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Dysregulation of Nrf2 Signaling in Diabetes: An Opportunity for a Multi-target Approach

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Abstract

Oxidative stress is a central feature of diabetes and plays a causal role in the pathogenesis of diabetic complications. The nuclear factor-like 2 (Nrf2) transcription factor mediates the induction of antioxidant and cytoprotective genes and is a major regulator of the endogenous antioxidant and detoxification systems. Multiple aspects of the Nrf2 signaling pathway are aberrant in diabetes and simultaneously targeting the dysregulated aspects of the Nrf2 signaling pathway ought to be considered.

Keywords: Oxidative stress; Nrf2; Pharmacotherapy

Introduction

Diabetes affects over 360 million people worldwide and increases the risk for cardiovascular disease (CVD), nephropathy, and neuropathy [1,2]. Compelling evidence exists to suggest that oxidative stress plays a causal role in the pathogenesis of diabetic complications such as nephropathy, neuropathy, and cardiac hypertrophy, suggesting oxidative stress is a central feature of the disease [3-5]. Improving the endogenous cellular antioxidant and detoxification system is under investigation as a therapeutic approach to reducing oxidative stress and attenuating complications in diabetes. Nrf2 is a Cap 'n' Collar (CNC) basic-region leucine zipper transcription factor that functions as a major regulator of the endogenous antioxidant and detoxification system and provides cells the ability to adapt to oxidative stress and electrophiles by mediating the induction of the cytoprotective genes. It is expressed in all tissues of the human body, and is essential in maintaining cellular redox status [6].

Nrf2 is decreased in diabetic mice and patients with type 2 diabetes mellitus (T2DM), which contributes to increased oxidative stress, endothelial dysfunction, insulin resistance, nephropathy, and increased cardiac insult [7-9]. Genetic overexpression of Nrf2 prevents the onset of T2DM in mice and small molecule activation of Nrf2 reduces oxidative stress, and a myriad of diabetic complications, including cardiovascular complications, nephropathy, and neuropathy [9-11]. The potential of the Nrf2 pathway as a panacea for the features of the diabetic milieu has given rise to research aimed at developing pharmacological therapies that target the Nrf2 pathway. Nrf2 is regulated through a multi-stage signaling process involving cytosolic regulation, nuclear translocation, DNA binding, and nuclear export. Recent evidence has demonstrated that the diabetic milieu results in dysregulation in multiple aspects of the Nrf2 signaling pathway [7,11-13]. Current therapeutic approaches focus on targeting a single aspect of the Nrf2 pathway. Developing an optimal therapeutic strategy for improving Nrf2 function would involve simultaneous targeting of the dysregulated aspects of the signaling pathway. Therefore, the purpose of this paper is to review the effect of diabetes on Nrf2, the dysregulation of Nrf2 signaling in diabetes, and current therapeutics that differentially target the Nrf2 pathway.

Nrf2 in type 2 Diabetes Mellitus

Hyperglycemia, the hallmark feature of T2DM, causes increased production of Reactive Oxygen Species (ROS) and other cytotoxic molecules such as methylglyoxal and advanced glycation end products. Initial cell culture studies demonstrated that Nrf2 was critical in reducing hyperglycemia induced production of ROS, and methylglyoxal [14]. Xue and colleagues found that incubating human microvascular HMEC-1 endothelial cells with 25 mmol/l glucose resulted in a three-fold increase in ROS and methylglyoxal, and administering the Nrf2 activator sulforaphane (SFN) attenuated the hyperglycemia induced increased in ROS and methylglyoxal [14]. Additionally, when Nrf2 was knocked down with siRNA, the protective effect of SFN was removed; indicating Nrf2 was indeed responsible for reducing the hyperglycemia induced oxidative stress *in vitro* [14].

He and colleagues also demonstrated that Nrf2 is activated in response to the oxidative and chemical stress caused by hyperglycemia [15]. They showed that *Nrf2* mRNA was significantly increased after 24 hours when treated with glucose at concentrations of 20 mM for 6 hours or 40 mM for 18 hours in neonatal cardiomyocytes obtained from Nrf2 WT and that this effect was not present in Nrf2 knockout (KO) mice [15]. In Wild Type (WT) cells, hyperglycemia increased mRNA levels of the downstream Nrf2 gene products NQO1 and HO-1 almost three fold and two-fold, respectively. Additionally, ROS levels and apoptosis were significantly increased in the Nrf2 KO cells compared to WT. He and colleagues also demonstrated that 2 weeks after inducing type I diabetes in C57BL/6 mice with a single dose of streptozotocin (STZ) there is a significant upregulation of Nrf2 downstream genes NQO1 and HO1 mRNA expression.

While acute hyperglycemia causes an increase in Nrf2 function, the chronic hyperglycemic milieu of the diabetic condition results in decreased Nrf2 function [7,16-18]. Tan and colleagues demonstrated that at the onset of diabetes in mice Nrf2 protein expression was increased at 2 months but decreased at 5 months and that expression

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of Nrf2 was decreased in humans with late-stage T2DM. Furthermore, immunochemical staining in normal and T2DM human hearts showed that Nrf2 was significantly decreased in late stage, failing, and diabetic hearts compared with the non-diabetic hearts [7]. Two further studies have validated these findings when they demonstrated that Nrf2 protein content and mRNA levels of NQO1 and HO-1 were increased at 3 months but decreased at 6 months in STZ-induced diabetic mice [11,16]. Diet-induced models of T2DM in C57BL/6J mice significantly reduced Nrf2 in skeletal muscle and the downstream product of Nrf2, HO-1, when compared with controls [17]. Additionally, Nrf2 along with several downstream products, NQO1, GSR, GSTA2, TXNDR1, GCLC, and GCLM, is reduced in the arterioles of adult *db/db* mice when compared to controls [19].

Research in humans has also shown that Nrf2 function is decreased in subjects with T2DM [18]. Siewart and colleagues compared pro-oxidant status and mRNA expression of Nrf2 and HO-1 in 40 patients with T2DM and 30 age-matched controls. Using the thiobarbituric acid-reactive substances (TBARS) method, the authors found that blood from patients with T2DM exhibit a roughly 100% increase in oxidative stress compared to healthy controls. Furthermore, Nrf2 and HO-1 gene expression was significantly lower in leukocytes from patients with T2DM when compared to healthy controls.

The initial increase in Nrf2 occurs concomitantly with increases in ROS suggesting Nrf2 increases in response to the cytotoxic insult of hyperglycemia, while long-term studies demonstrate that after the initial increase in Nrf2 function there is a marked decrease in both content and activity of Nrf2 and its downstream antioxidant products. These *in vivo* and *in vitro* experiments provide substantial evidence for the following hypothesis: expression of Nrf2 is initially increased in response to the onset of the diabetic milieu, which is then decreased in late stage diabetes. This suggests that therapies targeting the Nrf2 pathway in late-stage diabetes should be aimed at increasing Nrf2 activity.

Mechanisms of Nrf2 Signaling and Dysregulation in Diabetes

Decreased Nrf2 content and activity is associated with the diabetic state and plays a critical role in diabetic complications. For over a decade, Nrf2 has been investigated in cancer research; it is only more recently that Nrf2 has been the focus of diabetic research. Given the highly coordinated nature of Nrf2 signaling, it is reasonable to assume that signaling dysregulation is involved with Nrf2 dysfunction observed in T2DM. As such, several reviews have been published describing the mechanisms of Nrf2 signaling in the context of cancer cells, however, no reviews have been published describing the current research on Nrf2 signaling in T2DM. Therefore, one aim of this review is to discuss the relevant mechanisms of Nrf2 signaling in non-diabetic and diabetic states. Furthermore, Nrf2 is currently being intensely researched as a therapeutic target for pharmaceuticals, thus it is highly salient that we develop an understanding of Nrf2 signaling dysregulation in T2DM.

