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Nuclear Factor Erythroid-2 Like 1 (NFE2L1): Structure, function and regulation

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Abstract

Nrf1 (also referred to as ***NFE2L1***) is a member of the CNC-bZIP family of transcription factors that are characterized by a highly conserved CNC-domain, and a basic-leucine zipper domain required for dimerization and DNA binding. *Nrf1* is ubiquitously expressed across tissue and cell types as various isoforms, and is induced by stress signals from a broad spectrum of stimuli. Evidence indicates that *Nrf1* plays an important role in regulating a range of cellular functions including oxidative stress response, differentiation, inflammatory response, metabolism, and maintaining proteostasis. Thus, *Nrf1* has been implicated in the pathogenesis of various disease processes including cancer development, and degenerative and metabolic disorders. This review summarizes our current understanding of *Nrf1* and the molecular mechanism underlying its regulation and action in different cellular functions.

Keywords

transcription factor; CNC-bZIP; cellular stress response; antioxidant; proteasome regulation; proteostasis; antioxidant response element

Introduction

The *Nuclear Factor Erythroid-2 Related Factor 1 (Nrf1)* is a transcription factor belonging to the CNC-bZIP subfamily (Chan et al., 1993). Basic-leucine-zipper proteins structurally share a bZIP domain comprised of a leucine zipper domain, a leucine–rich amphipathic α-helical region composed of 30–40 amino acids that is crucial for dimerization with other bZIP proteins, and a DNA-binding domain containing basic amino acid residues (Alber, 1992). The N-termini of CNC-bZIP proteins are divergent, but share a conserved 43 amino acid CNC domain (Chan et al., 1995). The CNC domain was first identified in the *Drosophilia* cap and collar gene, and is located directly N-terminal to the bZIP DNA-binding domain (Mohler et al., 1991). The CNC-bZIP subfamily consists of four closely related

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members that began with the identification of *p45Nfe2*, the founding member that was isolated for its ability to bind to the tandem NF-E2/AP1 element in the DNaseI hypersensitive site-2 of the human beta-globin locus control region (Andrews et al., 1993). Independent efforts to isolate proteins that bind and regulate the NF-E2/AP-1 cis-element led to the cloning of other CNC-bZIP proteins. *Nrf1* (also known as NFE2L1) was isolated and characterized by several laboratories and given different names. The first cloning of *Nrf1* was performed in yeast using a genetic screening method (Chan et al., 1993). The screening of human erythroid cDNA libraries using the NF-E2/AP1 consensus sequence as a recognition site led to the isolation of the *Nrf2* gene, as well as *Lcrf1* and *Tcf11*, which are two isoforms of *Nrf1* (Caterina et al., 1994; Moi et al., 1994; Luna et al., 1995). An expressed sequence tag (EST) database search to identify a predicted CNC-bZIP gene on human chromosome 7 led to the identification of *Nrf3* (Kobayashi et al., 1999). In addition to these four closely related members, the CNC family also includes the *C. elegans* SKN-1 transcription factor and two distantly related factors, BACH1 and BACH2, which function as transcriptional repressors (Sun et al., 2002; An and Blackwell, 2003).

Gene Structure and Expression Profile -

The human *Nrf1* gene maps to chromosome 17q21.3, whereas the mouse gene is located on the distal end of chromosome 11 (Chan et al., 1995; McKie et al., 1995). Alternative splicing and alternative use of translation initiation codons in the same mRNA molecule produce different sized polypeptides in mouse and human cells. The human *Nrf1* locus spans 15 kb of genomic DNA, and has nine exons with alternate first exons (1a and 1b) and terminal exons (6 and 6a), and two potential polyadenylation sites (Luna et al., 1995). The tissue and cell expression pattern of *Nrf1* has been determined largely at the mRNA level. In contrast to the erythroid-specific expression of *p45Nfe2*, *Nrf1* expression is ubiquitous (Chan et al., 1993). *In situ* hybridization analysis showed that *Nrf1* is widely expressed during development in mouse (Murphy and Kolsto, 2000). In adult tissues, high levels of *Nrf1* transcript are detected in the heart, kidney, skeletal muscle, fat and brain, while lower levels are seen in the liver, pancreas, and other organs. Expression is also seen in neutrophils, macrophages, and myofibroblasts during the inflammatory and early proliferative phase of skeletal muscle tissue in response to injury, suggesting an involvement of *Nrf1* in regeneration of muscle (Zhang et al., 2013).

Isoforms of *Nrf1* –

Multiple NRF1 protein isoforms corresponding to full-length and amino terminal truncated proteins are expressed in the cell. The longest isoform characterized, termed TCF11, consists of 772 amino acids (Luna et al., 1995). Another long isoform, referred to as NRF1a, is comprised of 742 amino acids, and is derived from an alternatively spliced mRNA missing exon 4 that is present in the *Tcf11* transcript (Luna et al., 1995). It is interesting to note that the mouse *Nrf1* locus does not appear to encode a *Tcf11* equivalent product. These long isoforms generate products that are detected on Western blots with apparent weights of approximately 120-kDa or greater. LCRF1 (also referred to as p65NRF1 and NRF1β) is a short isoform made up of 572 amino acids with an apparent weight of 65kDa (Caterina et al., 1994). While an alternate transcript encoding LCRF1 is present in the mouse-Ensembl database, LCRF1 has also been suggested to be an alternate translation product derived from

