Minireview

Mechanisms of NRF2 activation to mediate fetal hemoglobin induction and protection against oxidative stress in sickle cell disease

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Impact statement

Sickle cell disease (SCD) is a group of inherited blood disorders caused by mutations in the human β-globin gene, leading to the synthesis of abnormal hemoglobin S, chronic hemolysis, and oxidative stress. Inhibition of hemoglobin S polymerization by fetal hemoglobin holds the greatest promise for treating SCD. The transcription factor NRF2, is the master regulator of the cellular oxidative stress response and activator of fetal hemoglobin expression. In animal models, various small chemical molecules activate NRF2 and ameliorate the pathophysiology of SCD. This review discusses the mechanisms of NRF2 regulation and therapeutic strategies of NRF2 activation to design the treatment options for individuals with SCD.

Abstract

Individuals with sickle cell disease have severe anemia due to the production of abnormal hemoglobin S, chronic red blood cell hemolysis, and increased oxidative stress leading to endothelial cell dysfunction, vasculopathy, and progressive organ damage. The transcription factor NRF2 (erythroid-derived 2)-like 2) is a master regulator of antioxidant proteins; under low oxidative stress, NRF2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1, β-transducin repeat-containing protein or HRD1, and directed to the proteasome for degradation. When cells are exposed to oxidative stress, NRF2 is released from these repressor proteins, translocates to the nucleus, and activates antioxidant genes to suppress cellular reactive oxidant species and inflammation. In erythroid progenitors, NRF2 also modulates fetal hemoglobin expression through direct binding in the γ-globin promoter and modification of chromatin structure in the β-globin locus. In sickle erythroid cells, NRF2 provides unique benefits through fetal hemoglobin induction to inhibit hemoglobin S polymerization and protection against oxidative stress due to chronic hemolysis. Thus, development of small chemical molecules that activate NRF2 has the potential to ameliorate the clinical severity of sickle cell disease. In this review, we discuss progress towards understanding NRF2 regulation and strategies to develop agents for the treatment of sickle cell disease.

Keywords: Sickle cell disease, fetal hemoglobin, KEAP1, NRF2, oxidative stress

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Introduction

Sickle cell disease (SCD) is a group of inherited blood disorders caused by mutations in the human β-globin gene, which encodes the β-chain subunit of adult hemoglobin A. The A to T mutation in codon 6 of β-globin leads to the synthesis of hemoglobin S (HbS), which under deoxygenated conditions undergoes polymerization. Red blood cells (RBCs) containing polymerized HbS become sickled shape causing chronic hemolysis, anemia, and vascular occlusion in vital organs including the brain, lungs, and kidney producing complications such as stroke and acute chest syndrome among others. The pathophysiology of organ damage involves the generation of inflammation and oxidative stress by sickle RBC formation.[1]

Hemolysis and oxidative stress in SCD pathophysiology

The hallmark of SCD is the high levels of plasma-free hemoglobin and heme from chronic hemolysis leading to increased serum lactate dehydrogenase and bilirubin. Intravascular hemolysis is associated with splenic dysfunction, leg ulcers, and priapism. Haptoglobin is a cytoprotective protein that neutralizes the toxic effects of heme; however, this protein becomes overwhelmed due to high heme levels,[2] producing vascular endothelial dysfunction, nitric oxide (NO) depletion, and hypoxia–reperfusion injury.[3]

The bioavailability of NO in SCD is impaired due to excess free radical superoxide, and its consumption by cell-free heme generated during hemolysis. The limited bioavailability of NO impairs the scavenging of arginine normally metabolized to ornithine and urea by arginase. Several mechanisms contribute to the high oxidative burden in SCD including: (1) autoxidation of HbS; (2) RBC sickling and hemolysis; (3) hypoxia/reperfusion injury, (4) recurrent vaso-occlusion tissue injury; and (5) chronic inflammation. Reactive oxygen species (ROS) and reactive nitrogen species counter balance the sophisticated antioxidant protein systems to maintain redox homeostasis in cells. Many classes of antioxidant proteins exist including reduced glutathione, vitamins C and E, bilirubin and urate; the non-catalytic antioxidant proteins, such as thioredoxin, glutaredoxin, and metallothioneins; and the superoxide dismutase, catalase, peroxiredoxin, and glutathione peroxidase enzymes. Ultimately, redox reactions in cells are enabled from oxidized and reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH) and nicotinamide adenine dinucleotide (NAD^+/NADH), respectively. NADPH reduces oxidized thioredoxin and glutathione (GSH) through the actions of thioredoxin and glutathione reductase, respectively.[4]

The pathophysiology of SCD involves activation of multiple antioxidant genes to accomplish cellular protective mechanisms. For instance, high plasma-free heme stimulates heme oxygenase 1 (HMOX1)[5] expression to convert heme to carbon monoxide and biliverdin producing vasodilation and cytoprotection.[6] The high oxidant burden in SCD activates NAD(P)H quinone oxidoreductase 1 (NQO1) expression as a scavenger of superoxide anion to prevent sickle vasculopathy.[7–9]

Central role of nuclear factor NRF2 (erythroid-derived 2)-like 2) in controlling oxidative stress

The transcription factor NRF2 was originally identified as a DNA binding protein in the β-like globin gene (*HBB*) locus and later characterized as the major regulator of oxidative stress.[10] Systematic and complementary genome wide analyses showed NRF2 binds the consensus antioxidant response element (ARE) 5′-TGACnnnGC-3′ in the proximal promoters of a wide variety of genes (Figure 1(a)).[11] The ARE motif resembles the DNA-binding elements of other basic leucine zipper domain proteins such as AP-1, NFE2, and MAF.[12] Protein–protein interaction studies demonstrated that NRF2