The Nrf2 Transcription Factor

Nrf2 is a cap 'n' collar (CNC) basic-region leucine zipper transcription factor that is highly conserved amongst vertebrates, is constitutively expressed, and homozygous KO mice develop normally, indicating it is dispensable for mouse development [20,21]. Nrf2 contains six highly conserved Nrf2-ECH homology (Neh) domains (Figure 1). The Neh 1 domain is the DNA-binding domain that interacts with small Maf proteins and incurs post-translational modifications, such as acetylation, that influence DNA-binding affinity [17,22,23]. The Neh2 domain serves as the binding domain for the Kelch domain of Keap-1 and contains seven lysine residues for ubiquitin conjugation, thus allowing negative regulation of Nrf2 via proteasome-mediated degradation [24,25]. The Neh 3 domain lies at the C-terminus and has been shown to be requisite for transcription through the recruitment of CHD6, a coactivator that contains a helicase and chromodomain [21]. Neh 4 and 5 are transcription transactivation domains which bind to p300-CBP and act synergistically to optimize activation of reporter gene expression [26]. The Neh 6 domain functions to regulate protein stability through the formation of a phosphodegron via GSK-3 mediated phosphorylation of serine residues and β-TrCP mediated ubiquitination [27-30].

Nrf2 is Negatively Regulated by Keap1

Keap1-dependent Cul3-Rbx1 degradation

Nrf2 is located in the cytosol where it is associated with a negative regulator, Kelch-like ECH-associated protein 1 (Keap1). The Kelch domain of Keap1 binds to an ETGE motif in the NEH2 amino-terminal regulatory domain of Nrf2, and in unstressed conditions, the Nrf2 protein is rapidly turned over in through Keap-1 dependent manner Cul3-Rbx1 ubiquitination and proteasomal degradation [31]. Thus, under basal conditions, the Neh2 domain of Nrf2 functions as a redox-sensitive degron via Keap1, which associates with Cul3 and Rbx, forming an E3 ubiquitin ligase complex that actively targets the lysine residues of Nrf2 for ubiquitination [25] (Figure 2).

When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 escapes Keap1-mediated repression, allowing it to translocate from the cytosol to the nucleus, and induce the antioxidant response element (ARE) response. Based upon our current understanding, it appears that cellular stress or pharmacological therapy mainly disrupts the Keap1-Nrf2 complex, thereby increasing Nrf2 protein stability through direct modification of Keap1 and Nrf2 residues. Keap1 is rich in cysteine residues, which often act as a molecular switch triggered by changes in intracellular redox status. *In vitro* studies demonstrated that formation of the Keap1-Nrf2 complex is dependent upon the presence of strong reducing agents and that thiol-reactive reactive compounds can alter the Keap1-Nrf2 interaction, suggesting that cysteine residues in Keap1 may play a role in Keap1 mediated repression of Nrf2 [32]. Keap1 mutation studies demonstrated that C273 and C288 are essential for Keap1-dependent repression of Nrf2 under basal conditions. Additionally, these studies demonstrated C151 is required for Keap1-dependent inhibition of Nrf2 by pharmacological agent, and electrophilic and oxidative stress [33].

The Toledano lab elucidated a molecular mechanism through which Keap1-Nrf2 interaction is inhibited under oxidative conditions. They demonstrated that under basal conditions, Keap1 carries a disulfide bridge between Cys^{226} and Cys^{613}, and exposure to H_2O_2, increases this disulfide bridge and initiates formation of an intermolecular disulfide linking two KEAP1 molecules via Cys^{151}, which then allows for increased Nrf2 Stabilization [34]. Independently, the Hayes lab demonstrated that Cys^{226} and Cys^{613} are required for the $Zn2^{+}$ sensor, which allows Keap1 to sense Cd^{2+}, As^{3+}, Se^{4+}, and Zn^{2+}. This finding indicates that the Keap1-Nrf2 complex is involved not only in sensing oxidative stress but also as a metal (oid) sensor [35]. When considered together, these studies support the hypothesis that the cysteine residues of Keap1 act as a molecular switch, thereby enabling Keap1 to regulate Nrf2 and respond appropriately to changes in the intracellular redox status and other cellular stresses.

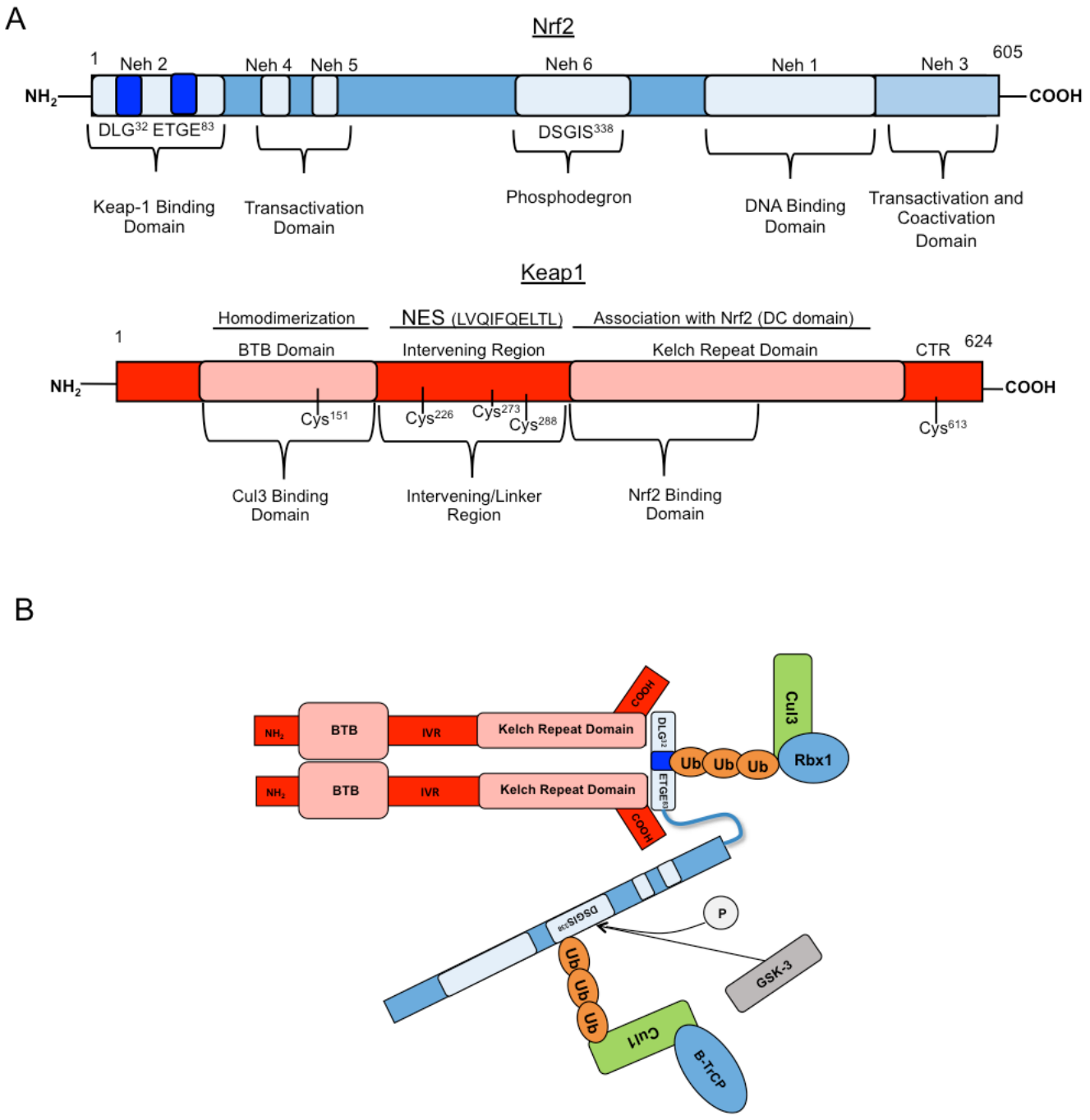

Figure 1: Structures of Nrf2 and Keap1 and Keap1-mediated ubiquitination and GSK-3/β-TrCP mediated ubiquitination. A) Human Nrf2 contains 589 amino acids and six highly conserved Nrf2-ECH homology (Neh) domains. Neh 1 domain is the DNA-binding domain that interacts with small Maf proteins, HATs, and HDACs. The Neh2 domain contains seven lysine residues for ubiquitin and serves as the binding domain for the Kelch domain of Keap-1. The Neh 3 domain is requisite for transcription through the recruitment of CHD6, a coactivator that contains a helicase and chromodomain. Neh 4 and 5 are transcription transactivation domains that bind to p300/CBP and act synergistically to optimize activation of reporter gene expression. The Neh 6 domain is a serine rich domain that is believed be involved in regulation of degradation and regulation through phosphorylation. Human Keap1 contains 624 amino acids and five domains. The BTB domain functions with the N-terminus to mediate homodimerization and binding of Keap1 to Cul3. The Kelch domain functions with the C-terminus to mediate the binding of Nrf2 to Keap1. B) Degradation of Nrf2 is regulated through the Keap1-Cul3-Rbx E3 ubiqutin ligase complex in the Neh2 domain and by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain.