internal ATG codons located in transcripts encoding the long isoforms of *Nrf1* (Chan et al., 1993; Husberg et al., 2001). Although LCRF1/p65NRF1/ NRF1β has been shown to be a weaker activator of ARE-driven genes relative to NRF1a, this shorter isoform has also been demonstrated to function as a competitive inhibitor of NRF2 by competing for small MAF dimerization partners and binding to ARE sites of ARE-driven genes (Wang et al., 2007; Zhang et al., 2014). Another isoform that has been characterized is NRF1b (Kwong et al., 2012). The *Nrf1b* transcript is generated through an alternate promoter and contains a unique N-terminus encoded by an alternate first exon, and it encodes for a protein of 583 amino acids that has an apparent weight of approximately 100kDa. Additional smaller isoforms of *Nrf1*, termed *Nrf1γ* and *Nrf1δ* that encode 36-kDa and 25-kDa protein products, respectively, have also been described (Zhang et al., 2015). Transient transfection studies in COS-1 cells using wild-type and mutant NRF1 expression constructs suggest that NRF1γ and NRF1δ may be derived from proteasome-mediated proteolytic processing of full-length NRF1, and transactivation studies using reporter genes suggest that these small isoforms can function as dominant-negative inhibitors of longer NRF1 isoforms and NRF2 (Zhang et al., 2015). However, whether NRF1γ and NRF1δ could result from in-frame translation of internal ATG codons within the longer *Nrf1* transcript has yet to be verified. Nonetheless, it is clear that there are multiple NRF1 isoforms, and further research is required to determine the functions of these different isoforms, their target genes in the cell, and how they are regulated. For the sake of simplification, the term *Nrf1* (or NRF1) is used throughout this review unless the data pertains to a specific isoform.

NRF1 binds DNA as heterodimers with other bZIP proteins -

Basic-leucine zipper proteins bind to target DNA sites as dimeric complexes, and evidence indicates that CNC-bZIP proteins form dimers mainly with small-MAF proteins for DNA-binding (Toki et al., 1997). Three *small-Maf* genes, *Maf-F, Maf-G* and *Maf-K*, that encode highly homologous bZIP proteins of approximately 18 kDa in molecular weight devoid of transactivation function have been identified (Blank, 2008). The *small-Mafs* are expressed in a wide variety of tissues. Because of the conservation of the bZIP domain of NRF1 with other CNC-bZIP proteins and the presence of charged amino acid residues in the dimerization interface of the leucine-zipper domain, NRF1 is predicted to form heterodimers with small-MAF proteins as well (Chan et al., 1993). Indeed, experimental data showed that TCF11 preferentially forms heterodimers with MAFF, MAFG, and MAFK instead of TCF11 homodimeric complexes (Johnsen et al., 1996), and transient transfection studies showed that TCF11-MAFG and NRF1a-MAFG heterodimers bind and activate transcription through the NF-E2/AP1 site (Marini et al., 1997; Johnsen et al., 1998). In addition to small-MAF proteins, both TCF11 and LCRF1 have been demonstrated to interact with ATF4 (activating transcription factor 4) (Murphy and Kolsto, 2000; Masuoka and Townes, 2002). ATF4 is a bZIP containing transcription factor that belongs to the cAMP response element binding protein family, and has been shown to play a role in regulating stress response in cells (Ameri and Harris, 2008). Consistent with this data, a comprehensive analysis using protein arrays to identify pairings between bZIP proteins indicates that ATF/CREB proteins, in addition to c-MAF, are potential partners of NRF1 (Newman and Keating, 2003). NRF1 isoforms have been found to interact with AP1 transcription factors, namely FOSB, JUN, JUND, and ATF2, to form AP1 complexes found at the duplicated NF-E2 binding site and

extended κ3/AP1 site of the *TNFα* (tumor necrosis factor α) promoter in induced mast cells (Novotny et al., 1998; Venugopal and Jaiswal, 1998). C/EBP-β (CCAAT Enhancer-binding protein) was also identified as a bZIP partner of NRF1 (Narayanan et al., 2004).

Functional domains of NRF1 -

Similar to other transcription factors, NRF1 contains distinct domains for various activities including DNA binding, dimerization, transcriptional activation and subcellular localization. The bZIP domain of NRF1 is located near the carboxyl terminus of the protein. The basic-zipper domain is characterized by heptad repeats of leucine and hydrophobic residues within an amphipathic helical domain of 40 amino acids, and is preceded by 30 amino acid region that is rich in arginine and lysine residues (Chan et al., 1993). Immediately N-terminal to the bZIP domain is a highly conserved 43 amino acid domain. This homology region is referred to as the "CNC" domain after the *Drosophila* cap-n-collar protein, and it is not present in JUN, FOS or other bZIP proteins. The CNC domain has been shown to be also required for DNA binding (Bean and Ney, 1997). The similarity between CNC proteins is most remarkable in the basic-DNA binding region, which suggests that CNC proteins bind to similar DNA recognition-sites.

Mapping of functional domains also showed that multiple regions of NRF1 are required for full transactivation function (Husberg et al., 2001; Zhang et al., 2009; Zhang et al., 2014). These regions include two acidic domains (AD1 and AD2) near the N-terminus, an asparagine/serine/threonine-rich domain (NST) that separates AD1 and AD2, and a serine-rich domain located near the CNC motif of NRF1. AD1 appears to possess strong transactivation activity—its absence results in weaker capacity of the LCR-F1/p65NRF1/NRF1β isoform in activating gene expression (Zhang et al., 2014). In addition, deletion studies have shown that the C-terminal domain (CTD) at the carboxyl-end of the NRF1 polypeptide, which shares 53% identity with the Neh3 domain of NRF2, also contributes to activation function (Zhang et al., 2014).

