Regulation of NRF1, a master transcription factor of proteasome genes: implications for cancer and neurodegeneration

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ABSTRACT The ability to sense proteasome insufficiency and respond by directing the transcriptional synthesis of de novo proteasomes is a trait that is conserved in evolution and is found in organisms ranging from yeast to humans. This homeostatic mechanism in mammalian cells is driven by the transcription factor NRF1. Interestingly, NRF1 is synthesized as an endoplasmic reticulum (ER) membrane protein and when cellular proteasome activity is sufficient, it is retrotranslocated into the cytosol and targeted for destruction by the ER-associated degradation pathway (ERAD). However, when proteasome capacity is diminished, retrotranslocated NRF1 escapes ERAD and is activated into a mature transcription factor that traverses to the nucleus to induce proteasome genes. In this Perspective, we track the journey of NRF1 from the ER to the nucleus, with a special focus on the various molecular regulators it encounters along its way. Also, using human pathologies such as cancer and neurodegenerative diseases as examples, we explore the notion that modulating the NRF1-proteasome axis could provide the basis for a viable therapeutic strategy in these cases.

INTRODUCTION

The highly regulated ubiquitin proteasome system (UPS) is responsible for degrading the majority of intracellular proteins to maintain cellular proteostasis, health, and, ultimately, cell survival (Kleiger and Mayor, 2014; Finley and Prado, 2020). At the heart of the UPS is the 26S proteasome, a 2.5 MDa multicatalytic protease complex built from at least 33 different protein subunits. Structurally, the 26S proteasome is composed of a 20S catalytic core, which is capped at one or both ends by a 19S regulatory particle (Pickart and Cohen, 2004; Ciechanover, 2005; Finley, 2009). Although it was initially assumed that the flux through the UPS is determined largely by the rate of substrate ubiquitination, it is now increasingly clear that the levels and activity of the proteasome could also be important rate-limiting factors. The abundance of the proteasome is especially important when cells are subjected to proteotoxic stress or challenged with proteasome inhibitors.

In *Saccharomyces cerevisiae*, proteasome levels are under the control of the transcription factor Rpn4. The promoter region of the proteasome subunit (PSM) genes were found to contain a consensus motif termed "proteasome-associated control element (PACE)" that can be bound and activated by Rpn4 (Mannhaupt et al., 1999). Interestingly, Rpn4 is also a short-lived substrate of the 26S proteasome (Xie and Varshavsky, 2001). Thus, stabilization of Rpn4 by diminished proteasome activity leads to activation of PSM gene expression, de novo proteasome assembly, and a rescue of proteasome activity. As proteasome activity recovers, degradation of Rpn4 is correspondingly increased, thereby creating a negative feedback loop that allows Rpn4 to act as a sensor for decreased proteasome activity (Dohmen et al., 2007).