Most evidence suggests the majority of the disruption occurs through direct modification of the Keap1-Nrf2 complex; however, several studies have shown that the protein kinase C (PKC) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathways can also cause disruption of the Keap1-Nrf2 complex (Figure 2). Two independent groups have demonstrated that

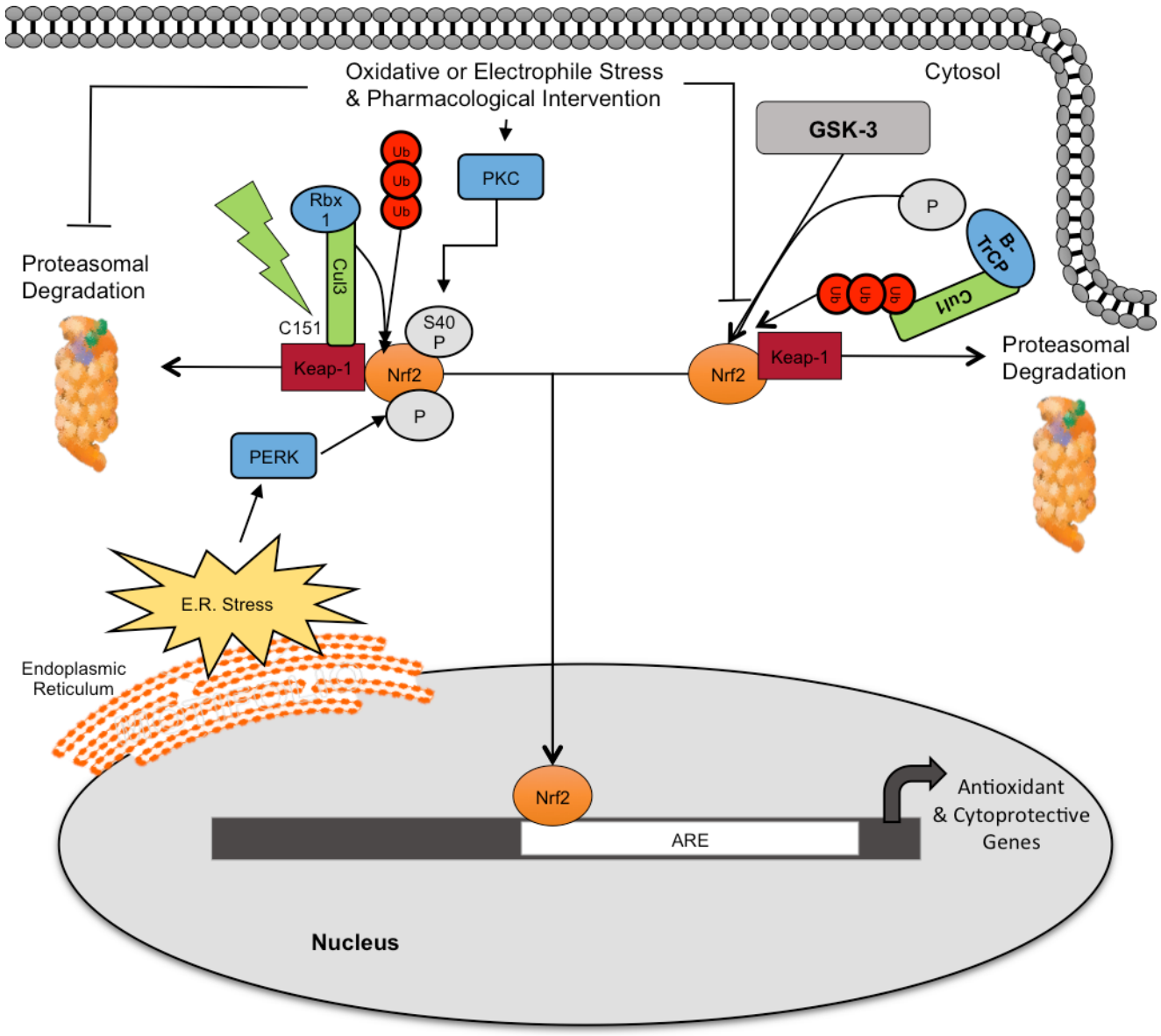

Figure 2: Cytosolic regulation of Nrf2. Under unstressed conditions, Nrf2 is located in the cytosol where it is associated with a negative regulator Kelch-like ECH-associated protein 1 (Keap1), which is associated with Cul3 and Rbx, forming an E3 ubiquitin ligase complex that actively targets the lysine residues of Nrf2 for ubiquitination. Nrf2 protein is rapidly turned over in a Keap1-dependent manner through Cul3-Rbx1 ubiquitination and proteasomal degradation. When cells are exposed to oxidative stress, electrophiles, or pharmacological agents, Nrf2 escapes Keap1-mediated repression, allowing it to translocate from the cytosol to the nucleus. Cellular stress can disrupt the Nrf2-Keap1 complex directly through directly modifying cysteine residues, the PKC pathway, or via the PERK pathway as a result of ER stress. Degradation of the Nrf2 transcription factors also occurs in a Keap1-indepdent manner by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain [28-30]. Briefly, GSK3 phosphorylates Ser^{335} and Ser^{338} in the $DSGIS^{338}$ of Nrf2 which increases β-TrCP binding and ubiquitination.

antioxidant therapy modifies the complex through the PKC pathway [36,37]. Huang and colleagues, together with Bloom and Jaiswal, demonstrated that PKC phosphorylates Nrf2 at Ser40, which lies in the Neh2 domain that interacts with the Kelch-domain of Keap1, and releases Nrf2 from Keap1. Bloom and Jaiswal also demonstrated that Ser40 phosphorylation was not required for protein stability or nuclear accumulation [36]. The Unfolded Protein Response (UPR) and endoplasmic reticulum (ER) stress has also been shown to disrupt the Keap-Nrf2 complex and activate Nrf2 and its target genes [38]. Immunoprecipitation of PERK from 3T3 cells transduced with a retrovirus-encoding PERK demonstrated that Nrf2 was a direct PERK substrate. In the same paper, The Diehl Lab demonstrated that the UPR and ER stress activated PERK rapidly phosphorylates Nrf2 and is necessary and sufficient for PERK-dependent dissociation of Nrf2 from Keap1, nuclear import, and prevents reassociation with Keap1 [38].

This data suggests that stability of Nrf2 is primarily controlled by the Nrf2-Keap1 complex, which is regulated through 2 distinct mechanisms: 1) Nrf2 can be released from Keap1 through modification of cysteine residues in response to thiol-reactive compounds, 2) through signaling pathways that sense cell stress through oxidative

stress-independent mechanisms (i.e. ER stress via the PERK pathway or chemopreventative therapy via the PKC pathway) (Figure 2).