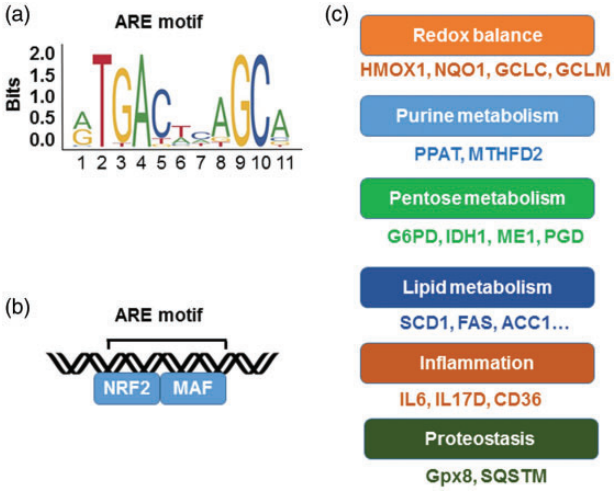


Figure 1. NRF2 as the master regulator of cytoprotective responses. (a) Shown is the NRF2 consensus binding sequences, known as the antioxidant response element (ARE). (b) NRF2 heterodimerizes with MAF family proteins through the basic leucine zipper (bZip) domain to bind the ARE of target genes. (c) NRF2 regulates a wide variety of genes involved in redox metabolism, inflammation, and protein hemostasis among other cellular processes. ACC1: acetyl-coenzyme A carboxylase 1; CD36: cluster of differentiation 36; FAS: fatty acid synthase; G6PD: glucose-6-phosphate dehydrogenase; Gpx8: glutathione peroxidase 8; HMOX1: heme oxygenase-1; GCLC: glutamate-cysteine ligase catalytic subunit; GCLM: glutamate-cysteine ligase: modifier subunit; HMOX1: heme oxygenase 1; IDH1: isocitrate dehydrogenase 1; IL6: interleukin 6; IL17D: Interleukin 17D; NQO1: NAD(P)H dehydrogenase (quinone) 1; ME1: malic enzyme 1; MTHFD2: methylenetetrahydrofolate dehydrogenase 2; PGD: phosphogluconate dehydrogenase; PPAT: phosphoribosyl pyrophosphate amidotransferase; SCD1: stearoyl-CoA desaturase; SQSTM: equestosome-1. (A color version of this figure is available in the online journal.)

heterodimerizes with small MAF proteins to bind DNA and activate gene transcription (Figure 1(b)). Moreover, NRF2 competes with the transcription factor BACH1 (tBTB Domain and CNC Homolog 1),[13–] a member of cap 'n' collar protein, for binding in the ARE motifs of antioxidant genes to regulate the cellular oxidative stress levels.[16,17]

The multi-functional transcription factor NRF2 possesses complementary and overlapping characteristics to control gene expression (Figure 1(c)). For example, NRF2 regulates the enzymes involved in GSH production including glutamate–cysteine ligase complex modifier subunit (GCLM), GCL catalytic subunit (GCLC), cysteine/glutamate transporter XCT, and glutathione reductase.[18] Other proteins required to synthesize GSH such as glutathione S-transferases and glutathione peroxidase 2 are also regulated by NRF2.[11] In addition, several genes involved in the production, regeneration, and utilization of thioredoxin such as thioredoxin reductase 1 and peroxiredoxin 1 are regulated by NRF2.[19,20] Enzymes required for NADPH production including glucose-6-phosphate dehydrogenase, phosphoglycerate dehydrogenase, malic enzyme 1, and isocitrate dehydrogenase 1 are targets of NRF2 regulation,[21] along with the major antioxidant response proteins NQO1, HMOX1, and ferritin.[22] NRF2 also activates the expression of a large number of genes necessary for purine and lipid metabolism, inflammation, and proteostasis discussed below.[21]

Regulation of NRF2

Role of the ubiquitin proteasome system in NRF2 regulation

One of the key mechanisms controlling NRF2 expression is protein stability, involving three signaling pathways comprising KEAP1 (Kelch-like ECH-associated protein 1), HRD1, and β-TrCP (β-transducin repeat-containing protein). These proteins facilitate NRF2 proteasomal degradation by different mechanisms. The structure of NRF2 contains six conserved domains, Neh1–Neh6 (Figure 2 (a)).[23] The Neh1 domain is a basic leucine zipper motif critical for NRF2 dimerization with small MAF proteins and facilitates DNA binding. KEAP1 interacts in the Neh2 domain containing the DLG and ETGE motifs. The Neh4 and Neh5 domains are important for the transactivation activity of NRF2 and serve as the site for HRD1 binding. The Neh6 domain of NRF2 is a serine-rich region containing the DSGIS and DSAPGS motifs that interact with β-TrCP.

Canonical KEAP1–Cul3–Rbx1 E3 ubiquitin ligase. The KEAP1–Cul3–Rbx1 E3 complex is the major regulator of NRF2 degradation (Figure 2(b)). Under normal cellular stress conditions, NRF2 is sequestered in the cytoplasm by KEAP1 and directed for ubiquitination by the E3 ligase complex. RING box protein 1 recruits the catalytic function of ubiquitin-conjugating enzyme E2 after binding the C terminal region of Cul3. The polyubiquitination of NRF2 on the lysine residues in the Neh2 domain is catalyzed by E2 and KEAP1, which promotes rapid NRF2 degradation. Several cysteine residues including C151, C273, and C288 of KEAP1 are critical for interaction with NRF2. Modifications of these critical cysteine sites by electrophiles lead to conformational changes in KEAP1 disrupting interaction with NRF2, thus inhibiting its silencing effects.