Different groups have shown that NRF1 is localized in the endoplasmic reticulum as an integral membrane protein. The N-terminus of NRF1 contains an additional 155 amino acid polypeptide that is absent in NRF2. Wang et al. first demonstrated that the N-terminus of NRF1 is necessary and sufficient for ER localization (Wang and Chan, 2006). The N-terminus is comprised of hydrophobic residues and sequence analysis predicts an amphipathic α-helical structure located between amino acids 7 and 24 that is also observed in other membrane-associated proteins (Wang and Chan, 2006; Zhang et al., 2006). Another group also obtained similar results, and this sequence has been termed NHB1 for N-terminal homology box-1 (Zhang et al., 2006). NHB1 does not appear to function as a signal peptide, but it mediates retention of NRF1 in the ER. After entering the ER, NRF1 undergoes N-linked glycosylation at the NST domain, and NRF1 remains in the ER until it is degraded or transported to the nucleus to mediate activation of target genes. It has also been proposed that full-length NRF1 is a multi-pass protein with several topogenic determinants distributed throughout its sequence (Zhang et al., 2009; Zhang and Hayes, 2010). Interestingly, NRF3 also contains NST-like and NHB1-like sequences, and NRF3 has been demonstrated to be N-linked glycosylated and localized to the ER compartemnt (Nouhi et al., 2007). In addition,

NRF1 and NRF3 also share the NHB2, but the exact function of this homology domain is currently not known (Chevillard and Blank, 2011).

Multifaceted Role of *Nrf1* in Cellular Homeostasis

Although NRF1 was identified based on its ability to bind and activate through the NF-E2/AP1 element in the *β-globin* gene locus control region, studies conducted using *Nrf1* knockout mice and cells do not support a role for *Nrf1* in globin gene expression (Chan et al., 1998). Instead, *Nrf1* regulates a wide variety of cellular responses, several of which are related to important aspects of protection from stress stimuli.

Functions of *Nrf1* in Antioxidant Defense -

Antioxidant genes provide cellular protection against oxidative stress, and essential to the basal and stress-induced expression of these genes is the antioxidant response element (ARE) present in their regulatory DNA sequences. The ARE, also known as the electrophile responsive element (EpRE), is comprised of the RTGACnnnGC core sequence, and has been shown to mediate transcription of genes involved in glutathione synthesis and conjugation, phase 2 xenobiotic metabolism, ROS elimination and drug transport (Wasserman and Fahl, 1997; Nguyen et al., 2003). Although a number of different transcription factors bind to the ARE, AP1 complexes made up of JUN/FOS dimers were initially thought to mediate ARE function (Li and Jaiswal, 1992). A study to identify proteins in the regulation of ARE-mediated expression of the *Nqo1* gene, which encodes a reductive enzyme involved in ROS metabolism, showed that while AP1 proteins bind the ARE of the *Nqo1* promoter, they have no effect on its expression. Instead, expression of *Nrf1*, as well as *Nrf2*, was shown to activate expression. These findings were the first demonstration that *Nrf1* can activate expression through the antioxidant response element (Venugopal and Jaiswal, 1996).

Subsequent studies have shown that various genes involved in the oxidative stress response are also under the control of *Nrf1*. *Nrf1* was shown to perform a crucial role in protecting cells against oxidative stress through its involvement in the glutathione (GSH) synthesis pathway and with other redox-related genes. GSH synthesis is catalyzed by glutamate-cysteine ligase, which contains the catalytic *Gclc* and regulatory *Gclm*, and glutathione synthetase (*Gss*) (Lu, 2009). Although germline knockout of *Nrf1* leads to embryonic lethality in mice, Kwong et al. showed that cells derived from *Nrf1* knockout embryos have increased sensitivity to oxidative stress-induced cytotoxicity, and that *Nrf1* regulates *Gclm* and *Gss* expression in mouse fibroblasts (Chan et al., 1998; Kwong et al., 1999). This role for *Nrf1* was further substantiated in double *Nrf1 and Nrf2* knockout cells, which showed severe oxidative stress under ambient air that can be rescued by culturing under reduced oxygen tension, or by addition of antioxidants. In addition, expression of antioxidant defense genes was severely impaired in compound mutant cells compared with single mutant cells. These findings indicate that *Nrf1* and *Nrf2* have overlapping functions in regulating antioxidant gene expression during early embryogenesis (Leung et al., 2003). Results from ChIP assays and EMSA demonstrated that *Gclm* is a direct target of Nrf1, which imparts its activity through the ARE in the *Gclm* promoter. Subsequent studies also showed *Nrf1* plays a role in regulating *Gclc* expression (Myhrstad et al., 2001). It was demonstrated that *Nrf1*

regulates the rat *Gclc* gene via an indirect mechanism by modulating steady-state levels of AP-1 and NF-κB proteins, which in turn activate NF-κB and AP-1 binding sites in the *Gclc* gene promoter (Yang et al., 2005).

In addition to its role in cellular GSH homeostasis, other redox-related genes are potentially under Nrf1 control. Fetal livers derived from Nrf1 knockout mice showed down-regulation of *Gpx1* and *Hmox1*, and Nrf1-deficient hepatocytes from liver-specific *Nrf1* knockout mice showed decreased expression of various *Gst* genes (Chen et al., 2003; Xu et al., 2005). However, it is yet to be determined whether these genes are direct *Nrf1* targets. *Nrf1* has also been demonstrated to protect cells against cytotoxicity induced by toxic metals. Metallothionein-1 (*Mt-1*) and metallothionein-2 (*Mt-2*) genes encoding low molecular weight, cysteine-rich proteins with potent metal-binding and redox capabilities are down-regulated in *Nrf1*-deficient hepatocytes, and have been revealed as direct targets of *Nrf1* (Ohtsuji et al., 2008). Song et al. demonstrated fibroblasts deficient in *Nrf1* or *Nrf2* are sensitized to copper-induced cytotoxicity, and gene expression analysis done on *Nrf1* null and *Nrf2* null cells over time indicated that both *Nrf1* and *Nrf2* are involved in mediating *Mt-1* transcription after acute exposure to copper, while *Nrf1* is required for *Mt-1* expression in chronic exposure (Song et al., 2014). Inorganic arsenic (iAs^{3+}) induces oxidative stress and leads to the nuclear accumulation of NRF1 in human keratinocytes to promote expression of *Nqo1, Gclc* and *Gclm* (Zhao et al., 2011).