Keap1 dependent nuclear export

Recent studies have demonstrated that Keap1 also plays a role in regulating post-transcriptional nuclear export of Nrf2 [39-41]. Immunofluorescence studies have shown that Keap1 detaches from the cytoskeleton and translocates to the nucleus after exposure to oxidative stress [40]. Velichkova and Hasson demonstrated that Keap1 possessed a nuclear export signal (NES), a LVQIFQELTL motif within the intervening region, and that mutation in the NES of Keap1 resulted in nuclear accumulation of both Keap1 and Nrf2, suggesting that the NES of Keap1 plays a primary role in nuclear export of Nrf2. The Zhang lab confirmed these findings and demonstrated that Keap1 nuclear translocation is independent of Nrf2 and the Nrf2-Keap1 complex does not bind to the ARE [41]. These findings suggest that in the presence of oxidative stress Nrf2 escapes Keap1, translocates to the nucleus wherein it transcribes antioxidant genes and once cellular redox homeostasis is recovered, Keap1 translocates into the nucleus and complexes with the disengaged Nrf2. The Keap1-Nrf2 complex is then exported from the nucleus where it associates with the E3 ubiquitin ligase, thereby resulting in degradation of Nrf2 [41]. The Jaiswal lab identified a specific residue for this mechanism of nuclear export when they demonstrated that phosphorylation of Tyr^{85} was required for Keap1-mediated nuclear export of Nrf2 [42].

Keap1 regulates Nrf2 protein stability via Cul3-Rbx1 ubiquitination and proteasomal degradation by binding an ETGE motif in Neh2 domain. Additionally, Keap1 contains a NES, a motif LVQIFQELT within the intervening region, which regulates nuclear export of Nrf2 through interaction with a phosphorylated Tyr85, which is then marked for degradation by the E3 ubiquitin ligase. Thus, Keap1 plays a critical role in governing Nrf2 function.

Keap1 is increased in diabetes

When bound to Keap1, Nrf2 is ubiquitinated and degraded by the proteasome in a rapid manner, effectively preventing Nrf2 from transcribing Nrf2-dependent phase 2 gene products. Furthermore, Keap1 regulates post-transcriptional repression of Nrf2-via nuclear export and subsequent proteasomal degradation. Together, these mechanisms reduce Nrf2 protein stability and transcription of Nrf2-mediated genes.

Keap1 levels are elevated in fibroblasts taken from diabetic rats and co-immunoprecipitation analysis has demonstrated that association of Nrf2 with Keap1 is significantly increased in the diabetic fibroblasts when compared to non-diabetic controls. Furthermore, Nrf2 is degraded by the 26S proteasome in diabetic fibroblasts at a greater rate than in control fibroblasts [12]. Additionally, Keap1 mRNA is elevated in high-fat diet induced models of diabetes in mice [43].

The Shinohara lab has recently demonstrated that increased Keap1 in diabetes may be due to epigenetic mechanisms. They found that CpG islands in Keap1 promoter were demethylated in cataractous lenses from diabetic patients and that treating human lens epithelial cells with 5-aza-2′deoxycytidine (5-Aza), a demethylation agent, induces a 10-fold increase in *Keap1* mRNA, a 3-fold increase levels of *Keap1* protein, and reduces Nrf2 function. These results indicated that demethylation of the CpG islands in the *Keap1* promoter will activate the expression of *Keap1* protein, which then increases the targeting of Nrf2 for proteosomal degradation. Decreased Nrf2 activity represses the transcription of many antioxidant enzyme genes and alters the redox-balance towards lens oxidation. Thus, demethylation of the CpG islands in the Keap1 promoter may be a contributor factor to increased Keap1 levels observed in diabetes and that Nrf2 protein stability is reduced in T2DM, at least in part, through increased levels of Keap1.

Targeting Keap1-Nrf2 Dysregulation

The antioxidants sulforaphane (SFN) and tetrahydroxyquinone (tBHQ) disrupt the Keap1-Nrf2 complex and have been shown to be effective in reducing or preventing diabetic complications by increasing Nrf2 content and activity in animal models [8,11,44]. It was originally believed that SFN and tBHQ increased Nrf2 activity by modifying cysteine residues of the Nrf2-Keap1 complex and caused dissociation in a manner similar to ROS [32]. This theory would present a paradox as the evidence has shown that increased ROS causes Nrf2 to dissociate and translocate to the nucleus and that late T2DM is associated with increased ROS but decreased Nrf2. The evidence that treating oxidative stressed diabetic cells and animals with tBHQ or SFN increases Nrf2 signaling suggests these compounds have a different mechanism of action. Several research groups have been attempting to elucidate exactly how SFN and tBHQ increase Nrf2 content and activity. Based upon current evidence, SFN and tBHQ disrupt the Nrf2-Keap1 complex by inhibiting the activity of the Keap1-Cul3 ubiquitin ligase. Kobayashi and colleagues demonstrated that tBHQ blocked ubiquitination of Nrf2 [45]. Zhang confirmed this and demonstrated that SFN functions in the same manner [46]. This presents an interesting question, how does preventing ubiquitination of Nrf2 increase its activity? Zhang presented the following saturation model to answer that question: during unstressed, basal conditions, large amounts of Keap1 molecules exist in relation to Nrf2, and as such the majority of newly synthesized Nrf2 binds to Keap1 and is degrading through proteasomal degradation [46]. Upon administration of tBHQ or SFN, Nrf2 degradation is suppressed by blocking ubiquitination and its subsequent degradation. The amount of Nrf2 in the cell eventually saturates the binding capacity of Keap1, resulting in free Nrf2, which is capable of transcription. This makes SFN and tBHQ an attractive target for improving Nrf2 function in diabetes by reducing the ubiquitination caused by increased levels of Keap1 present in the diabetic state [12].

The triterpenoid derivative of dihydro-CDDO-trifluorethyl amid (Dh404) has also been shown to directly disrupt Keap1 association and reduce Keap1-dependent suppression of Nrf2 [47]. Ichikawa and colleagues used a balanced Keap1-Nrf2 expression system and residue mutations of C151S, C273S, and C288S to examine whether Dh404 increases Nrf2 by directly disrupting the Keap1-Nrf2 complex. They found that Dh404 increased Nrf2 activity in all cells except those with Nrf2 and C151S Keap1 mutant. Furthermore, Dh404 increased Nrf2 activity and inhibited the formation of ROS in in H9C2 cardiomyocytes. Together, this evidence indicates that Dh404 directly disrupts the Keap1-Nrf2 complex by interacting with the C151 residue in Keap1, thereby increasing Nrf2 stability. Importantly, the mechanism of Dh404 is independent of SFN and tBHQ, suggesting they may have synergistic effects when administered in combination.

Targeting the Keap1-Nrf2 complex with SFN, tBHQ, and Dh404 *in vivo* has shown promise in attenuating diabetic complications in animal models. Bai and colleagues demonstrated that administration of SFN to diabetic mice upregulated Nrf2 expression and its downstream genes and concurrently preserved ejection fraction, reduced protein expression and mRNA of the hypertrophic marker atrial natriuretic peptide (ANP), attenuated cardiac hypertrophy, reduced fibrosis and TGF-β expression, decreased inflammation and TNFα, and reduced

oxidative stress in the myocardium. SFN has been shown to have similar effects in diabetic nephropathy. 3 months of SFN treatment attenuated loss of kidney function, fibrosis and TGF-β expression, reduced inflammation and TNFα, and oxidative stress in the kidneys of diabetic mice [47,48]. The improved renal function in the diabetic mice by SFN treatment occurred concomitantly with increased Nrf2 expression in the kidneys of the diabetic mice. Bai et al. demonstrated that SFN upregulates renal expression of Nrf2 and its downstream gene products at both mRNA and protein level. Importantly, *in vitro* studies from the Cui et al. paper demonstrated that silencing Nrf2 abolished SFN's prevention of hyperglycemia induced fibrosis, indicating the therapeutic benefits of SFN treatment occur predominately via Nrf2. Additionally, a recent randomized double-blind placebo controlled trial found that a supplemental source of SFN, broccoli sprout powder, reduced markers of oxidative stress in patients with T2DM [49].

tBHQ disrupts the Keap1-Nrf2 complex via the same mechanism as SFN and has shown similar results as SFN in animal models of diabetic nephropathy. Feeding diabetic mice a diet containing 1% tBHQ significantly reduced the levels of serum and glomerular malondialdehyde, kidney weight and proteinuria, as well as decreased levels of fibronectin while concomitantly increasing Nrf2 protein expression and nuclear accumulation and expression of HO-1 and γ-GCS [8].