Cysteine residues in KEAP1 are modified by endogenous metabolites such as itaconate and succinate. Itaconate regulates macrophage function and activates NRF2 to produce an anti-inflammatory effect by modifying KEAP1 cysteine residues via alkylation. Once activated, NRF2 stimulates the expression of downstream antioxidant and anti-inflammatory genes.[24] Cysteine residues in KEAP1 are also modified through succination to enhance NRF2 protein stability.[25,26] Other mechanisms of KEAP1-inactivation involve autophagy-related protein p62[27,28] which is a scaffold protein that sequesters KEAP1 in the autophagosome to inhibit its function.[29]

HRD1 E3 ubiquitin ligase in endoplasmic reticulum. The protein synoviolin is an endoplasmic reticulum (ER) membrane-associated E3 ubiquitin ligase recently identified as a negative regulator of NRF2 during cirrhosis.[30] Cirrhotic livers are characterized by increased ROS production and ER stress, causing perturbation of the unfolded protein response.[31] The XBP1–HRD1 arm of the ER stress pathway when activated, upregulates HRD1 expression, producing enhanced NRF2 ubiquitylation and degradation to attenuate the antioxidant response (Figure 2(c)).

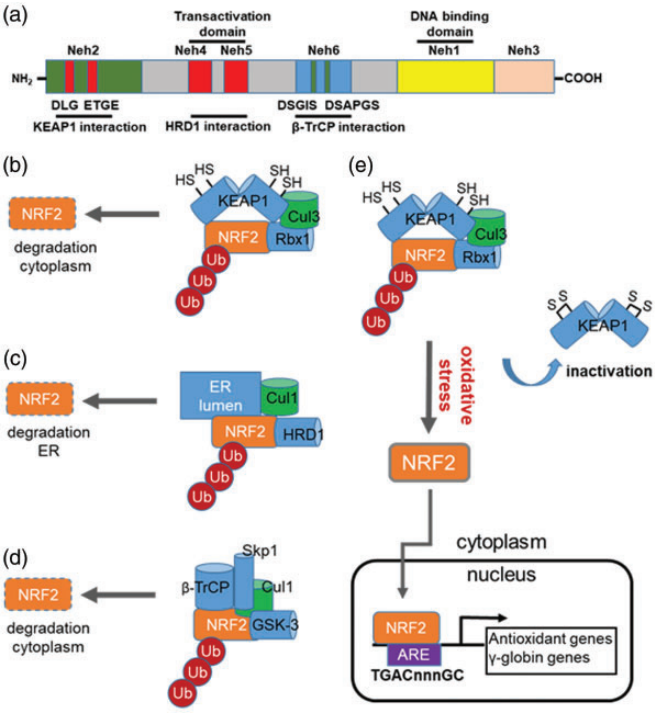


Figure 2. Regulation of NRF2 stability by different E3 ubiquitin ligase complexes. (a) Shown is a schematic structure of the NRF2 protein domains such as Neh1 involved in small MAF heterodimerization and the transactivation domains Neh4 and Neh5 for interacting the co-activators CBP/p300. Also depicted are the individual domains in NRF2 required for interaction with KEAP1, HRD1, and β-transducin repeat-containing protein (β-TrCP). (b) KEAP1-mediated NRF2 repression. Under basal conditions, NRF2 activity is repressed through ubiquitination (Ub) via cullin-dependant E3 ubiquitin ligase Cul3, bound to Kelch-like ECH associated protein 1 (KEAP1). Multiple cysteines residues in KEAP1 including C151, C273 and C288 are required for its interaction with the DLG and ETGE motifs in the Neh2 domain of NRF2. When cysteine residues are modified by electrophiles or ROS, the conformation structure of KEAP1 is altered and interactions with NRF2 disrupted; the net result is reversal of NRF2 proteasomal degradation. (c) HRD1-mediated NRF2 repression. HRD1 is the E3 ubiquitin ligase, which resides in the ER. HRD1 ubiquitylates NRF2 after interacted with the Neh4 and Neh5 domains. (d) β-TrCP-mediate NRF2 repression. Shown is a schematic of NRF2 repression by β-TrCP, which interacts in the Neh6 domain of NRF2 comprising DSGIS and DSAPGS. The DSGIS motif contains a functional GSK-3 phosphorylation site that mediates NRF2 degradation. (e) Mechanism of KEAP1 inactivation that induce γ-globin gene expression. Several cysteine residues in KEAP1 are critical for interaction with NRF2; once modified through various mechanisms, disruption of interaction between KEAP1 and NRF2 occurs, which inhibits polyubiquitination of NRF2. Stabilized NRF2 undergoes nuclear translocation to activate target genes containing the antioxidant response element (ARE) including the antioxidant and γ-globin genes among others. (A color version of this figure is available in the online journal.)