This phenomenon of PSM gene activation in the face of diminished or inhibited proteasome activity, dubbed the "proteasome bounce-back response," is well conserved in evolution (Mitsiades et al., 2002; Meiners et al., 2003; Lundgren et al., 2005; Radhakrishnan et al., 2010). Akin to Rpn4 in yeast, the transcription factors Cnc-C and SKN-1A mediate the transcriptional proteasome bounce back response in *Drosophila* and *Caenorhabditis elegans*, respectively
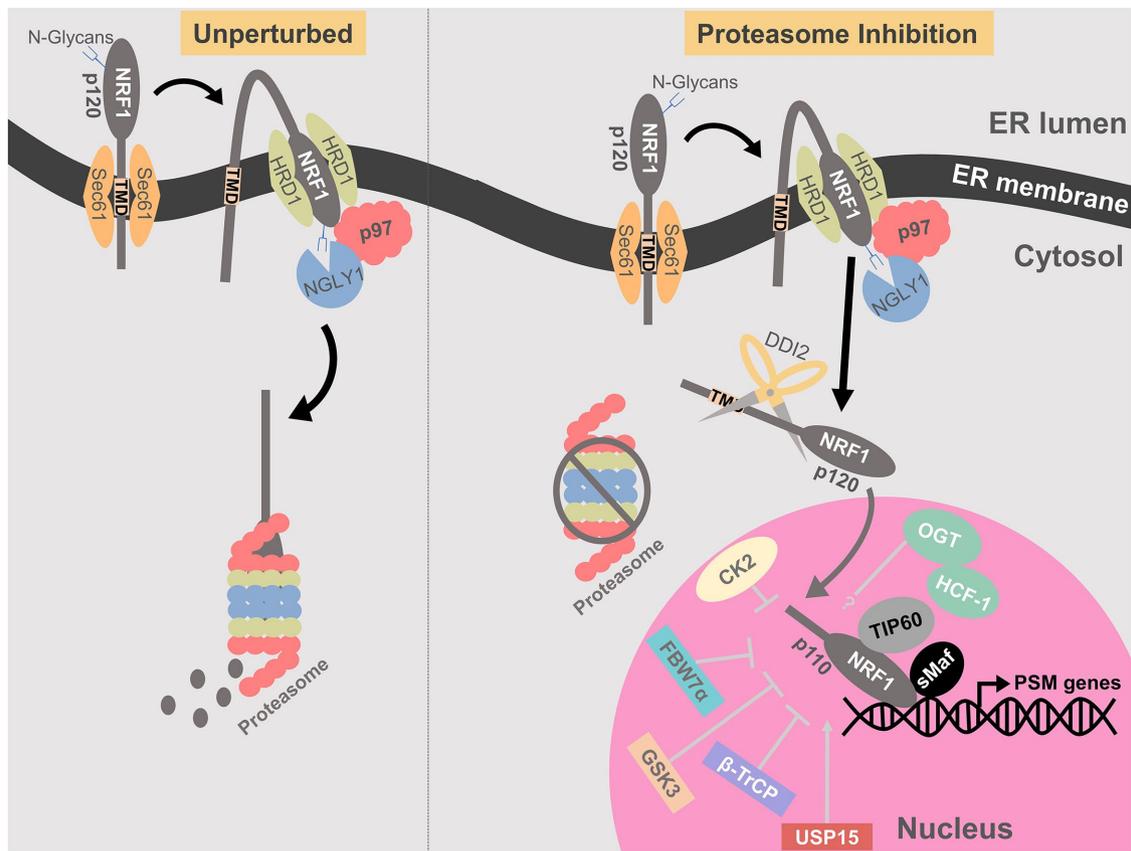
FIGURE 1: Molecular players involved in the NRF1 pathway. This model depicts our current understanding of the Nrf1 pathway. During biosynthesis, Nrf1 p120 is inserted into the endoplasmic reticulum (ER) membrane via the Sec61 pathway in a type II (C_{lumen}/$N_{cytosol}$) orientation. The domains close to the C-terminus of p120 are glycosylated in the ER lumen. Under unperturbed conditions, p120 is ubiquitinated by HRD1, retrotranslocated by the action of ATPase p97, deglycosylated by NGLY1, and degraded by the proteasome. When the proteasome is inhibited, retrotranslocated p120 is deglycosylated and additionally cleaved by the protease DDI2 and the active fragment p110 migrates to the nucleus to induce transcription of proteasome subunit (PSM) genes. The activity of Nrf1 p110 in the nucleus is influenced by cofactors such as small Maf (sMaf) proteins, TIP60, FBW7α, GSK3, β-TrCP, and USP15. Although CK2 is depicted in the nucleus, the compartment where it phosphorylates NRF1 is currently unclear. Details and references are provided in the text.

