that might impinge on NRF2 regulation has been done to date in two relevant studies. Using a fluorescence correlation spectroscopy-based screening system, two of 1633 drugs significantly increased NRF2 protein levels in HepG2 cells: chlorophyllin and bonaphton (Yoshizaki et al., 2017). In another study, the connectivity map database that comprises gene expression profiles for human cell lines treated with 1309 agents (Lamb et al., 2006; Iorio et al., 2010) was analyzed (Zhang et al., 2017) in search for potential redox regulators through activation of NRF2 (Xiong et al., 2014). This study found astemizole, a potent antihistamine drug, used in allergic conditions, as a novel NRF2 activator.

VI. Biomarkers as Nuclear Factor (Erythroid-Derived 2)–Like 2 Signature and for Monitoring Target Engagement

The evaluation of the redox status in patients or population studies is hampered by the short half-life of ROS, in the range of milliseconds or nanoseconds (Ghezzi et al., 2017b). Therefore, biomarkers of pathologic ROS formation are based on measuring the traces left by ROS, which are normally terminal oxidation products of cellular molecules, many of them being nonspecific (Frijhoff et al., 2015). On the contrary, activation of NRF2 and subsequent expression of its target genes is an indirect but reliable estimation of the total exposure of the organism to pathologic ROS formation. Because NRF2 activation is a well-established cellular response to environmental stressors, it has been considered as biomarker of exposure to xenobiotics. In lung, a data mining of several transcription studies followed by Ingenuity pathway analysis reported that the NRF2 signature is upregulated in healthy smokers, therefore suggesting that NRF2-regulated antioxidant genes play a central role in protection against toxic effects of tobacco smoke (Comandini et al., 2010). Similarly, the levels of NQO1, an enzyme regulated by NRF2, were 15-fold higher in liver tissue obtained from acetaminophen-overdosed patients (Aleksunes et al., 2006). The association between disease and nutrition is frequently based on unreliable self-reporting (Archer et al., 2015). Measuring biomarkers of response to nutrients, supposedly having beneficial effects by activating NRF2, could provide a reliable method to validate nutritional studies. However, this possibility is still unexplored.

The changes associated with NRF2 transcription could be useful as biomarker for monitoring the efficacy of drugs aimed at reducing pathologic ROS formation by inhibitors of xanthine oxidase and NADPH oxidase. Similarly, exposure to environmental chemicals could be detected and monitored by defining a global protein and gene expression profile (Ghezzi et al., 2017a). This approach is similar to the use of phase I drug-metabolizing enzymes, in which cytochrome P450, which is induced by various xenobiotics through the Ah receptor, can be used as indicator of marine pollution (Cajaraville et al., 2000). Daily oral administration of fumaric acid esters over 12 weeks was associated with the increased expression of NRF2 target genes in the skin of patients with psoriasis (Onderdijk et al., 2014). Similarly, a fivefold increase in the mRNA level of NQO1 has been reported in peripheral blood mononuclear cells obtained from cancer patients that received a daily dose of CDDO-Me for 3 weeks (Hong et al., 2012).

The use of the transcriptional signature of NRF2 as a biomarker requires a good knowledge of the mechanisms involved in activation of ARE genes, as most of the NRF2 targets are regulated by additional transcription factors. It is therefore important to analyze the expression of several ARE genes. For instance, a study using NRF2 as predictor for response to treatment in lung squamous cell carcinoma has proposed the use of 28 genes to define a NRF2 activation profile (Cescon et al., 2015).

VII. Conclusions

Systems medicine together with network pharmacology highlights a cluster of chronic disease pathophenotypes in which NRF2 plays a fundamental role. These diseases share common mechanisms, including oxidative, inflammatory, and metabolic alterations. The NRF2 interactome, the NRF2 diseasome, and the NRF2 drugome presented in this work are still in an early stage of development, but they represent a first attempt to structure NRF2 as a common therapeutic and systems medicine approach. The forthcoming refinement of current databases and upcoming clinical outcome data will further improve the accuracy of this new approach to pharmacology and mechanism-based drug repurposing. This paper provides a road map for a comprehensive strategy for drug discovery to activate or inhibit NRF2 and highlights the need of translational efforts toward the development of de novo drugs or the

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repurposing of drugs that target NRF2 as a common element in chronic diseases.

Authorship Contributions

Participated in research design: Cuadrado, Schmidt.

Wrote or contributed to the writing of the manuscript: Manda, Oliva, Guney, Alcaraz, Barbas, Valverde, Daiber, Ghezzi, López, León, Pajares, Rojo, Robledinos-Antón, Hassan.

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