Dh404 has been efficacious in attenuating diabetes-associated nephropathy via increasing Nrf2 function. Tan and colleagues demonstrated that Dh404 improves diabetic kidney disease and atherosclerosis in an inverse dose-dependent manner [50]. A low dose but not a high dose of Dh404 lessened diabetes-associated atherosclerosis while concomitantly decreasing oxidative stress (markers) and TNF-α, ICAM-1, VCAM-1 and MCP-1. They also demonstrated that Dh404 attenuates loss of renal function and glomerular and renal tubular injury in diabetic mice. Corresponding in vitro studies in NRK cells showed that low doses Dh404 increased the Nrf2 gene products HO-1, NQO1 and GSH-S transferase, and inhibited TGFβ-mediated pro-fibrotic fibronectin and IL-6 [50]. It is pertinent to note that analogues of Dh404, namely bardoxolone methyl, have shown to have adverse effects. However, it was believed that impure compounds were responsible for the adverse effects in those studies [51]. Follow up studies demonstrated that using structural analogs lacking the impurities and at appropriate doses were unable to reproduce the adverse effects and that Dh404 is in fact well tolerated and exhibits efficacy in rodent models of T2DM [52]. It is believed these negative side effects were due to impure compounds and that Dh404 is both efficacious and well tolerated in several animal models and in humans. As Dh404 has shown promise in animal models and human trials have already been conducted, both the dose and source of Dh404 need to be given thorough consideration in future research and human trials.

SFN and tBHQ increase Nrf2 stability by preventing Keap1-mediated ubiquitination and degradation of Nrf2. Dh404 increases Nrf2 protein stability through a dissimilar mechanism by causing dissociation of the Nrf2 complex. In combination therapy, Dh404 may increase Keap1-Nrf2 disassociation while SFN or tBHQ may reduce ubiquitination and improve Nrf2 protein stability. Therefore, a therapy utilizing both approaches may prove synergistic in improving Nrf2 function in diabetes and research exploring this is needed.

Nrf2 is negatively regulated by Glycogen Synthase Kinase 3

Degradation of the Nrf2 transcription factor also occurs in a Keap1-independent manner by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain [28-30]. Rada and colleagues initially demonstrated that Glycogen Synthase Kinase 3 (GSK-3) regulates Nrf2 protein levels independently of Keap1[31]. Administration of SB216763, a GSK-3 inhibitor, to $Keap1^{-/-}$ mice and $Keap1^{+/+}$ mice SB216763 increased Nrf2 protein levels by increasing its half-life in both the $Keap1^{-/-}$ and WT mice, suggesting GSK-3 alters protein levels through regulation of Nrf2 stability independently of Keap1 [29]. In the same paper, utilizing bioinformatics and a series of elegant experiments, the authors were able to demonstrate that GSK-3 phosphorylates a serine residue cluster (335, 338, 342, 347, 351, and 355) in a β-TrCP destruction motif within the Neh6 domain of Nrf2, and that serine to alanine mutation of these residues prevented GSK-3/β-TrCP dependent degradation. These initial findings indicate that the Neh6 domain in the Nrf2 transcription factor functions as a phosphodegron in which GSK-3 phosphorylates serine residues within a β-TrCP destruction motif that is then ubiquitinated by the β-TrCP E3 ligase.

Recently the Hayes lab identified the binding site for the GSK-3 phosphorylation site through which β-TrCP serves as a receptor for the Skp1-Cul1-RBX/Roc1 ubiquitin ligase complex [30]. Utilizing biotinylated-peptide pull-down assays, they identified $DSGIS^{338}$ as the binding site for β-TrCP, and that phosphorylation of Ser^{335} and Ser^{338} increased β-TrCP binding. Together, these findings indicate that GSK-3 represses Nrf2 by phosphorylating Ser^{335} and Ser^{338} within a phosphodegron in the Neh6 domain, which increases binding of β-TrCP and subsequent ubiquitination and degradation.

GSK-3 activity is, at least in part, negatively regulated via the PI3K and PKB/Akt pathway. Several studies have demonstrated that inhibition of PI3K and PKB/Akt pathway increases GSK-3 activity and downregulates Nrf2 [29,30]. As there is aberrant PI3K and PKB/Akt signaling in diabetes, the GSK-3/ β-TrCP axis is likely to be involved in Nrf2 dysregulation in Diabetes.

GSK-3β-Fyn Axis Regulates Nuclear Export of Nrf2

Deacetylation of Nrf2 causes transcriptional termination and disengages it from the ARE [53]. Upon disengagement from the ARE Nrf2 must then be exported from the nucleus where it is degraded by the proteasome. Currently, there are two major mechanisms for nuclear export of Nrf2; the GSK3B-Fyn pathway and Keap1-mediated nuclear export.

Upon completion of Nrf2 activation, the Src kinase, Fyn, phosphorylates Nrf2 at Tyr^{568}, and causes nuclear export by Crm1 and ultimately degradation of Nrf2 [39]. When treated with xenobiotics or hydrogen peroxide, Fyn is exported out of the nucleus, thereby allowing Nrf2 to bind to the ARE and induce transcription. Allowing accumulation of Fyn by removing its NES motif renders Nrf2 ineffective and increases susceptibility to cellular death. Nuclear accumulation of Fyn is regulated by Glycogen synthase kinase -3β (GSK-3β). GSK-3β phosphorylates Fyn, causing it to localize to the nucleus where it phosphorylates Nrf2 at Tyr^{568}, resulting in nuclear export and degradation.

Exposing granule neurons to oxidative stress by administrating H_2O_2 inhibits GSK-3β activation and promotes nuclear accumulation of Nrf2 [54]. Furthermore, inhibiting GSK-3β with the compound TDZD-8 resulted in large increases of nuclear Nrf2. These studies, along with several others suggest that in the presence of oxidative stress GSK-3β-regulated nuclear translocation of Fyn is inhibited, thereby reducing nuclear export of Nrf2 [55]. It has been hypothesized that

there may be two prominent phases of the Nrf2 stress response: 1) an early phase (0-4 hrs.) in which Nrf2 translocates to the nucleus and the oxidative stress suppresses the GSK-3β pathways, thereby reducing nuclear export, 2) a delayed response (5-8 hours) in which unknown kinases activate the GSK-3β pathways [56]. This hypothesis poses several novel questions and potentially rewarding areas of investigation. I propose there may be an alternative hypothesis that explains GSK-3β-regulated control of Fyn. I propose the GSK-3β-Fyn axis works on a feedback mechanism whereby GSK-3β constitutively activates Fyn and causes nuclear translocation under basal conditions and when exposed to oxidative and/or cellular stress, GSK-3β-regulated activation of Fyn is inhibited, and Nrf2 is allowed to accumulate in the nucleus and transcribe antioxidant genes. When the antioxidant products of Nrf2 transcription have attenuated the cellular stress, the GSK-3β-inhibitory signal is removed and Fyn translocation is restored, exporting Nrf2, thereby attenuating the activity of the endogenous antioxidant system. Such a mechanism is parsimonious and would be effective in maintaining the redox status of the cell.

GSK-3 functions to downregulate Nrf2 through two distinct mechanisms, 1) through the formation of a phosphodegron via the GSK-3/ β-TrCP axis, and 2) by enhancing nuclear export of Nrf2 via the GSK-3β-Fyn axis. The dual role of GSK-3 in regulating Nrf2 makes it an attractive potential target for improving Nrf2 function.