(Grimberg *et al.*, 2011; Li *et al.*, 2011; Lehrbach and Ruvkun, 2016). In the case of mammals, this pathway is orchestrated by the transcription factor NRF1 (also called NFE2L1 or TCF11) of the CNC-bZIP family (Radhakrishnan *et al.*, 2010; Steffen *et al.*, 2010). Given that NRF1 starts out as an endoplasmic reticulum (ER)-anchored protein, its translocation to the nucleus, where it can act as a functional transcription factor, is accomplished via an elaborate activation pathway (Figure 1). In the following sections, we chart the mobilization of NRF1 from the ER to the nucleus via the cytosol and point to the various regulators that it encounters in these different cellular compartments. We also discuss how manipulating this NRF1-proteasome axis could be beneficial in the treatment of human diseases, such as cancer and neurodegeneration.

NRF1 IN THE ENDOPLASMIC RETICULUM
The full-length p120 precursor form of NRF1 is cotranslationally inserted into the ER via the classical Sec61-dependent pathway (Steffen *et al.*, 2010). ER-embedded NRF1 is oriented with the bulk of its polypeptide, including the C-terminus, residing in the ER lumen and a small portion of the N-terminus protruding into the cytosol (C_{lumen}/$N_{cytosol}$) (Wang and Chan, 2006; Zhang and Hayes, 2010; Radhakrishnan *et al.*, 2014). This type II membrane orientation is

facilitated by an N-terminal homology box-1 transmembrane domain (TMD) that is enriched with hydrophobic residues. Within some of its luminal domains, NRF1 undergoes extensive N-glycosylation, adding as much as 10–15 kDa to its final size. Under steady-state conditions, when proteasomes are active, NRF1 is subjected to ER-associated degradation (ERAD), a well-established pathway that specializes in the turnover of misfolded ER proteins (Wu and Rapoport, 2018). This involves ubiquitination of NRF1 by the ER-associated E3 ubiquitin ligase HRD1 and extraction from the ER membrane by the homohexameric AAA ATPase p97 (also known as valosin containing protein; VCP) to be degraded by the proteasome in the cytosol (Steffen *et al.*, 2010; Radhakrishnan *et al.*, 2014). However, under proteotoxic stress or proteasome inhibition, this retrotranslocated NRF1 is activated in the cytosol (described in the next section). Since ERAD is dedicated to degrading misfolded ER proteins, it is currently unclear how NRF1 is perceived as such. However, this seemingly futile cycle of synthesis and immediate destruction by the ERAD does ensure a short half-life for NRF1 (~12 min) and thus precludes additional synthesis of proteasomes when not actually necessary.

Another relevant question in this context is why NRF1 is localized to the ER in the first place. If NRF1 is simply a sensor for proteasome capacity, this could be achieved easily by a cytosolic or nuclear

localization where proteasomes are in abundance. In fact, the related CNC-bZIP transcription factor NRF2 that responds to oxidative stress is primarily localized in the cytosol, where it is subjected to constant degradation until it is needed in the nucleus (Ma, 2013). In the case of NRF1, there is some evidence suggesting its involvement in the ER stress response, partly justifying its presence in the ER. For instance, ER stress–causing agents tunicamycin and thapsigargin induce the expression of Herpud1 (a protein involved in ERAD) in a NRF1-dependent manner (Ho and Chan, 2015). Also, NRF1 protects the liver from ER stress via its ability to induce PSM genes (Lee et al., 2013). Despite these insights, it is unclear whether NRF1 can directly sense and defend against ER stress or whether its activation is simply a result of overloaded proteasomes in this context. Further studies are necessary to distinguish between these models. Perhaps the most convincing reason for NRF1's ER presence could be unrelated to its role in proteostasis. In a recent work, Hotamisligil and colleagues demonstrated that NRF1 functions as a cholesterol sensor at the ER and mediates an adaptive response designed to protect the ER from excess cholesterol exposure (Widenmaier et al., 2017).

NRF1 IN THE CYTOSOL

The fate of the p120 precursor form of NRF1 that emerges from the ER is decided in the cytosol. As mentioned above, it is either subject to degradation when proteasome capacity is adequate or activated and mobilized to the nucleus in the event of proteasome insufficiency. In the case of degradation, NRF1 is treated like a typical ERAD substrate and is transferred from the ATPase p97 to the proteasome. It is not clear whether there are one or more intermediate ubiquitin shuttle proteins involved in this transfer.