Interaction of HRD1 with the Neh4 and Neh5 domains of NRF2 mediates degradation, which is independent of KEAP1 promoting high ROS level in cirrohosis.[30]

GSK-3β/β-TrCP–Skp1–Cul1–Rbx1 E3 ubiquitin ligase. The discovery of a redox-insensitive degron in the Neh6 domain of NRF2 led to the identification of a new E3 ubiquitin ligase complex involved in degradation.[32] The Neh6 domain is recognized by the F box WD40 substrate adaptor β-TrCP[33]; phosphorylation of β-TrCP by GSK3β greatly increases its affinity for NRF2 (Figure 2(d)).[34,35] Once activated, β-TrCP binds to the SKP1-CUL1-RBX1 E3 ubiquitin

ligase complex and ubiquitinates NRF2 in a KEAP1-independent manner to promote degradation.[33,34,36]

Although three pathways play a role in NRF2 post-translational regulation, KEAP1 has been investigated most extensively for its role in erythroid differentiation and hemoglobin switching (Figure 2(e)). Small chemical compounds such as tert-Butylhydroquinone (tBHQ), diethylmaleate, and 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid (CDDO-Im) modify KEAP1 cysteine residues,[37] to disrupt interactions with NRF2. Once stabilized, NRF2 translocates to the nucleus to activate downstream genes containing ARE motif such as γ-globin and the antioxidant genes.

Regulation of NRF2 at the protein translation levels by microRNA genes

The miRNA genes encode small non-coding RNA molecules that function in mRNA silencing and post-transcriptional regulation. Several miRNA genes are involved in NRF2 silencing including miR-153, miR-27a, miR-142-5p, miR-144, miR-28, and miR-34a.[38–42] Related to erythroid development, miR-144 decreases NRF2 expression in K562 cells, human erythroid progenitors, and SCD reticulocytes.[43] The net effect of increased miR-144 expression and reduction of NRF2 protein levels is the altered expression of GSH[44] and the antioxidant response capacity.[45,46] Narasimhan *et al*.[47] demonstrated that ectopic expression of miR-144, miR-153, miR-27a, and miR-142-5p decreased NRF2 levels in neuronal cells, leading to reduced GSH. Other miRNAs such as miR-27b repressed NRF2 expression during intracerebral hemorrhage observed in rat brain injury.[48] These findings suggest a possible role for these miRNA genes in controlling cellular oxidative stress through NRF2 regulation.

Regulation of NRF2 gene transcription

The transcriptional regulation of NRF2 involves the oncogenes K-Ras, B-Raf, and Myc[49] although the precise mechanism is unknown. Recently, two groups demonstrated activation of NRF2 by K-rat sarcoma through a 12-O-tetradecanoylphorbol-13-acetate-responsive element in the NRF2 gene promoter.[50,51] In addition, there are three xenobiotic response-like elements in the NRF2 gene that are bound by the aryl hydrocarbon receptor. NRF2 transcription is activated by aryl hydrocarbon receptor binding, while siRNA-mediated gene silencing of the receptor reduced NRF2 mRNA levels and activation of downstream antioxidant genes.[52,53]

NRF2 in SCD mouse models

NRF2 loss exacerbated the SCD phenotype

Since NRF2 activation inhibits age-related progression of intravascular hemolysis, vascular inflammation, and pulmonary damage in mice, it follows that NRF2 deficiency would accelerate these complications in SCD. To test this hypothesis, Ofori-Acquah and colleagues[54] created chimeric SCD mice lacking NRF2 expression in non-hematopoietic tissues that caused early death. In addition, these mice had high plasma hemoglobin, heme, and lactate dehydrogenase levels contributing to progressive intravascular hemolysis,[55] and pulmonary edema to exacerbate the SCD phenotype.

To elucidate the role of NRF2 in erythropoiesis, our group established an NRF2 knockout SCD mouse model[56] where impaired *in utero* survival of SCD pups and fetal hemoglobin (HbF) expression was observed in embryonic day 13.5- and 18.5-day fetal liver, adult spleen, and bone marrow. As expected, NRF2 loss led to an increase of ROS and RBC sickling under hypoxic conditions and greater splenomegaly with red pulp expansion.[56] In addition, NRF2 loss in SCD mice reduced the expression of antioxidant proteins NQO1, HMOX1, and catalase, causing increased pro-inflammatory cytokines IL6, IL1β, and TNFα, and the adhesion molecules ICAM1 and VCAM-1 levels. These observations demonstrated a role of NRF2 in the developmental regulation of γ-globin and its ability to control the oxidative stress and phenotypic severity of SCD.

Genetic and chemical NRF2 activation in the SCD mouse model

Recently, KEAP1 ablation to produce constitutive NRF2 activation was achieved in the SCD mouse model.[57] KEAP1 ablation improved the SCD phenotype as demonstrated by a decrease in pro-inflammatory cytokines and adhesion molecules levels. Notably, after KEAP1 ablation, heme levels were reduced and oxidative stress was inhibited. The inflammatory cytokines such as interleukin-6 and interleukin-1β were suppressed, while liver fibrosis was reversed. Moreover, when SCD mice were treated with the NRF2 inducer CDDO-Im, a reduction of inflammation was observed along with improved organ function.[57] Similarly, Ghosh *et al*.[55] demonstrated that D3T (3H-1, 2-dithiole-3-thione), another small chemical inducer of NRF2, decreased acute chest syndrome and mortality in SCD mice. Our group treated SCD mice with the NRF2 inducer, dimethyl fumarate (DMF), which induced HbF expression and reduced RBC sickling under hypoxia conditions.[58] Chronic DMF administration induced expression of NRF2-dependent antioxidant genes to detoxify heme and suppressed inflammation. These results support the notion of NRF2 induction as a therapeutic approach to induce HbF expression, reduce oxidative stress, and preserve organ function in SCD.[57]

Mechanisms of globin gene regulation by NRF2

NRF2 binds the *HBB* locus to alter chromatin structure and γ-globin gene expression