Nrf1 in Proteasome Homeostasis -

Accumulating evidence underscores the role of *Nrf1* in proteasome homeostasis. The ubiquitin proteasome system (UPS) is an important pathway for degradation of intracellular proteins in eukaryotic cells. Proteins destined for degradation by the UPS are tagged with ubiquitin moieties, and subsequently targeted for proteolysis by the 26S proteasome consisting of two sub-complexes—the 20S core and the 19S regulator, each of which is made up of multiple protein subunits encoded by *Psm* genes (Goldberg, 2003; Groll et al., 2005). In yeast, expression of the *Psm* genes is regulated by the RPN4 transcription factor via binding to the Proteasome Associated Control Element, and homeostatic levels of proteasome are maintained through feedback mechanisms where RPN4 is either up-regulated or degraded depending on proteasome levels (Xie, 2010). Similarly, proteasome genes are coordinately regulated in mammalian cells, and a feedback mechanism also occurs (Meiners et al., 2003). The importance of *Nrf1* in proteasome gene expression was discovered through analysis of *Nrf1* knockout mice. Brains of mice with conditional knockout of *Nrf1* in neuronal cells showed decreased proteasome activity and accumulation of ubiquitin-conjugated proteins, and down regulation of genes encoding the 20S core, and 19S regulatory complex (Lee et al., 2011). A similar effect on proteasome gene expression and function was observed in livers of mice with *Nrf1* conditional knockout in hepatocytes (Lee et al., 2013). While loss of *Nrf1* did not appear to significantly affect basal expression of proteasome genes in MEF cells, induction of these proteasomal genes in response to MG132 inhibition of proteasome function and recovery of proteasome activity after transient treatment with proteasome inhibitors was impaired in *Nrf1* deficient fibroblasts (Radhakrishnan et al., 2010). Consistent with these findings, induction of proteasome genes was also lost in brains and livers of *Nrf1* conditional knockout mice (Lee et al., 2011; Lee et

al., 2013). Re-establishment of *Nrf1* function in *Nrf1* null cells rescued proteasome expression and function indicating *Nrf1* was necessary for induction of proteasome genes (bounce-back response) in response to proteasome inhibition (Radhakrishnan et al., 2010). This compensatory up-regulation of proteasome genes in response to proteasome inhibition has also been demonstrated to be *Nrf1*-dependent in various other cell types (Steffen et al., 2010; Sha and Goldberg, 2014). Sequence analysis revealed AREs are present in the promoter regions of proteasome subunit genes (Radhakrishnan et al., 2010; Lee et al., 2011). By transient transfection assay, *Nrf1* was demonstrated to activate the promoter of the *PsmB6* gene, which encodes one of the catalytic subunits of the 20S core, and ChIP assay indicated that NRF1 binds near the transcription start site of the *PsmB6* promoter, which on sequence inspection revealed a consensus ARE (Radhakrishnan et al., 2010; Lee et al., 2011). In addition to proteasome subunit genes, *Nrf1* also controls aspects of the endoplamic reticulum-associated degradation (ERAD) pathway. *Nrf1* was shown to regulate expression of *Herpud1* and *Vcp/p97*, which are components of the ERAD pathway (Sha and Goldberg, 2014; Ho and Chan, 2015). *Nrf1* was found to be necessary for *Herpud1* basal expression and induction under ER stress in both mice and human cells (Ho and Chan, 2015). Transient transactivation studies showed *Nrf1* acts through antioxidant response elements located in the *Herpud1* promoter, and chromatin immunoprecipitation demonstrated that NRF1 binds directly to the *Herpud1* promoter. These findings suggest that NRF1 may be the functional homolog of RPN4 in mammalian cells.

Nrf1 in Inflammation and Regeneration -

In addition to mediating antioxidant genes, *Nrf1* has been linked to cellular immune responses. Inducible nitric oxide synthase (*iNos*) is a transcriptionally regulated enzyme that synthesizes nitric oxide, which is important in regulating the vascular response during inflammation and injury. TGF-β has been shown to be a negative regulator of *iNos* expression. TGF-β treatment of human smooth muscle cells leads to increased expression and binding of NRF1/MAFG heterodimers to an ARE-like site in the *iNos* promoter (Berg et al., 2007). Knockdown of *Nrf1* obstructs the suppression of *iNos* expression by TGF-β. In addition, Prieschl et al. showed that stimulation of FCγRIII in the γ chain of the immunoreceptor tyrosine-based activation motif (ITAM) results in the γ chain activating a splice variant of *Nrf1* with a 411 bp deletion in the serine-rich region to bind to an extended κ3 site of the *Tnf-α* promoter to trans-activate expression in dendritic cells (Prieschl et al., 1998). Novotny et al. (1998) investigated *Nrf1* trans-activation of the *Tnf-α* promoter in stimulated mast cells, and found *Nrf1* splice variants bind to the extended $\kappa 3/AP1^{+}$ site of the *Tnf-α* promoter as a complex with AP1 proteins (FOSB, JUN, JUND, ATF2) (Novotny et al., 1998). A notable difference was that the latter group found NRF1 did not independently bind to an extended $\kappa 3/AP1^{-}$ site, but rather to an extended $\kappa 3/AP1^{+}$ site as an AP1 component, signifying that in mast cells, *Tnf-α* promoter induction necessitates an intact AP1 binding site.

Nrf1 and Metabolic Homeostasis -

There is growing evidence that *Nrf1* also plays a role in metabolic processes. Loss of hepatic *Nrf1* has been shown to result in lipid accumulation, hepatocellular damage, cysteine accumulation, and altered fatty acid composition (Xu et al., 2005; Tsujita et al., 2014).