The activation of NRF1 that escapes degradation is more elaborate. One of the critical steps is the N-deglycosylation of NRF1 by the action of the enzyme N-glycanase 1 (NGLY1), a cofactor of p97 (Tomlin et al., 2017). An elegant study by Ruvkun and colleagues investigated this aspect in SKN1-A, the orthologue of NRF1 in *C. elegans*, and demonstrated that the importance of deglycosylation lies not in the removal of glycans per se, but rather the deamidation of the glycosylated-asparagine to aspartic acid (Lehrbach et al., 2019). Given the importance of acidic residues in the function of some transactivation domains (Triezenberg, 1995), the introduction of Asp residues, dubbed "protein sequence editing" in this case, is thought to enhance the transactivation potential of SKN1-A. Consistent with this notion, an earlier study indicated that mutation of potential N-glycosylation sites to Asp in NRF1 increased its transactivation ability (Zhang et al., 2014).

Another event important for NRF1 activation is cytosolic proteolytic processing to convert the precursor p120 into the active p110 form that is devoid of the N-terminal TMD. Our earlier work precisely delineated the cleavage site to be between Trp-103 and Leu-104, although the identity of the relevant protease remained unknown (Radhakrishnan et al., 2014). Sha and Goldberg (2014) proposed that proteasome could act as a site-specific protease to cleave and activate NRF1. However, we rigorously tested this hypothesis and demonstrated that NRF1 is processed in a proteasome-independent manner (Vangala et al., 2016). This controversy was put to rest when the aspartic protease DDI2 was reported to cleave and activate NRF1 (Koizumi et al., 2016). Likewise, the orthologous protease DDI1 was shown to process SKN-1A in *C. elegans* (Lehrbach and Ruvkun, 2016).

The identification of DDI2 as the relevant protease for NRF1 has led to further mechanistic understanding of this pathway. For instance, using DDI2-knockout cells, we showed that NRF1 is completely pulled out into the cytosol to be processed (Northrop

et al., 2020), in contrast to the previous model where NRF1 cleavage was thought to facilitate its release from the ER membrane (Radhakrishnan et al., 2014). This observation predicts a possible role for one or more unidentified chaperones that could transiently shield the TMD of NRF1 to prevent aggregation in the cytosol until the N-terminal domain with the TMD can be cleaved off by DDI2.

A recent report by Rapoport and colleagues (Yip et al., 2020) has important implications for our understanding of the DDI2-NRF1 axis. Using Ddi1, the yeast counterpart of mammalian DDI2, it was demonstrated that this protease recognizes and cleaves a model substrate only when it is tagged with an extraordinarily long ubiquitin chain (more than eight ubiquitins). The activity of the Ddi1 protease domain was dependent on the adjacent helical domain and stimulated by the N-terminal ubiquitin-like domain, which was found to mediate high-affinity interaction with the polyubiquitin chain. Thus, it is likely that under conditions of proteasome insufficiency, NRF1 is decorated with one or more long ubiquitin chains that enable its recognition and subsequent proteolytic cleavage by DDI2.

NRF1 IN THE NUCLEUS

The processed and active p110 form of NRF1 that arrives in the nucleus is subjected to regulation by a multitude of factors. Some of the earliest identified regulators of NRF1 are the bZIP domain–containing small MAF (MafF, MafG, and MafK) cofactors (Johnsen et al., 1996; Kim et al., 2016). Using its bZIP domain, NRF1 heterodimerizes with one of the small MAF proteins to bind the antioxidant response elements (ARE) found upstream of PSM and other target genes. More recently, our work pointed to the RUVBL1-containing TIP60 chromatin regulatory complex as a necessary cofactor for NRF1 in enabling its transcriptional activation of PSM genes in response to proteasome inhibition (Vangala and Radhakrishnan, 2019). Unlike the small MAFs that dictate NRF1's DNA binding, the effect of TIP60 is restricted to maintaining its transcriptional activity.

