INTRODUCTION

Within the past half-century, extensive efforts to overcome cancer, including DNA sequencing of human cancer tissues and bioinformatics analyses, have led directly to the characterization of significant functional mutations in genes and pathways in tumors and the development of new cancer therapies against these targets, in other words, precision medicine. These results further highlight...
that human tumors develop in a multistep process through sequentially acquiring the hallmarks of cancer, comprising at least 8 biological capabilities: sustained proliferative signaling, growth suppressor evasion, cell death resistance, replicative immortality, angiogenesis, invasion and metastasis, energy metabolism reprogramming, and immune destruction evasion. These hallmarks are endowed to cells by mutations of pivotal genes. Moreover, new concepts of gene mutations related to carcinogenesis have emerged: “driver gene mutations” and “passenger gene mutations.” Driver gene mutations confer selective growth advantages to tumor cells, and more than 100 driver genes, such as PIK3CA, SMAD4, and TP53, have been identified. Passenger gene mutations are considered to be just “passengers” and have no effect on the tumorigenic process.

The transcription factor NRF2, which plays crucial roles in cytoprotection against oxidative stress and electrophiles, is one of the cancer driver genes. NRF2 mediates the expression of genes involved in the oxidative stress response and metabolic reprogramming, such as glutaminolysis, and its functional activity is regulated by KEAP1, which is a dual functional protein that acts as both an oxidative stress sensor and a ubiquitin E3 ligase. Cancer cells depend, namely “addict” these biological functions of NRF2 for their aberrant growth. Accordingly, these insights also suggest that the KEAP1-NRF2 axis provides an attractive target for anticancer drug development, and numerous pharmaceutical companies are struggling to generate both NRF2 inhibitors and activators.

We recently discovered a new cancer addiction to NFE2L3, which is entirely different from NRF2. In this review, we summarize recent breakthroughs in understanding the physiological function of NRF3 in tumors, especially through ubiquitin-independent proteolysis by the 20S proteasome.

2 IDENTIFICATION OF NRF3 ADDICTION IN CANCER

2.1 Remarkable upregulation of the NRF3 gene in several cancer tissues

Human cancer databases, such as The Cancer Genome Atlas and Oncomine, strongly suggest the biological relevance of NRF3 in tumors because of the following 4 points:

1. A substantial number of cancer tissues, namely bladder urothelial carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, colon adenocarcinoma, lymphoid neoplasm diffuse large B-cell lymphoma, esophageal carcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma, kidney chromophobe, ovarian serous cystadenocarcinoma, PAAD, rectum adenocarcinoma, stomach adenocarcinoma, testicular germ cell tumors, thyroid carcinoma, thymoma, uterine corpus endometrial carcinoma, and uterine carcinosarcoma, show high upregulation of the NRF3 gene compared to adjacent normal tissues (Figure 1).

2. Although it is not currently considered as a driver gene, NRF3 is one of 127 significantly mutated genes among 12 cancer types.

3. High NRF3 expression is correlated with poor prognosis in PAAD, in terms of both overall survival and disease-free survival.

4. Moreover, recent evidence indicates that NRF3 regulates the growth and metastasis of thyroid, testis, and breast cancer.

NRF3, which was initially identified by our group, belongs to the CNC-type bZip transcription factor family, including NRF2 and NRF1 (NFE2L1). As described, NRF2 is well-known for its cytoprotective effects against oxidative stress, and NRF1 sustains protein homeostasis (e.g., proteostasis) by mediating proteasome gene expression following proteasome inhibition. These transcription factors augment gene expression by binding to the ARE in genes by heterodimerizing with small Maf proteins. Considering their protein structures and amino acid sequences, it is likely that NRF3 is a close homologue of NRF1 but not NRF2 (as discussed in Section 4).

Remarkable upregulation of the NRF3 gene in various cancer tissues (Figure 1). Intriguingly, remarkable NRF3 expression is induced in several types of cancer cells (Figure 1). This expression profile of NRF3 is clearly distinct from those of both NRF1 and NRF2, which are expressed ubiquitously and are not significantly upregulated in cancer cells. Regarding the role of NRF2, cancer cells activate their selective growth advantages mainly by somatic mutations of the NRF2 and KEAP1 genes, as well as aberrant posttranslational modifications and protein-protein interactions. These observations imply unique roles of NRF3 in tumors from those of NRF2 and NRF1.

The physiological function of NRF3 remains unclear, in part because Nrf3-deficient mice, which were generated independently by our and another laboratories, do not show apparent abnormalities under physiological conditions. In chemical carcinogenesis experiments, benzo[a]pyrene highly induces T-cell lymphoblastic lymphoma in Nrf3 KO mice, implying a protective role of Nrf3 in hematopoietic malignancies. We herein describe the new mechanisms in tumors in which NRF3 induces tumor growth and malignancy by degrading the tumor suppressor proteins p53 and Rb through activating 20S proteasome assembly.