Multiple transcription factors are involved in the regulation of the five major globin genes located in the *HBB* locus on chromosome 11. To elucidate the mechanisms of drug-mediated γ-globin activation, studies conducted by Lowrey and colleagues[59] demonstrated enhanced NRF2 binding in the γ-globin promoter after tBHQ and simvastatin treatment. Deletion of a critical region 100 bp upstream

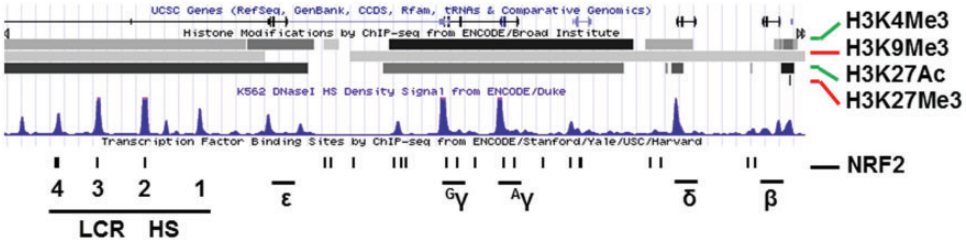


Figure 3. Predicted NRF2 binding sites across the human β-like globin (*HBB*) locus. ENCODE ChIP-seq data generated using K562 cells were downloaded from the UCSC server to discover NRF2 consensus binding motif across the *HBB* locus (https://genome.ucsc.edu/cgi-bin/hg).[61] The location for human β-like globin genes (ε-, ^{G}γ-, ^{A}γ-, δ- and β-globin) and locus control region (LCR) are shown. The histone active chromatin marks H3K4Me3 (histone 3 lysine 4 trimethylation) and H3K27Ac (lysine 27 acetylation) and the repressive marks H3K9Me3 (histone 3 lysine 9 trimethylation) and H3K27Me3 (lysine 27 trimethylation) are shown by the black and gray horizontal lines. The blue peaks represent DNaseI hypersensitivity sites (DNaseI HS). The ENCODE data were modified with predicted antioxidant response element (ARE) motifs (black bar) with the general consensus sequence 5′-TGACnnnGC-3′. (A color version of this figure is available in the online journal.)

of the γ-globin transcription start site, 5′-TGACAAGGC-3′, abolished the HbF induction by these agents. Our group investigated the ability of DMF to activate γ-globin expression; we demonstrated HbF induction in human erythroid progenitors through NRF2 binding in the γ-globin gene ARE.[60] These small chemical compounds alter NRF2 protein stability by different mechanisms to induce HbF expression in erythroid progenitors. Through a JASPAR[61] software search, we identified 23 NRF2 consensus ARE motifs – TGAnnnnGC in the *HBB* locus (Figure 3). Subsequently, NRF2 was demonstrated to bind the *HBB* locus control region and γ-globin promoter (Figure 4), which correlates with gene transcription through long-range chromatin looping to regulate globin gene expression during hemoglobin switching.[60]

Indirect effects of NRF2 in globin gene regulation

Other than direct regulation in the *HBB* locus, NRF2 and drug inducers alter the expression of the transcription factors KLF1 and BCL11A.[62] KLF1 plays a major role in developmentally regulated globin gene switching and activates adult β-globin expression, while BCL11A is the major repressor of γ-globin during erythropoiesis. Treatment with tBHQ induced HbF expression via NRF2 activation with significant downregulation of KLF1 and BCL11A. In addition, using HOMER software, Lessard *et al.*[63] illustrated that NRF2 binding sites are enriched near CpGs hypomethylated dinucleotides in adult erythroblasts with an increase of hydroxymethylcytosine marks during commitment to the erythroid lineage. These studies support the direct and indirect regulation of globin gene expression by NRF2 through DNA binding in the γ-globin promoter and locus control region, and silencing of the major repressors KLF1 and BCL11A, and epigenetic DNA methylation.

NRF2 regulation of multiple cellular protein targets influence SCD phenotype

Heme biosynthesis

A major contributor to the pathophysiology of SCD is the elevated plasma heme levels due to chronic RBC hemolysis, and as a consequence, there is a significant increase in HMOX1 levels in adults with SCD.[64] HMOX1 is the rate

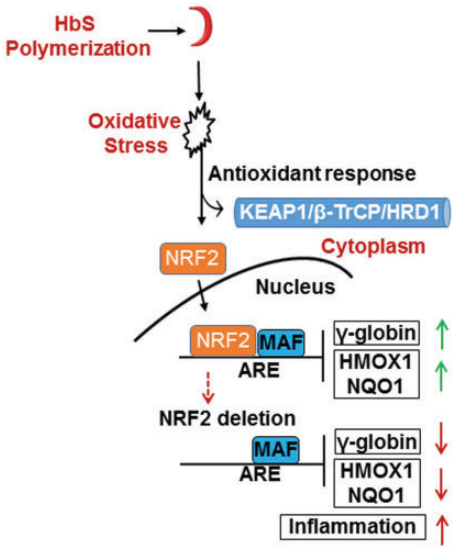


Figure 4. Proposed beneficial effects of NRF2 function in SCD. Shown is a model of the role of oxidative stress in SCD. High reactive oxygen species occur in SCD due to HbS polymerization, RBC sickling, chronic hemolysis, and high reactive oxygen species. The net result is inactivation of KEAP1, HRD1 or β-TrCP, which promote NRF2 stabilization and nuclear translocation. Once bound to the ARE, NRF2 regulates a wide variety of genes such as γ-globin to induce HbF and the antioxidant genes (e.g. HMOX1, NQO1) to suppress oxidative stress and ameliorate the pathophysiology of SCD. Under NRF2 knockout conditions, preclinical data generated in SCD mice demonstrated exacerbation of oxidative stress and γ-globin gene silencing, leading to increased severity of the pathophysiology of SCD. (A color version of this figure is available in the online journal.)