Diabetes increases GSK-3 activity

GSK-3 decreases protein stability through the formation of a phosphodegron and ubiquitination via β-TrCP. GSK-3 is constitutively active at basal conditions and is inhibited via the IRS-1/PI3K and Akt pathways, and inhibition of the PI3K/Akt pathway increases Nrf2 degradation [29,30]. Hayes and Dinkova-Kostova noted that this mode of regulation has been overlooked because GSK-3 is inhibited under conventional cell-culture conditions [57]. As such, no studies have explicitly demonstrated that GSK-3/TrCP mediated degradation of Nrf2 is increased in diabetes. However, GSK-3 is elevated in rodent models and humans of diabetes [58,59]. Furthermore, PI3K/Akt signaling is diminished in insulin-resistant rodents models and in the skeletal muscle of obese humans with T2DM [60,61]. This suggests increased degradation of Nrf2 via the GSK-3/β-TrCP axis is a plausible, and highly probable mechanism for decreased Nrf2 function in diabetes. Research is needed to establish the state of this mechanism in the function of Nrf2 in diabetes.

Gsk-3 is also involved in reducing nuclear accumulation of Nrf2 via the GSK-3β/Fyn axis and evidence from animal models of diabetes suggest this mechanism is increased in the diabetic state. Bitar and Al-Mulla demonstrated that GSK-3β/Fyn mediated nuclear export of Nrf2 was increased in diabetes when they showed increased GSK-3β activity and expression of Fyn alongisde diminished accumulation of nuclear Nrf2 and Nrf2 gene products in primary fibroblasts from diabetic mice [12]. Furthermore, they demonstrated that siRNA-mediated downregulation of GSK-3 restored nuclear accumulation of Nrf2 signaling in the diabetic fibroblasts; suggesting inhibition of GSK-3β can improve Nrf2 function in diabetes. Independently of Bitar and Al-Mulla, the Calvert lab has also demonstrated that nuclear expression of Fyn is increased in hearts of *db/db* mice when compared to controls [13]. Together, these findings indicate that augmentation in GSK-3B-Fyn signaling reduces nuclear accumulation of Nrf2 in T2DM.

Increased GSK-3 in diabetes can reduce Nrf2 protein stability through the phosphodegron in the Neh6 domain and decrease nuclear accumulation through nuclear export via the GSK-3β-Fyn mechanism. As such, GSK-3 inhibition is an attractive target to improve Nrf2 function in diabetes.

GSK-3 Inhibition in Diabetes

GSK-3 inhibitors have shown efficacy in treating diabetes, primarily for their role in restoring defects in glycogen synthesis and glucose uptake [62,63]. While the majority of GSK-3 inhibition in diabetes has focused on improving insulin sensitivity, inhibition of GSK-3 has also been shown to improve Nrf2 function in cell culture and in non-diabetic and diabetic animal models.

In initial *in vitro* studies, Rojo and colleagues treated N2A neuroblasts with the GSK-3 inibitors10 mM lithium or 30 μM TDZD8 and then submitted them to SFN or tBHQ. Lithium or TDZD8 increased luciferase activity of an ARE-LU/c reporter 2-fold [54]. Rada and colleagues found that the GSK-3 inhibitor SB216763 increased Nrf2 protein levels in the liver and hippocampus of C57/BL6 mice [28]. *In vitro* work by Bitar and Al-Mulla showed that GSK-3 inhibition with lithium normalizes basal and inducible levels of Nrf2 and transcriptional activity in diabetic rat fibroblasts [12].

When TDZD8 and lithium are combined with SFN or tBHQ, there is a synergistic effect in increasing Nrf2 transcription [12,28,54]. Rojo and colleagues demonstrated that combination of either lithium or TDZD8 with SFN increased HO-1 levels to a greater extent than any treatment alone, suggesting that combination therapies targeting different mechanisms of Nrf2 signaling have additive effects [28]. Furthermore, Rojo et al. demonstrated that combining inhibition of GSK-3 with the Keap1-Nrf2 disrupter tBHQ induced a roughly 4-fold increase in Nrf2 protein levels and transcription in the diabetic fibroblasts, which was greater than either treatment in isolation [54].

GSK-3 regulates Nrf2 stability and nuclear export in a Keap1-independent manner. Recent evidence has demonstrated that combination therapy of the GSK-3 inhibitor TDZD-8 with the Keap1-Nrf2 modifying tBHQ has a synergistic effect on Nrf2 transcriptional activity in diabetic rat fibroblasts[13]. This provides a strong case for combining therapies that target different aspects of the Nrf2 signaling pathway in diabetes.

Transcriptional Regulation of Nrf2 by Acetylation/Deacetylation

Nrf2 forms heterodimers with small v-maf musculoaponeurotic fibrosarcoma oncogene family (Maf) proteins and activates gene expression directly through the ARE [64-66]. Transcriptional activity of Nrf2 and its nucleo-cytoplasmic localization are regulated via acetylation and deacetylation of lysine residues by the histone acetyltransferase P300/CREB-binding protein (CBP) and histone deacetylase (HDAC) proteins

Katoh and colleagues found that two transactivation domains, Neh4 and Neh5, bind to CREB-binding protein (CBP) and are critical in attaining maximal activation of Nrf2-dependent gene expression as measured by reporter assays [26]. CBP induces acetylation of Nrf2, which increases binding of Nrf2 to the gene promoter in the ARE, and increases dependent Nrf2-dependent transcription [53]. Mutation of Lys^{588} and Lys^{591} to alanine or arginine of Nrf2 reduces Nrf2-dependent gene transcription and abolishes the acetylation and transcription activating effect of CREB-binding protein, identifying Lys^{588} and Lys^{591} as critical sites of acetylation and transcription of Nrf2-dependent genes.

Kawai et al. demonstrated that the deacteylase sirtuin 1 (SIRT1) decreases acetylation of Nrf2 and Nrf2-dependent transcription [53]. Furthermore, acetylation of Nrf2 is recapitulated when transfected the cells with negative SIRT1 or administered the SIRT1 inhibitors nicotinamide or EX-527 [53]. In addition to SIRT1, HDAC proteins have been shown to regulate nuclear Nrf2 function. Chromatin immunoprecipitation (ChIP) studies in vascular endothelial cells have shown that association of class I HDACs with Nrf2 results in deacetylation and inhibits its *in vivo* binding to the ARE and NQO1 expression [67]. Conversely, Nrf2 was able to bind to the ARE and maintain NQO1 expression after transfecting the cells with HDAC-1/2/3-specific siRNA. This indicates that HDAC proteins, specifically class I HDACs, associate with Nrf2 and modulate its transcriptional activity.

Kawai and colleagues showed that acetylation/deacetylation also regulates nucleo-cytoplasmic localization. Acetylation of Nrf2 increases nuclear localization while deacetylation conditions promote cytoplasmic localization [53]. The authors hypothesized that nuclear acetylation of Nrf2 results in binding of to the ARE and augments transcription, while deacetylation disengages Nrf2 from the ARE, terminating transcription, and encourages nuclear export [53]. When considered together, these findings indicate that hyperacetylation results in increased expression of Nrf2 regulated genes and hypoacetylation results in decreased expression of Nrf2 regulated genes (Figure 3). This evidence suggests that Nrf2 activity is modulated by class I HDACs in a variety of cells and types of cell stress and that Nrf2 is primarily regulated by HDACs by the following mechanisms: 1) HDACs associate with Nrf2 and deacetylate it, thereby reducing binding of Nrf2 to DNA for transcription, 2) HDACs modify histone proteins and modulate chromatin to condense it, thereby reducing the availability of the DNA for Nrf2 to bind and engage in transcription.

HDAC inhibitors to improve Nrf2 in diabetes

Abnormalities in epigenetic regulation by class I HDACs have been associated with T2DM [68]. Class I HDAC activity is elevated in diabetic mice and is associated with metabolic dysfunction, hypertrophic cardiomyopathy, renal impairment and are considered to be regulators of diabetic complications [68-70]. Small molecule inhibitors of class I HDACs have therapeutic potential in improving metabolic dysfunction, and attenuating diabetic cardiomyopathy, nephropathy, and neuropathy in animal models of diabetes [68,71].