2.2 Proteasome-mediated proteostasis in cancer

Before discussing the pathological roles of NRF3 in tumors, we would like to introduce the pivotal roles of the proteasome in cancer. Increasing evidence suggests that cancer cells have a heightened dependence on mechanisms of protein homeostasis (proteostasis) or protein quality control because cancer cells synthesize many WT and mutant proteins (Figure 2). To sustain proteostasis, the ubiquitin-proteasome system is activated for protein degradation mechanisms as well as autophagy. Thus, cancer cells are more susceptible to proteasome inhibition than normal cells, and proteasome
inhibitors, such as bortezomib, are consequently utilized to treat multiple myeloma and mantle cell lymphoma.\textsuperscript{29}

The proteasome in the ubiquitin-proteasome system is the 26S proteasome.\textsuperscript{29,30} The 26S proteasome is an unusually large complex that consists of 66 subunits expressed from 33 genes and is composed of 2 portions, the 20S proteasome and the 19S-RP: the 20S proteasome is a catalytic domain for proteolysis, and 19S-RP recognizes polyubiquitin chains conjugated to substrate proteins and unfolds their structure by using ATP for proteolysis through the 20S proteasome. The molecular mechanisms underlying proteasome gene expression in mammals remained obscure for a long time. Recently, several transcription factors have been reported to mediate gene expression.\textsuperscript{31} For example, NRF3-related NRF1 mediates the gene expression of most 26S proteasome subunits in response to proteasome inhibition\textsuperscript{32-35} (it is very confusing that “NRF1” stands for 2 distinct transcription factors, “NFE2L1” and “nuclear respiratory...
factor 1). This cellular response is called the “proteasome bounce-back response” (or proteasome recovery) and sustains proteostasis despite proteasome dysfunction. Similarly, NRF2 directly induces 26S proteasome gene expression under oxidative stress conditions, implying that it supports the degradation of proteins damaged by reactive oxygen species.36

### 2.3 NRF3-POMP-20S proteasome axis attenuates p53/Rb function by degrading in a ubiquitin-independent fashion

Let us return to the question of the physiological function of NRF3 in cancer cells. Based on the fact that NRF1 is a regulatory factor of 26S proteasome expression, we hypothesized that NRF3 also regulates 26S proteasome gene expression; however, no alteration of proteasome gene expression was observed in NRF3 knockdown cancer cells. Alternatively, NRF3 directly augments the expression of POMP as a chaperone for the assembly of the 20S proteasome complex (Figures 2 and 3).30 NRF3 and NRF1 complementarily regulate basal 26S proteasome activity through a translational mechanism in cancer

Although NRF3 does not likely contribute to proteasome gene expression, we found that both NRF1 and NRF3 complimentarily regulate the basal gene expression of the 26S proteasome in cancer cells.39

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FIGURE 2 Imbalance of proteostasis in cancer cells. Top panel, In normal cells, the 26S proteasome sustains protein homeostasis (ie, proteostasis) by degrading protein in a ubiquitin-dependent manner.29 In cancer cells, high levels of normal or mutated proteins are expressed for higher proliferation, consequently causing an imbalance where the degradation load exceeds the ubiquitin-proteasome system capacity. Bottom panel, Molecular mechanisms of the assembly of the 20S and 26S proteasome complexes. The proteasome maturation protein (POMP) chaperone promotes the assembly of the 20S proteasome along with PAC1-4 chaperones. The 26S and 20S proteasomes degrade proteins in a ubiquitin (Ub)-dependent and Ub-independent manner, respectively.30 PAC, proteasome assembly chaperone; Rpn, regulatory particle non-ATPase; Rpt, regulatory particle triple-A protein.
Intriguingly, NRF3 suppresses NRF1 function at the translational level (Figure 3). The underlying molecular mechanism is due to NRF3-mediated gene induction of CPEB3, which is known to mediate protein translation both positively and negatively by regulating mRNA polyadenylation.\(^{40,41}\) NRF3 augments CPEB3 gene expression by binding directly to a species-conserved ARE site in the gene. Consequently, induced CPEB3 represses NRF1 translation through a cytoplasmic polyadenylation element in the 3′-UTR of the NRF1 gene. This finding could explain why Nrf3 null mice do not show apparent abnormalities, because Nrf1 could rescue the loss of Nrf3 function in mice.\(^{26,27}\) Moreover, the existence of a mechanism to switch from NRF1 to NRF3 might suggest that cancer cells favor NRF3 rather than NRF1, or different function of NRF3 from that of NRF1 in transcription is indispensable for sustaining aberrant growth of cancer cells, irrespective that they share similar protein structures.