limiting enzyme in the degradation of heme, converting heme to biliverdin, with the release of iron and carbon monoxide, which is regulated by NRF2 in multiple cellular systems.[16] In SCD mouse models, NRF2 activation significantly increased HMOX1 expression,[57] while studies from our group showed NRF2 knockout silenced HMOX1 expression and increased severity of the SCD phenotype (Figure 4).[56] Moreover, in SCD mice treated with DMF,[58] HMOX1 expression was activated and amelioration of the SCD phenotype was observed. In a Phase I clinical trial, treatment with broccoli sprout homogenate containing the NRF2 activator, sulforaphane, increased the peripheral blood mRNA levels for HMOX1 in SCD patients.[65]

There are other proteins regulated by NRF2 involved in heme biosynthesis such as the ATP binding cassette

subfamily B member 6 (ABCB6)[66] and ferrochelatase.[11] ABCB6 imports porphyrins, such as coproporphyrinogen III, from the cytosol to the mitochondria to support heme synthesis, while ferrochelatase participates in the final step of heme biosynthesis.[67] By microarray analysis, it was demonstrated that ABCB6 gene is transcriptionally regulated by NRF2 in arsenic exposed human lymphoblastoid cells[68] and hepatocytes cells.[66] Ferrochelatase catalyzes the insertion of ferrous iron into protoporphyrin IX to produce heme and is regulated by NRF2 in a gene dose-response model.[69,70] While NRF2 controls the biological utilization of oxygen and iron by facilitating the transcription of genes that incorporate iron into heme, NRF2 also regulates the mobilization of iron during hemoglobin degradation. Expression of the heme-responsive transporter gene was decreased in NRF2 knockout mice, while drug-mediated activation of NRF2 increased its expression at the mRNA and protein levels.[71–73]

Apart from heme-bound iron, cells maintain a pool of labile iron to accomplish other biosynthetic processes such as iron–sulfur cluster generation. Individuals with SCD on chronic transfusion regimens suffer hemosiderosis from iron overload due to inability of the body to excrete iron. This leads to the formation of oxygen-derived free radicals, such as hydroxyl radical, through the Fenton reaction. While upregulation of ferritin gene transcription by NRF2 alters iron homeostasis by increasing storage and decreasing labile iron, NRF2 also buffers labile iron by altering its flux across cell membranes.[74,75]

NRF2 regulates GSH synthesis

NRF2 controls the expression of enzymes responsible for producing GSH, which is the most abundant antioxidant cofactor in cells.[76] NRF2 drives the expression of two GSH subunits including the GCL complex comprising GCLM and catalytic GCLC components. GCL catalyzes the reaction of glutamate with cysteine to form the dipeptide gamma-glutamylcysteine, which is the rate-limiting step in GSH synthesis. In addition, NRF2 controls the abundance of cysteine in cells through activation of the solute carrier family 7 members 11, which encodes the cysteine/glutamate transporter XCT.[77]

NRF2 involved in glutamine metabolism

Glutamine is a nonessential amino acid converted to glutamate to provide anti-oxidative activity that plays an important role in cellular energy homeostasis. Glutamine is converted to α-ketoglutarate, a citric acid cycle intermediate that is a cofactor for dioxygenase proteins such as ten-eleven translocation enzymes,[78] and prolyl hydroxylases among others.[79] Moreover, glutamine is required for the synthesis of nicotinamide adenine dinucleotide in response to ROS stress in sickle RBCs.[80,81] Recent clinical trials demonstrated the beneficial effects of glutamine in preventing SCD vaso-occlusive crises.[81] SCD patients treated with L-glutamine showed significant increases in NADH and NAD redox potential. Furthermore, L-glutamine decreased the endothelial adhesion of sickle RBCs *in vitro*, suggesting the clinical improvement most likely occurred through oxidative stress mechanisms. The regulation of glutamine by NRF2 was also investigated in cancer cells where NRF2 activation increased dependency on exogenous glutamine through enhanced glutamate consumption to achieve GSH synthesis.[82,83] The ability of NRF2 to regulate glutamine metabolism is another reason to support the development of small molecule activators for the treatment of SCD.

NRF2 regulation by microRNA genes during erythropoiesis

Recently, the miR-144 and miR-451 (miR-144/451) gene locus was demonstrated to play a major role in erythroid differentiation, homeostasis, and fine-tuning gene expression in erythroid cells.[84,85] Interestingly, in adults with SCD increased miR-144 expression decreased NRF2 and GSH levels, and was associated with severe anemia.[46] Moreover, low NRF2 expression was associated with a lack of the antioxidant proteins GCLC/M and superoxide dismutase 1.[86] We performed genome-wide analysis to discover miRNA genes associated with HbF expression in individuals with SCD. We observed an 8-fold up-regulation of miR-144 in patients with low HbF group compared to those with high HbF.[87] Functional studies in adult erythroid cells showed NRF2 gene silencing by miR-144 and concomitant repression of γ-globin expression; by contrast, treatment with antagomir that inhibits miR-144 reversed its silencing effects.[88] Additional studies have shown miR-28 inhibits NRF2 through a KEAP1-indepenedent mechanism and similarly miR-153, miR-27a, and miR-142-5p down-regulate NRF2 expression.[38–40] These studies support miRNA-mediated mechanisms of NRF2 regulation in γ-globin expression.