Currently the effect of class I HDAC inhibitors on Nrf2 function have not been explored in diabetic models; however, evidence from non-diabetic studies indicate that HDAC inhibitors are efficacious in increasing Nrf2 function [69-71]. Suberoylanilide hydroxamic acid (SAHA), a class I and II HDAC inhibitor, upregulates transcription of the Nrf2 gene products GCLC and GLCM, without increasing expression of Nrf2 in U937 cells [72]. The pan HDAC inhibitor trichostatin A (TSA) restores the expression of Nrf2 as well as transcription of NQO1 in a TRAMP C1 cell line [73]. TSA has also been shown to increase Nrf2 binding to the ARE and transcription of HO1. Wang and colleagues demonstrated that TSA increases Nrf2 nuclear translocation, enhances Nrf2-ARE binding, and upregulates expression of HO1 in cortical neuronal cells. [74]. Additionally, Correa and colleagues demonstrated that increased HDAC activity decreased levels of Nrf2 and the downstream target γGCL-M in astroglial cells and valproic acid (VPA) restored the Nrf2 function and protected against oxidative-stress induced cell death in astroglial cells [75]. *In* vivo experiments have show that TSA protects against cerebral ischemia in mice, at least in part, through increased Nrf2 activity and that the protective effect of TSA was abolished in Nrf2-deficient mice [74].

As previously mentioned, SFN has shown to be efficacious in attenuating diabetic cardiomyopathy and nephropathy in animal models of diabetes via improving Nrf2 function. In addition to the ability of SFN to repress Keap1-mediated degradation of Nrf2, it is also an HDAC inhibitor. Thus, it is likely that SFN improves Nrf2 function in diabetes, at least it part, through its ability to act as an HDAC inhibitor. Future experiments exploring SFN as a potential therapeutic for diabetes and diabetic complications ought to explore this mechanism of action.

The role of HDAC proteins on Nrf2 function has been overlooked in the diabetic condition. Based upon the evidence that class I HDACs reduce Nrf2 function and inhibition of these HDACs restore Nrf2 function, HDAC inhibition may provide a powerful therapeutic target in the Nrf2 signaling pathway (Figure 4).

Disparate Evidence on Nrf2 Activators in Diabetes

Activation of Nrf2 is a promising therapeutic target for diabetes. However, there is conflicting data regarding the effect of Nrf2 on mitigating the diabetic milieu. A few papers have provided preliminary evidence that Nrf2 agonists may exacerbate insulin resistance. For example, Xu and colleagues have suggested that enhanced NRf2 activity induces insulin resistance and impairs glucose metabolism [76]. They demonstrated that genetic overexpression of Nrf2, via Keap1-knockdown (Keap1-KD) has been shown to augment hyperglycemia and hyperlipidemia at 16 weeks in the $Lep^{ob/ob}$ mouse. However there were no differences in serum glucose, free fatty acids, or triglycerides in the normal Keap1- knockdowns. Furthermore, they show that the $Lep^{ob/ob}$ Keap1-KD mouse exhibited impaired glucose tolerance and insulin-induced glucose removal at 6 weeks; however, at 8 weeks there was no difference in either measure. The Keap1-KD mice also showed increased hepatic steatosis and increased lipid deposition in liver; however, obesity and high-fat diet-induced lipid accumulation in white adipose tissue was decreased in Keap1-KD mice. Zhang and colleagues demonstrated that Nrf2-null mice were resistant to high-fat diet induced glucose intolerance, while Keap1-KD mice exhibited glucose intolerance on both the high-fat and standard chow diet [77].

While these data hint at the possibility that Nrf2 may exacerbate insulin resistance in the presence of obesity, there are other plausible explanations. In regards to the study by Xu and colleagues, complete loss of leptin, in the presence of aberrant Nrf2 function, may account for the metabolic derangement observed in these mice, not merely overexpression of Nrf2. The data from Zhang and colleagues present a challenge to other data whereby Nrf2 activation, genetically and/or pharmacologically, prevents diet-induced obesity and hyperglycemia. In the studies that demonstrate that activation of Nrf2 worsens insulin resistance and serum markers of metabolism, the mechanism of Nrf2 activation was constitutive overexpression via Keap1-KD [76,77]. Conversely, insulin resistance and metabolism show positive changes in studies whereby Nrf2 is activated by drugs in a dose response manner [10]. This suggests that activation of Nrf2 via drug or gene knockout have disparate effects on insulin-resistance, and ultimately diabetic complications. Further studies that examine the effect of Nrf2 activating drugs in these same models would provide further clarification on the potential of Nrf2 activators to worsen insulin resistance and metabolic derangement.

In regards to human data, Nrf2 was the intended target of the phase 3, Bardoxolone Methyl Evaluation in Patients with Chronic