Transcriptome profiling in livers of hepatic-specific *Nrf1* gene knockout mice showed reduced expression of genes related to amino acid metabolism, as well as transcriptional regulators, *Ppara, Lipin1* and *Pgc-1β* that are involved in lipid metabolism (Hirotsu et al., 2012). ChIP analyses and promoter studies using a Luciferase reporter indicate that *Lipin1* and *Pgc-1β* are target genes of *Nrf1,* suggesting that *Nrf1* plays a direct role in hepatic lipid metabolism. In a separate study, *Nrf1* inactivation in hepatocytes was associated with increased expression of *Apoer2* and *Vldlr* lipoprotein receptor genes, the *Fads3 desaturase* gene, and *Alox5ap* gene that is involved in prostaglandin and leukotriene synthesis (Tsujita et al., 2014). In addition, it was shown that *Nrf1* negatively regulates the basal levels of cysteine uptake by system x_c^- through direct suppression of xCT expression (Tsujita et al., 2014). Through gain of function analysis in mice, it was demonstrated that *Nrf1* also plays a role in regulating glucose flux (Hirotsu et al., 2014). Increased expression of *Nrf1* led to development of insulin resistance in transgenic mice. While the basis of these abnormalities is not clear, insulin-regulated glycolytic genes—*Gck, Aldob, Pgk1*, and *Pklr,* hepatic glucose transporter gene — *Sl2a2*, and gluconeogenic genes — *Fbp1* and *Pck1* were found to be repressed in livers of *Nrf1* transgenic mice. In another study, Zheng et al. also showed a relationship between *Nrf1* and glucose homeostasis (Zheng et al., 2015). It was shown that loss of *Nrf1* in pancreatic β-cells leads to fasting hyperinsulinemia, reduced glucose-stimulated insulin secretion, and glucose intolerance. These abnormalities were associated with altered expressions of glycolytic proteins. *Glut2, Ldh1, Gapdh*, and *Hk1* were upregulated, whereas *Gck* expression was reduced. MIN6 β-cells depleted of *Nrf1* also showed elevated basal insulin release and reduced glucose-stimulated insulin secretion suggesting that these effects were specific to loss of *Nrf1*. Collectively, these results demonstrate that a role for *Nrf1* in lipid and glucose gene expression.

Control of Cellular Differentiation -

Nrf1 has been shown through various studies to inhibit or promote differentiation depending on the cellular context. Ascorbic acid has been demonstrated to be essential in osteoblast differentiation. A key downstream target of ascorbic acid action is *Osterix* (*Osx*), an osteoblast-specific transcription factor essential in controlling gene expression during differentiation and maturation of osteoblasts. It was demonstrated that siRNA-mediated depletion of *Nrf1* led to down-regulation of *Osx* expression and impairment in osteoblast differentiation, and that ascorbic acid induced *Osx* expression involves NRF1 nuclear translocation and NRF1 binding to the ARE element in the *osterix* promoter (Xing et al., 2007). *Nrf1*, along with *Zscan10*, *Atf1*, and *Srebf2*, was also identified as a positive regulator of osteoclastogenesis via a genome-wide analysis of open chromatin during RANKL (receptor activator of NF-κB ligand)-induced osteoclastogenesis using DNase-seq (Inoue and Imai, 2014). siRNA knockdown of *Nrf1* resulted in impaired osteoclastogenesis and the formation of TRAP (tartrate-resistant acid phosphatase)-positive multinucleated osteoclasts, demonstrating *Nrf1*'s role in early osteoclastogenesis.

Besides its role in bone formation, *Nrf1* also plays a role in tooth development. Odontoblasts initiate dentinogenesis, and once fully differentiated, synthesize type I collagen and two noncollagenous proteins, dentin phosphophoryn (DPP) and dentin sialoprotein (DSP), that are encoded by the *dentin sialophosphoprotein (Dspp)* gene. The TCF11 isoform of *Nrf1*

was shown to dimerize with C/EBP-β and repress *Dspp* expression during odontoblast differentiation (Narayanan et al., 2004). The TCF11 isoform was also shown to transactivate the *Dmp1* (*Dentin matrix protein 1*) promoter in odontoblasts and osteoblasts, suggesting that *Nrf1* also regulates this DSPP-related gene that is important in the formation of mineralized tissue (Jacob et al., 2014).

Other Functions -

Nrf1 may also play a role in maintaining chromosomal stability and genomic integrity by inducing expression of genes encoding components of the spindle assembly and kinetochore (Oh et al., 2012). Loss of *Nrf1* function leads to increased formation of abnormal nuclei and aneuploidy in cells, and down regulation of genes encoding kinetochore and mitotic checkpoint proteins including *Ndc80, Nuf2, Spc25* and *Sgo1*. Whether any of these genes are direct targets of Nrf1 has not been established; however, *Nrf1*'s role in maintaining mitotic checkpoint genes suggests *Nrf1* dysregulation may lead to tumorigenesis. *Nrf1* has also been reported to play an important role in DNA repair in response to UVB irradiation through regulating expression of *xeroderma pigmentosum C* (*Xpc*), a component of the nucleotide excision repair pathway (Han et al., 2012). In addition, *Nrf1* promotes survival of UVB-irradiated cells by suppressing expression of the proapoptotic protein BIK, a member of the Bcl-2 family that regulates antiapopototic family members MCL-1 and BXL-XL to promote apoptosis (Han et al., 2012). The mechanism of *Xpc* and *Bik* regulation by *Nrf1* appears to be indirect via maintaining cellular glutathione levels.