The protein level of NRF1 in the nucleus is regulated by the action of two different Cullin-RING ubiquitin ligases (CRLs) and perhaps offers a convenient mechanism to quench NRF1 activity when nuclear proteasome capacity is sufficient. Glycogen synthase kinase 3 (GSK3)-mediated phosphorylation of NRF1 in the phosphodegron domain promotes binding of the F-box protein Fbw7α, a nuclear-localized substrate-specifying component of the SCF (Skp1-Cul1-F-box protein)-type ubiquitin ligase, for ubiquitination and subsequent degradation of NRF1 via the proteasome (Biswas et al., 2011, 2013). In addition, SCF$^{\beta\text{-TrCP}}$ has been shown to recognize phosphorylated Ser residues in a DSGLS motif, leading to ubiquitination and degradation of NRF1 by the proteasome (Tsuchiya et al., 2011). The deubiquitinating enzyme ubiquitin-specific protease 15 (USP15), however, has been shown to counteract the effect of SCF$^{\beta\text{-TrCP}}$ by deubiquitinating and stabilizing nuclear NRF1 (Fukagai et al., 2016).

Apart from the N-linked glycosylation in the ER described above, NRF1 is also a substrate of O-linked glycosylation in the nucleus. In 2015, Chen et al. demonstrated O-linked N-acetylglucosamine transferase (OGT)-mediated O-GlcNAcylation of Nrf1 as a negative regulation, resulting in decreased protein stability and transcription factor activity, presumably due to increased ubiquitination, thereby promoting NRF1 degradation (Chen et al., 2015). However, two publications have since demonstrated that OGT-mediated O-GlcNAcylation, facilitated by the mutual binding partner host cell factor C1 (HCF-1), actually enhances NRF1 stability and transcription factor activity by disrupting the interaction between the CRL SCF$^{\beta\text{-TrCP}}$ and NRF1 to prevent ubiquitination and subsequent degradation (Han et al., 2017; Sekine et al., 2018). Overall, published

results on the regulatory effect of O-GlcNAcylation of NRF1, whether positive or negative, remain discordant.

Not surprisingly, like many transcription factors, NRF1 is also subject to regulation by phosphorylation. Casein kinase 2 (CK2)-mediated phosphorylation of NRF1 at residue Ser-497 has been shown to decrease the transcriptional activity of NRF1 (Tsuchiya et al., 2013). The precise mechanism behind this effect is unknown, but it was proposed that Ser-497 phosphorylation may induce a conformational change in NRF1 that compromises DNA binding (Tsuchiya et al., 2013). The cellular location of the CK2-mediated phosphorylation event remains elusive, as CK2 has been observed to exist in the nucleus and cytosol, as well as interacting with various organelles, including the ER membrane (Faust and Montenarh, 2000; Litchfield, 2003).