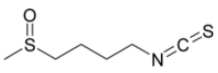
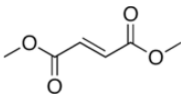
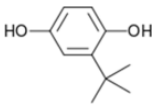
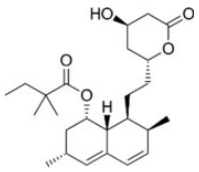
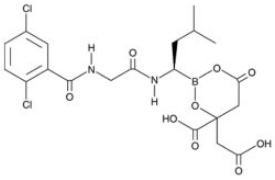
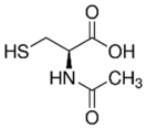
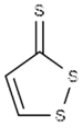
Development of NRF2 drug inducers to activate fetal hemoglobin expression

Various pharmacologic agents increase NRF2 levels via inactivation of KEAP1 resulting in NRF2 translocation into the nucleus and transcriptional activation of antioxidant genes.[89] Among these agents, several small chemical compounds have been explored to induce HbF expression in erythroid progenitors, SCD mice, and individuals with SCD through NRF2 activation (Table 1).

Sulforaphane is an organosulfur compound obtained from cruciferous vegetables such as broccoli sprouts and cabbages. Treatment of SCD erythroid progenitors with this agent activates NRF2 expression and improved GSH levels.[96] In a recent Phase I trial completed in adults with SCD,[65] sulforaphane increased the HMOX1 mRNA levels and produced a trend toward inducing HbF expression.

DMF is a methyl ester of fumarate which was originally approved as oral therapy for psoriasis[97] and later multiple sclerosis.[98] The primary metabolite of DMF is monomethyl fumarate that activates NRF2 and several antioxidant genes.[99] Our group demonstrated the ability of DMF to induce HbF in normal and sickle erythroid progenitors and SCD mice via NRF2 activation.[60] SCD mice treated with DMF displayed less vaso-occlusion with improved oxidative stress tolerance. DMF increased nuclear

Table 1. Summary of drugs that induce HbF through the Keap1/NRF2 pathway.

Drug	Structure	Effect	References
Sulforaphane (SFN)		Induce HbF expression in primary erythroid progenitors; increase mRNA levels of NRF2 target HMOX1 gene in SCD patients	Doss *et al*.[65]
Dimethyl fumarate (DMF)		Induce HbF in normal and sickle erythroid progenitors; induce HbF in SCD mice; decrease inflammation factor expression in SCD mice.	Zhu *et al.*[60] Krishnamoorthy *et al*.[58] Belcher *et al*.[90]
Tertiary butylhydroquinone (tBHQ)		Induces NRF2 activation and binding to γ-globin promoter; Increase γ-globin expression and HbF level in K562 and primary erythroid progenitors; increased NOx levels in SCD patients	Macari *et al*.[62] Hoppe *et al*.[91]
Simvastatin		Increase γ-globin expression and HbF level in K562 and primary erythroid progenitors by NRF2 activation and inhibits β-globin transcription by suppression KLF1 and BCL11A	Macari and Lowrey[59]
MLN9708		Inhibits proteasome mediated ubiquitination and degradation of NRF2; induces ROS generation and antioxidant response in B-CFU cells from SCD patient; induces NRF2 nuclear localization; Increases HbF level in K562 cells	Kupperman *et al*.[92] Pullarkat *et al.*[93]
N-acetylcysteine (NAC)		Induce GSH biosynthesis;	Dodd *et al.*[94]
3H-1,2-dithiole-3-thione (D3T)		Induce GSH biosynthesis; modulate inflammatory response in SCD mouse	Manandhar *et al*.[95] Ghosh *et al*.[54]

NRF2: Nuclear factor (erythroid-derived 2)-like 2; SCD: sickle cell disease; HbF: fetal hemoglobin.

translocation of NRF2 and transcription of NRF2-responsive genes in SCD mouse livers and kidneys.[58] DMF also increased the expression of genes associated with oxidative stress including HMOX1, haptoglobin, hemopexin, and ferritin heavy chain and decreased markers of inflammation, such as nuclear factor-kappa B phospho-p65, and toll-like receptor 4. Chronic DMF administration decreased the hepatic necrosis in SCD mice, and the levels of inflammatory cytokines,[97] supporting the development of DMF for the treatment of SCD.

The synthetic aromatic organic derivative of hydroquinone tBHQ induces the expression of NRF2, HMOX1, GCLC, and NQO1[92] and inhibits the NFκB DNA binding in peripheral blood mononuclear cells.[100] In normal erythroid progenitors, tBHQ induces HbF expression through NRF2 binding to the γ-globin ARE.[62] Moreover, tBHQ silences the adult β-globin gene, KLF1, and BCL11A when combined with simvastatin via the PI3K/AKT signaling pathway.[62,90]

Simvastatin (Zocor) is a statin drug developed to decrease cholesterol levels in humans by inhibiting the function of HMG-CoA reductase.[101] This agent modulates ROS levels, NRF2 activation, and PI3K/AKT signaling. It was also demonstrated using human erythroid progenitors that simvastatin treatment increased NRF2 binding in the *HBB* locus control region.[59] A recent Phase I/II clinical trial

with short-term simvastatin treatment in SCD patients showed increased NO levels, and decreased C-reactive protein and interleukin-6 expression. However, no effects on vascular endothelial growth factor, vascular cell adhesion molecule-1 or tissue factor protein levels were observed.[91]