Regulation of *Nrf1* expression -

Nrf1 is regulated under a variety of conditions through the actions of a number of factors, including cytokines, nutrients and agents that cause cellular stress. Understanding the mechanisms that are involved in the regulation of *Nrf1* during such conditions has been a focus of recent research. Various types of cellular stress have been shown to trigger *Nrf1* expression. Exposure of cells to inorganic arsenic and t-butyl hydroquinone (TBHQ), which are potent oxidative stress inducers, leads to increased NRF1 protein levels and accumulation in the nucleus (Zhang et al., 2009; Zhao et al., 2011). Treatment with tunicamycin, an ER stress inducer, also leads to increased nuclear accumulation of NRF1 suggesting that ER stress might be an activator of *Nrf1* function (Wang and Chan, 2006). Furthermore, hypoxia was also shown to significantly increase NRF1a expression while decreasing p65NRF1 expression (Chepelev et al., 2011). Aside from stress-related stimuli, *Nrf1* expression is also activated by growth factors through mTORC1-mediation of the SREBP1 transcription factor (Zhang and Manning, 2015). Although mTORC1 promotes anabolic processes, protein degradation mediated by proteasomes is also enhanced by mTORC1 activation thus ensuring amino acid building blocks are in adequate supply in metabolically active cells.

Regulation of NRF1 nuclear localization -

Current evidence indicates NRF1 is a type II membrane protein with a N_{cyt}/C_{lum} orientation (Zhang and Hayes, 2010; Radhakrishnan et al., 2014). How NRF1 is transported to the nucleus is not entirely clear. The ER-associated degradation pathway has been suggested to mediate translocation of NRF1 from the ER to the cytosol, and proteasome level has been

suggested to regulate this process (Steffen et al., 2010; Radhakrishnan et al., 2014; Sha and Goldberg, 2014). In this model, NRF1 initially undergoes ubiquitination by the HRD1 complex, and is repositioned by p97/VCP such that the C-terminus and a major part of NRF1 exits the ER lumen through the retrotranslocon complex on the ER membrane. Under conditions when proteasome activity is diminished (i.e. dimished proteasome levels), NRF1 escapes proteasome degradation and is proteolytically cleaved at the Leu-104 residue, thus freeing NRF1 from the ER membrane. Subsequently, the free form of NRF1 (p110) then migrates to the nucleus to activate expression of proteasome subunit genes to restore proteasome function in the cell. However, under normal levels of proteasome function, p97/VCP binds to full-length NRF1 and facilitates its degradation by the proteasome. TCF11 was also shown to utilize the ERAD pathway for translocation to the cytosol in response to proteasome inhibition (Steffen et al., 2010). It has also been proposed that NRF1 may gain access to the nucleus from the ER by diffusion through the nuclear pore membrane, which would enable NRF1 to interact directly with its target genes. However, the mechanism by which this might occur is not known (Zhang et al., 2007).

Regulation of NRF1 activity -

Casein kinase 2 (CK2) was shown to interact with and phosphorylate at serine-497 of NRF1 (Tsuchiya et al., 2013). CK2-mediated phosphorylation of NRF1 leads to down-regulation of proteasome gene expression. Proteasome dysfunction was rescued by the knockdown of CK2 and S497 alanine substitution suggesting that activation function of NRF1 is suppressed by CK2. In addition to CK2, phosphorylation of NRF1 at Ser-599 by protein kinase A (PKA) enables NRF1 and C/EBP-β to associate into a dimeric complex to repress *Dspp* expression during odontoblast differentiation (Narayanan et al., 2004).

Regulation of NRF1 stability -

NRF1 is a short-lived protein that is regulated via ubiquitin-dependent protein degradation. KEAP1 controls ubiquitination and proteasomal degradation of NRF2 through its Neh2 domain. Although NRF1 also contains a Neh2-like domain, and has been shown to bind KEAP1, KEAP1 does not appear to regulate NRF1 function (Wang and Chan, 2006; Zhang et al., 2006). Currently, three different E3 ubiquitin ligases including β-TRCP, 7 and HRD1, have been reported to act on NRF1. The F-box containing protein, FBXW7, a tumor suppressor frequently inactivated in human cancers, was demonstrated to negatively regulate NRF1 expression. FBXW7 facilitates ubiquitination and degradation of NRF1 through a Cdc4 phosphodegron (CPD) motif in NRF1 (Biswas et al., 2011). This process is promoted by GSK3β, which phosphorylates NRF1 in its CPD, and thus facilitating binding by FBXW7 for subsequent proteasome-mediated degradation (Biswas et al., 2013). Consistent with FBXW7 modulating NRF1 expression, enforced expression of FBXW7 leads to suppression of NRF1-mediated gene activation. Another F-box protein, β-TRCP, has also been shown to promote the degradation of NRF1 by binding to its DSGLC motif, a highly conserved region among NF-E2-related factors (Tsuchiya et al., 2011). Depletion of β-TRCP by siRNA-mediated gene silencing increases transcriptional activity of NRF1 and augments expression of NRF1 target genes, indicating β-TRCP downregulates NRF1 expression. In addition to F-box proteins, NRF1 stability is also impacted by the ERAD pathway (Steffen et al., 2010; Tsuchiya et al., 2011). Depletion of HRD1, an ER-associated

ubiquitin ligase, and VCP/p97, an ATPase protein that facilitates retrotranslocation of proteins from the ER, leads to stabilization of NRF1 and TCF11. The ERAD-dependent regulation of NRF1 has been suggested to function as a negative feedback loop that turns off expression of proteasome subunit genes through NRF1. The exact mechanism through which these E3 ligases regulate NRF1 levels in response to various stress stimuli is an important issue for future research. Finally, NRF1 also contains a Neh2-like domain, which is the binding site for KEAP1, a substrate adaptor protein in the Cul3-dependent E3 ubiquitin ligase complex that controls ubiquitination and proteasomal degradation of NRF2. Although NRF1 has been shown to bind KEAP1 via the Neh2-like domain, KEAP1 does not appear modulate NRF1 stability (Wang and Chan, 2006; Zhang et al., 2006). Thus, the biological significance of this interaction remains to be determined.