TARGETING THE NRF1-PROTEASOME AXIS TO TREAT HUMAN DISEASES

A number of diverse human diseases are thought to be due to dysregulation of proteasomal activity and the resultant aberrant protein degradation (Schmidt and Finley, 2014). For instance, cancer cells exhibit increased UPS activity, perhaps to provide a balance for their elevated protein synthesis needs and to promote procancer cellular activities (Kumatori et al., 1990; Chen and Madura, 2005; Bazzaro et al., 2006). Proteasome inhibition as a cancer therapeutic aims to exploit this dependence on proteasome activity to induce fatal proteotoxic stress (Deshaies, 2014). Proteasome inhibitors are currently used in the clinic to treat multiple myeloma (MM) and mantle cell lymphoma (MCL). However, even in patients who initially respond well to proteasome inhibitors, the development of drug resistance and disease relapse are far too common (Sherman and Li, 2020). Also, from a pharmacokinetic standpoint, rapid clearance of the proteasome inhibitor drugs from the blood of the patients is a major issue (Papandreou et al., 2004; Schwartz and Davidson, 2004; Wang et al., 2013; Albornoz et al., 2019). Given the central role of NRF1 in protecting cells from proteasome inhibition, it is conceivable that targeting this pathway could increase the efficacy of proteasome inhibitor drugs in MM and MCL and also enable expansion of this therapeutic modality to patients with other types of cancers, especially solid tumors. As is the case for most transcription factors, inhibiting NRF1 directly may not be easy. However, there are several enzymes in the pathway that play critical roles in NRF1 activation and could provide readily actionable targets. We and others have demonstrated the viability of this approach in cell culture and/or preclinical models using depletion/inhibition of NGLY1, p97, DDI2, RUVBL1, and TIP60 (Auner et al., 2013; Le Moigne et al., 2017; Tomlin et al., 2017; Vangala and Radhakrishnan, 2019; Northrop et al., 2020).

Conversely, there could be value in enabling the NRF1-proteasome axis in certain diseases. For example, neurodegenerative diseases, such as Alzheimer's, Huntington's, and Parkinson's diseases, are often marked by the accumulation of aggregate-prone proteins such as phosphorylated tau, huntingtin, and β-amyloid peptide, due to decreased proteasome-mediated protein degradation (Haass and Selkoe, 2007; Murphy and LeVine, 2010; Luk et al., 2012; Graham and Liu, 2017; Liu et al., 2017). While it is known that NRF1 is responsible for the proteasome bounce back response and is active when proteasome activity is diminished, there is emerging evidence to suggest that NRF1 is also responsible for regulating basal PSM gene expression in certain cell types, such as in neurons and hepatocytes (Lee et al., 2011, 2013). It was demonstrated that neuron-specific NRF1 knockout in mice caused impaired proteasome activity and neurodegeneration (Lee et al., 2011). Thus, boosting proteasome activity via manipulation of the NRF1 pathway could be an effective therapeutic strategy in these neurodegenerative diseases. Blocking the activity of negative regulators of NRF1, such as GSK3 and CK2, could be the key to such a strategy (Biswas et al., 2013; Tsuchiya et al., 2013).

CONCLUDING REMARKS

Until recently, the maintenance and expression of proteasome subunits was thought to be a housekeeping process in the cell. Mounting evidence now suggests that the expression of proteasome genes is tightly regulated and highly responsive to changes in the intracellular environment and protein degradation needs (Kwak et al., 2003; Radhakrishnan et al., 2010; Rousseau and Bertolotti, 2018; Thibaudeau and Smith, 2019).

Although the players and details differ, the basic transcriptional circuit that responds to proteasome insufficiency and thereby directs de novo proteasome synthesis is remarkably conserved in evolution and can be found in species ranging from yeast to humans. In the case of mammals, this function is fulfilled by the transcription factor NRF1. After its initial discovery, efforts to characterize the NRF1-proteasome axis has unraveled a complex pathway. Beginning its cellular journey embedded in the ER membrane, NRF1 is retrotranslocated into the cytosol, where it is either constitutively degraded by the 26S proteasome or mobilized through an activation pathway, culminating in the induction of proteasome genes to help restore diminished proteasome activity. There are a number of molecular regulators that aid NRF1 to fulfill its role as a transcriptional activator of proteasome genes. Fortunately, some of these regulators, such as p97, NGLY1, DDI2, RUVBL1, and TIP60, possess enzymatic activity that can be readily targeted to inactivate the NRF1 pathway. This could be a viable strategy to potentiate cytotoxic cancer cell killing by proteasome inhibitor treatments. On the other hand, we note a prominent paucity of druggable negative regulators of NRF1 function. Further identification of such factors could aid in the development of novel therapeutics that can be used to enhance the NRF1-proteasome axis in some neurodegenerative diseases where proteasome capacity is diminished.

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