MLN9708 is a second-generation oral proteasome inhibitor of MLN2239. This prodrug is hydrolyzed in plasma to the biologically active form, which inhibits a subunit of the 20S proteasome.[92] The oral bioavailable prodrug of MLN9708 regulates the NRF2/KEAP1/Cul3-Rbx1 pathway to induce HbF. To confirm the ability of this agent to induce HbF via NRF2 activation, erythroid progenitors from SCD patients were treated with NRF2 siRNA, which silenced HbF expression.[93]

Another potent antioxidant agent, N-acetylcysteine increases GSH[94] and NRF2 levels to promote nuclear translocation and activation of downstream target genes.[102] N-acetylcysteine is currently under investigation in adults with SCD to determine its effect on blood coagulation, specifically von Willebrands factor and oxidative stress (NCT01849016). Compared to steady-state levels, the concentration of cysteine and GSH in blood increased rapidly after N-acetylcysteine infusion with a decrease in RBC fragments and dense cells.[103,104] Since a limited number of SCD patients participated in this trial, additional data are needed to validate these findings.

The sulfur-containing compound dithiolethione D3T found in cruciferous vegetables was shown to induce NRF2 and GSH biosynthesis.[95] In addition, D3T modulates inflammatory diseases including endo-toxemic shock, multiple sclerosis, and light-induced retinal dysfunctions in animal models.[54] Although NRF2 pathway activation was discovered by D3T treatment,[95] this agent has not been investigated as an HbF inducer.

Summary and future directions

James Herrick reported the first case of sickle cell anemia in 1910, followed many years later by discovery of the genetic defect by Linus Pauling in 1949. After several decades of research, the first HbF inducing agent hydroxyurea was FDA-approved in 1998 for the treatment of vaso-occlusive pain in adults with SCD.[105] Almost two decades later, in 2017, Endari (L-glutamine) became the second FDA-approved drug for the treatment of pain episodes in children and adults with SCD.[81] Despite slow progress, the number of studies to develop new therapies for SCD has exploded, with over 40 active clinical trials registered in Clinical Trials.Gov (https://clinicaltrials.gov/). A wide variety of small molecules are under development that target the downstream detrimental effects of sickle RBC formation including chronic hemolysis, NO depletion, and vasculopathy.[55,57–59,65] For example, the Phase II SUSTAIN trial of crizanlizumab (SelG1) tested a humanized anti-P-selectin antibody that prolonged the time to first pain crisis in SCD patients[106]; subsequently, SelG1 was shown to decrease the number of sickle cell pain crises over time.[107] Several other agents should emerge from these widespread clinical efforts establishing a repertoire of effective agents to develop combination therapy for SCD.

Historically, the most successful treatment for SCD involved HbF induction and inhibition of HbS polymerization in RBCs to ameliorate the clinical severity. Two major classes of agents including histone deacetylase[108] and DNA methyl transferase[109] inhibitors are under development. For example, the histone deacetylase inhibitor arginine butyrate induced HbF in SCD and β-thalassemia patients; however, butyrate requires intravenous administration hindering further clinical development. The short chain fatty acid derivative 2,2-dimethylbutyrate (HQK-1001) induced HbF in individuals with β-thalassemia,[110] but failed to induce in SCD patients.[111] Currently, the most promising HbF inducer is the DNA methyl transferase inhibitor decitabine; however, clinical development was hampered by drug inactivation when given by oral administration. The recently completed Phase II trial demonstrated decitabine combined with tetrahydrouridine effectively blocks the metabolism to mediate HbF induction in SCD patients.[112]

Our group and others have focused on the development of chemical activators of NRF2 to produce antioxidant effects and HbF induction to ameliorate the clinical severity of SCD. Although transcription factors are difficult to target for clinical treatment, NRF2 is unique since it is sequestered in the cytoplasm by KEAP1, β-TrCP, and HRD1, allowing the development of agents that inactivate these negative regulatory proteins. For example, DMF modifies cysteine 151 in KEAP1 to inactivate the enzyme and mediate NRF2 stabilization and translocation to the nucleus where γ-globin gene activation occurs.[59,60] Tecfidera® (DMF) is FDA-approved and used routinely to treat multiple sclerosis where it mediates potent immune modulation and anti-inflammatory effects. Future clinical trials are required to establish whether DMF induces HbF in SCD patients.

Another, NRF2 activators sulforaphane was tested for *in vivo* effects in a Phase I clinical trial.[65] HbF induction was not observed in SCD patients; however, the lack of response might be due to low concentrations of sulforaphane in homogenized broccoli sprouts. Small chemicals that directly inhibit KEAP1 such as DMF might be more efficacious. Other potential agents for development as HbF inducers include CDDO-Im, a cancer chemotherapy agent[113] and D3T under preclinical development.[55] Both agents are potent NRF2 activators. Several miRNA genes that target NRF2 have the potential for targeted treatment of SCD. We recently demonstrated NRF2 activation and HbF induction by miR-144 antagomir in sickle erythroid progenitors[88]; other miRNA genes such as miR-153, miR-27a, miR-142-5p, miR-28, and miR-34a[38–42,114,] have the potential for clinical development.

The recent explosion of clinical trials to develop additional effective therapies for SCD has challenged patient recruitment efforts in major medical centers. In an effort to address this hindrance to moving forward new therapies for SCD, the American Society of Hematology (ASH) will establish the ASH Research Collaborative to accelerate progress in hematology. One such collaborative will be comprised of sickle cell disease treatment programs across the United States. We are hopeful that several new

therapies including NRF2 activators will achieve FDA-approval to make possible combination drug therapies for SCD.

Authors' contributions: XZ, ARO, LHN and BL conducted the literature search and contributed to writing the paper. BSP executed the conception and wrote this paper.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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