Unraveling the Role of *Nrf1 in vivo*

Mouse models developed in a number of labs provide an important step in determining the physiological significance of *Nrf1* in vivo. Genetic loss and gain of function studies in mice showed that misregulation of *Nrf1* leads to pathological states that could have relevance in human diseases. Global knockout of *Nrf1* leads to embryonic lethality that was attributed to anemia from impaired fetal liver erythropoiesis (Chan et al., 1998). Consistent with this idea, analysis of mouse chimeras revealed that *Nrf1* is not necessary for fetal liver development, but is important for the maintenance and survival of hepatocytes during development (Chen et al., 2003). The loss of *Nrf1* in adult mouse hepatocytes leads to development of steatosis, inflammation and tumorigenesis (Xu et al., 2005). Although the exact basis of liver pathologies in liver-specific *Nrf1* knockout mice is not clear and is likely to be multifactorial, *Nrf1*-deficient hepatocytes are characterized by both oxidative and ER stress (Xu et al., 2005; Ohtsuji et al., 2008; Lee et al., 2013). It is interesting in this regard that ER stress has emerged as a key player in regulating aspects of steatohepatitis including inflammation, oxidative stress and cell death (Urano et al., 2000; Rutkowski et al., 2008; Zhang et al., 2011). Hence, liver-specific *Nrf1* knockout mice may be a useful model for studying the physiological function of *Nrf1* in ER stress responses and liver diseases. *Nrf1* is also necessary for maintaining neuronal homeostasis. Neuron-specific *Nrf1* conditional knockouts generated using Cam-K2 Cre transgenic mouse to drive deletion of the *Nrf1* gene developed age-dependent forebrain atrophy as a result of progressive neurodegeneration (Lee et al., 2011). In addition to the neurodegenerative phenotype, brains in these mice showed accumulation of ubiquitinated proteins and apoptosis. In another study, deletion of *Nrf1* in neurons generated using Nestin-Cre, which is expressed in neuronal and glial precursor cells, led to motor ataxia and neurodegeneration, as well as chromatolysis in the spinal cord (Kobayashi et al., 2011). Conditional knockout of *Nrf1* in osteoblasts showed decreased bone size, trabecular bone, peak bone mass, and mechanical strength (Kim et al., 2010). Gene expression analysis suggests that bone formation may be secondary to decreased expression of *Osterix*, a zinc finger transcription factor essential for osteoblast differentiation. Currently, it is not known whether *Nrf1* is also required to maintain bone mass in response to hormonal or nutritional stress. *Nrf1* has also been implicated in glucose metabolism. In a genome-wide association study, a single nucleotide polymorphism rs3764400 in the 5’-flanking region of the human *NRF1* gene appears to be associated with

obesity (Speliotes et al., 2010). Analysis indicates rs3764400 is a regulatory SNP that is associated with increased *Nrf1* expression levels (Hirotsu et al., 2014). Contrary to expectations, transgenic mice with over-expression of *Nrf1* are characterized by weight loss and protection from diet-induced obesity. In addition, *Nrf1* transgenic mice developed insulin resistance through suppression of insulin signaling via AKT activation in liver and skeletal muscle (Hirotsu et al., 2014). Gene expression studies indicated that insulin resistance is not directly achieved through transcriptional repression of the insulin signaling pathway components, but rather through an indirect pathway possibly through elevated plasma β-hydroxybutyrate levels resulting from enhanced fatty acid oxidation in livers of transgenic mice. However, the basis for increased fatty acid utilization remains to be explored. Loss of *Nrf1* function is also associated with insulin resistance. Mice with deletion of *Nrf1* in pancreatic β-cells exhibited severe fasting hyperinsulinemia and glucose intolerance suggesting that *Nrf1* may play a role in the development of type-2 diabetes. Given the inherent limitation of Ins2-cre mouse used in this study, however, the role of *Nrf1* in glucose homeostasis requires further analysis using other Cre driver lines to generate β-cell-specific ablation of *Nrf1*.

Future Research

Maintaining cellular homeostasis is critical for normal functioning of the body, and disruption in this process can result in a variety of pathological conditions. In this review, we discussed the aspects of *Nrf1* in maintaining cellular, proteasomal, and metabolic homeostasis, and its role in inflammation and cellular differentiation. Regulatory pathways of *Nrf1* and phenotypes observed in *Nrf1* knockout animal studies were also outlined. Currently, there remain identified isoforms that have yet to be fully characterized. Further exploration of these other isoforms may reveal additional regulatory functions of *Nrf1*. NRF1 has been proposed as a Type II membrane protein with a hydrophobic transmembrane region attaching the protein to the ER membrane, with its topology oriented such that the DNA binding domain faces the ER lumen. Further research is needed however to validate other models, such as that proposed by Zhang et al., suggesting NRF1 is integrated into the ER via multiple membrane-spanning α-helices (Zhang and Hayes, 2010). Studies clarifying the membrane localization and orientation of NRF1 may reveal other pathways that regulate NRF1 function. Finally, a recent effort to develop NRF1-specific inducers highlights *Nrf1* as an emerging target (Tsujita et al., 2015). Therapeutic implications associated with *Nrf1* are provocative, particularly in in cancer, neurodegeration, and metabolic diseases. Additional approaches taken to identify interventions that encompass transcriptional regulation and partner proteins that activate or repress NRF1 function offer exciting opportunities to treat a diverse range of diseases associated with *Nrf1*.