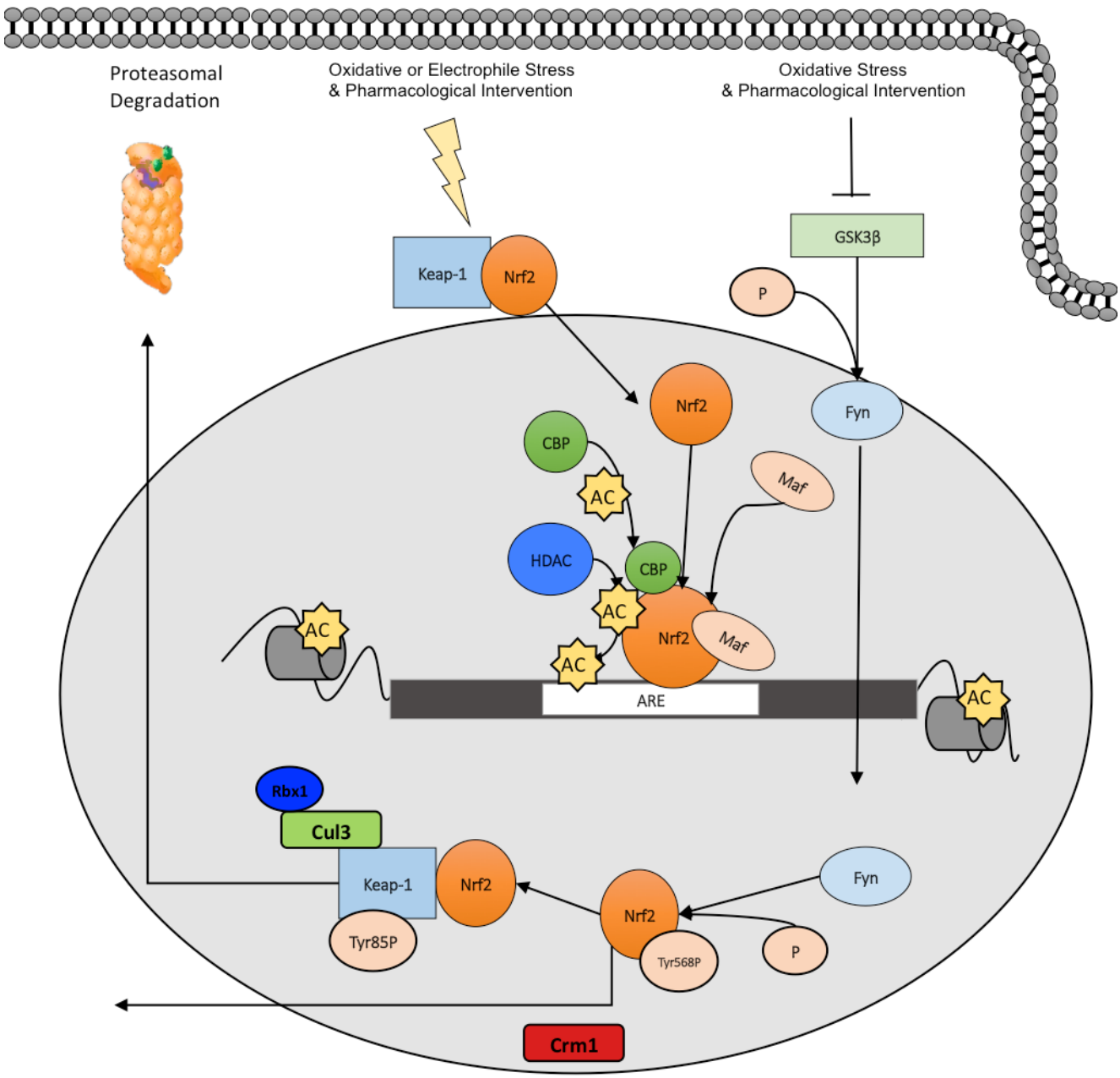

Figure 3: Nuclear regulation of Nrf2. Nrf2 forms heterodimers with Maf proteins to control differential gene regulation through the antioxidant response element (ARE). Acetylation of Nrf2 by the co-activator p300/CBP increased binding of Nrf2 to promoters in the ARE. Deacetylation by SIRT1 and other class I HDACs disengages Nrf2 from the ARE, thereby terminating transcription. Upon disengagement from the ARE, Fyn phosphorylates Tyr568 of Nrf2, resulting in Keap1 independent nuclear export via Crm1. Nrf2 can also complex with Keap1-Cul3-Rbx1, which is then ubiquitinated, exported, and degraded by the proteasome.

Kidney Disease and Type 2 Diabetes Mellitus: the Occurrence of Renal Events (BEACON) trial [78]. This trial was terminated prematurely due to severe adverse events including increased rates of cardiovascular events and heart failure, elevated albuminuria, heart rate, and blood pressure. A notable paper identified possible toxicity of bardoxolone methyl. Zoja and colleagues conducted experiments with two analogs of bardoxolone methyl (dh404 and RTA 405) to further elucidate the effect of Dh404 and RTA 405 in a ZDF rat model of diabetic nephropathy [51]. They found that RTA 405 increased diuresis and blood pressure, while also exacerbating proteinuria, tubular damage, and glomerulosclerosis. Additionally, RTA 405 increased levels of serum transaminase and caused liver injury in the rats. Follow up studies with Dh404, demonstrated no beneficial effect on diabetes-induced proteinuria, interstitial inflammation or glomerulosclerosis. In their paper, Zoja and colleagues noted that the RTA 405 used in their studies was contaminated with impure compounds and may have confounded their results. Chin et al. tested the identical compounds in the same animal DKD model (ZDF rat) [52]. The findings from their

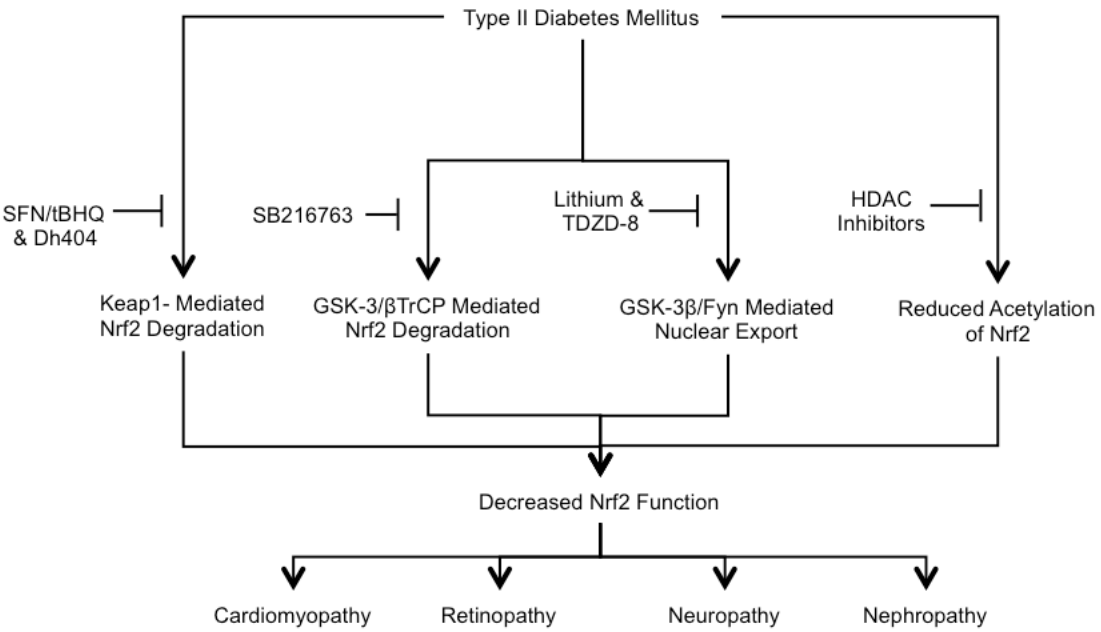

Figure 4: Effect of diabetes on Nrf2 signaling and current mechanism of action of current therapeutics. Sulforaphane and tert-Butylhydroquinone (tBHQ) prevents ubiquitination of Nrf2, thereby reducing proteasomal degradation. The triterpenoid derivative of dihydro-CDDO-trifluorethyl amid (Dh404) has been shown to directly disrupt Keap1 association and reduce Keap1-dependent suppression of Nrf2, thereby increasing Nrf2 stability. Inhibition of GSK-3 by SB216763 increases Nrf2 protein stability by preventing phosphorylation of Ser^{335} and $Ser^{338\ DSGIS338}$ and β-TrCP mediated ubiquitination. TDZD-8 and Lithium are GSK-3B inhibitors that prevents GSK-3B-Fyn mediated nuclear export of Nrf2 and normalizes basal and inducible levels of Nrf2 in diabetic rat fibroblasts [12]. Class I HDACs deacetylate Nrf2, which reduces DNA binding and increases nuclear export and cytosolic accumulation. Class I HDAC inhibitors reduce deacetylation of Nrf2, thereby promoting DNA binding and Nrf2 activity.

studies were in contrast with Zoja et al.; they found no toxicity and or exacerbation of renal histology in the RTA 405 or dh404 groups. They also found that RTA 405 improved renal function and glucose control in a STZ-induced diabetic rat model. Another follow up study with dh404 found that low doses of dh404 reduced oxidative stress and pro-inflammatory mediators (TNF-α, MCP-1, ICAM-1) with concomitant attenuation of UACR, mesangial expansion, and renal tubular injury [79]. Conversely, high doses of dh404 resulted in increases in inflammatory mediators. These data highlight the need to develop a more comprehensive understanding of Nrf2 biology and the safety and efficacy Nrf2 activators in the context of diabetes before the initiation of clinical trials.

Future Perspectives

The redox sensitive transcription factor Nrf2 is one of the major cellular defenses against the cytotoxic effects of oxidative stress and is currently being investigated as a therapeutic target for diabetic complications [6]. Evidence indicates that multiple aspects of the Nrf2 signaling pathway are dysregulated in T2DM, including: 1) increased expression of the Keap1, 2) increased GSK-3 activity, 3) upregulated nuclear Fyn expression, and 4) possibly decreased acetylation via increased HDAC activity. Currently most therapeutic strategies for increasing Nrf2 activity are aimed at targeting one aspect of the Nrf2 signaling pathway. While this approach has shown to be modestly effective in reducing diabetic complications such as nephropathy, hypertrophic cardiomyopathy, and neuropathy in animals and to reduce oxidative stress in humans, it is likely that combination therapy designed to target multiple aspects of Nrf2 signaling simultaneously may lead to more powerful outcomes. Data from *in vitro* data studies have shown that combination of a Keap1-Nrf2 disruptor with a GSK-3 inhibitor, were more effective in improving Nrf2 content and its downstream gene products in diabetic fibroblasts than either treatment in isolation [12]. Therefore, research aimed at developing a better understanding of Nrf2 signaling in diabetes and the development combination therapies to optimize Nrf2 activity in diabetes is warranted.

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