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Abbreviations:

Nrf1	Nuclear Factor Erythroid-2 Related Factor 1
CNC	cap ‘n’ collar
bZIP	basic leucine zipper
Maf	musculoaponeurotic fibrosarcoma oncogene
ARE	antioxidant response element
EpRE	electrophile responsive element
C/EBP-β	CCAAT Enhancer-binding protein
ROS	reactive oxygen species
NQO1	NAD(P)H: quinone oxioreductase 1
GSH	glutathione
GST	glutathione-S-transferase
Gpx	glutathione peroxidase
Hmox1	heme-oxygenase 1
Fth	ferritin heavy chain
Psm	proteasome
ERAD	ER-associated degradation
UPS	ubiquitin proteasome system
mTORC1	mammalian target of rapamycin complex 1

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Highlights:

- Nrf1is a CNC-bZIP transcription factor
- Nrf1 regulates expression of genes involved in cellular stress response
- Nrf1 is critical for maintaining proteostasis
- Nrf1 expression is controlled by its protein stability
- Knockout mouse studies suggest the importance of Nrf1 in cancer, neurodegenerative and metabolic disorders

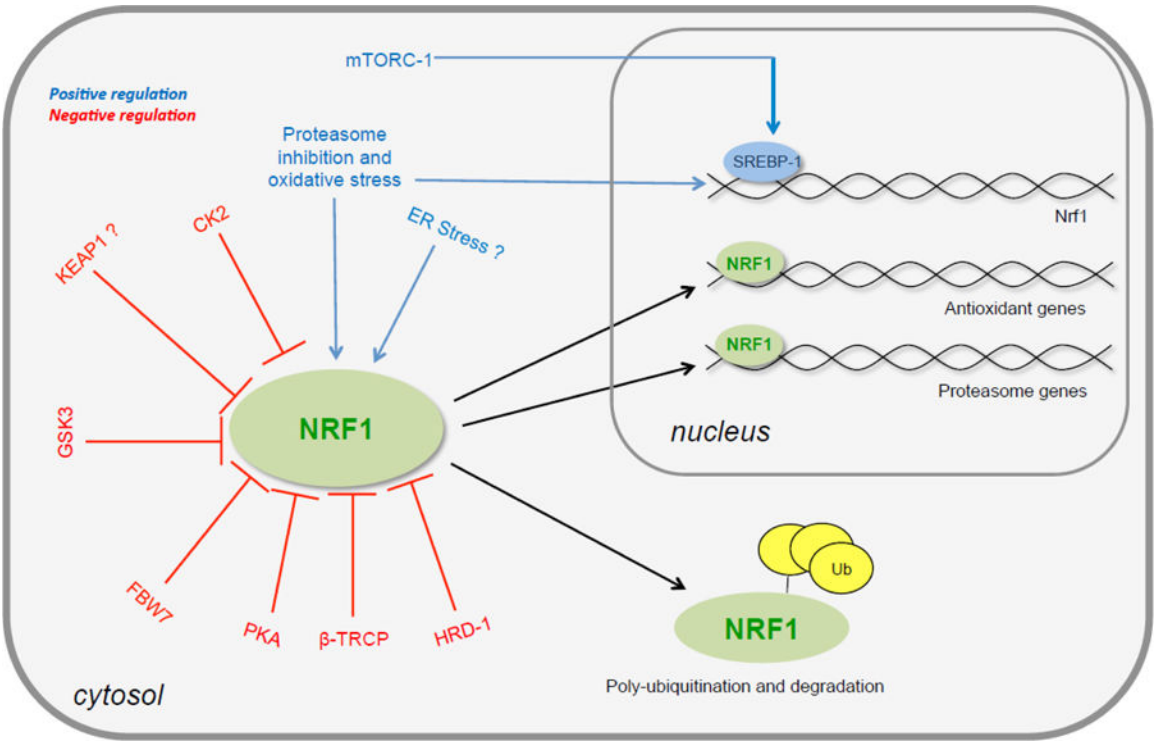

Figure 1.

Nrf1 function is subject to transcriptional and post-translational regulation. This diagram depicts the pathways that have been identified that control the expression, activity and stability of NRF1. See text for explanation.

Table 1.

Genes regulated by *Nrf1*.

Function	Gene	References
Antioxidant Defense	*Gclm, Gclc, Gss*	(Kwong et al., 1999; Myhrstad et al., 2001; Leung et al., 2003; Yang et al., 2005; Zhao et al., 2011)
	Gstp2, Gstm3, Gstm6	(Xu et al., 2005)
	Gpx1, Hmox1, Fth	(Chen et al., 2003; Leung et al., 2003)
	Mt1, Mt2	(Ohtsuji et al., 2008; Song et al., 2014)
	Nqo1	(Venugopal and Jaiswal, 1996; Zhao et al., 2011)
Proteasome Homeostasis	*Psm subunits*	(Radhakrishnan et al., 2010; Steffen et al., 2010; Lee et al., 2011; Sha and Goldberg, 2014)
	Herpud1, Vcp/p97	(Sha and Goldberg, 2014; Ho and Chan, 2015)
Inflammation	*iNOS*	(Berg et al., 2007)
	TNF-α	(Novotny et al., 1998; Prieschl et al., 1998)
Metabolic Homeostasis	*Ppar-α, Pgc-1β, Lipin1*	(Hirotsu et al., 2012)
	Xct	(Tsujita et al., 2014)
	Vldlr, Apoer2	(Tsujita et al., 2014)
	Gck, Aldob, Pgk1, Pklr, Sl2a2, Fbp1, Pck1	(Hirotsu et al., 2014)
	Glut2, Ldh1, Gapdh, Hk1, Gck	(Zheng et al., 2015)
Cellular Differentiation	*Osterix*	(Xing et al., 2007)
	DSPP	(Narayanan et al., 2004)
	DMP1	(Jacob et al., 2014)
Other	*Ndc80, Nuf2, Spc25, Sgo1*	(Oh et al., 2012)
	Xpc, Bik	(Han et al., 2012)