2.5 Other transcriptional networks of NRF3 in tumors

To comprehensively understand the physiological roles of NRF3 in tumors, genome-wide identification of NRF3 target genes is indispensable. To this end, we carried out ChIP sequence analyses along with transcriptome analyses using a microarray, and we discovered that the glucose transporter GLUT1 (SLC2A1) is one of the direct NRF3 target genes (Figure 3).\(^{42}\) Consistent with our findings, cancer cells reprogram the metabolic status from oxidative phosphorylation to anaerobic glycolysis by upregulating certain genes, including GLUT1 and GLUT3 (SLC2A3), to increase glucose uptake, which correlates with poor prognosis.\(^{43-45}\) This metabolic reprogramming of cancer cells is called the Warburg effect. Considering that ARE sites in the GLUT1 gene perfectly match the consensus sequence and are highly conserved among several species, it would be possible that NRF1 and NRF2 also regulate the gene expression. Indeed, we observed NRF1-mediated regulation under certain conditions. Furthermore, NRF3 has been recently reported to upregulate VEGFA expression, promoting angiogenesis and malignancy in pancreatic cancer.\(^{36}\) Researchers also found NRF3 involvement in the invasion and metastasis of pancreatic cancer. Although we previously reported UHMK1 as an NRF3 target gene,\(^{46}\) it might be regulated indirectly by NRF3 because there are no functional ARE sites in the gene.

2.6 Upregulation of NRF3 by β-catenin-TCF4 in cancer cells

We next addressed the molecular mechanisms underlying NRF3 upregulation in cancer cells and identified that the β-catenin-TCF4 complex in the Wnt signaling pathway.\(^{47}\) As a result, NRF3 augments the expression of several genes, such as POMP, 26S proteasome subunits, CPEB3, and GLUT1, in tumors. Bottom panel, Genetic alterations associated with colorectal tumorigenesis. Loss of APC is an initial event of tumorigenesis, thus activating β-catenin in the Wnt signaling pathway.\(^{3}\) Rb, retinoblastoma; VEGFA, vascular endothelial growth factor A
complex in the Wnt pathway directly promotes NRF3 expression.42 The Wnt-β-catenin pathway, which is essential for normal intestinal growth and development, plays a key role in the initiation of colorectal cancer progression.47-49 Alterations of the APC gene and the CTNNB1 gene encoding β-catenin are initial events leading to the development of adenoma in most sporadic cases (Figure 3). As a result, β-catenin protein stabilization promotes the expression of stemness-related genes, such as c-MYC, AXIN2, and LGR5, along with TCF/LEF family proteins, leading to hyperplastic epithelium.45,50,51 These insights give rise to the attractive hypothesis that NRF3 cancels the tumor suppressor function of p53 by activating the 20S proteasome following tumorigenesis initiation. Initially, acquiring resistance to apoptosis through gene mutations in tumor suppressors is essential for tumorigenesis, and p53 gene mutations occur at the last step in colon cancers.3

3 | MOLECULAR REGULATION OF THE BIOLOGICAL FUNCTION OF NRF3

3.1 | Multiple suppression mechanisms of the biological function of NRF3

Our findings regarding the deleterious effects of aberrant NRF3 activation in tumors strongly indicate that NRF3 activity (and probably its expression [Figure 1]) must be tightly regulated to sustain cellular homeostasis. Under physiological conditions (quiescent conditions), NRF3 function is suppressed by sequestration in the ER through the N-terminal NHB1 domain, and its proteasomal degradation is mediated by the ER-associated degradation-related E3 ubiquitin ligase HRD1 (Figure 4).46 In the nucleus, NRF3 is subjected to alternate proteasomal degradation by β-TRCP, which is an adaptor of Cul1-based E3 ubiquitin ligase. It has also been

![Figure 4](Distinct regulatory mechanisms of NFE2-related factor 3 (NRF3) and NRF2. Top panel, Schematic structures of NRF3, NRF1 and NRF2. Middle panel, Under quiescent (physiological) conditions, the transcriptional activity of NRF3 is suppressed by sequestration in the endoplasmic reticulum (ER) and HRD1-mediated proteasome degradation. Under stimulation conditions or in NRF3-addicted cancer, NRF3 is liberated from multiple suppression mechanisms and processed by the DNA damage-inducible protein 1 homolog 2 (DDI2) protease, thereby achieving liberation from the ER and activating gene expression in the nucleus.46 Bottom panel, Regulatory mechanisms of the oxidative stress response transcription factor NRF2 are completely different from those of NRF3. Under quiescent conditions, NRF2 function is suppressed by a KEAP1-mediated proteasomal degradation mechanism.9-12 The nature of the oxidative stress response by NRF2 is the derepression of this mechanism.62 β-TRCP, beta-transducin repeat containing E3 ubiquitin protein ligase; ARE, antioxidant response element; KEAP, Kelch-like ECH-associated protein 1; NHB, N-terminal homology box; sMaf, small Maf; Ub, ubiquitin; VCP, valosin-containing protein.
reported that NRF3 is degraded in a glycogen synthase kinase 3β-dependent manner by Fbw7, which is a famous cancer driver and adaptor of Cul1-based ubiquitin ligase.52 These 2 independent degradation systems should suppress the biological function of NRF3 under quiescent conditions to prevent its detrimental effects.

3.2 | Nuclear translocation of NRF3 and NRF1 requires DDI2 protease

The nuclear entry of NRF3 is suppressed by multiple mechanisms in the ER under physiological conditions. Thus, the molecular mechanisms underlying nuclear translocation play a fundamental role in NRF3 functional regulation (Figure 4). Regarding this hypothesis, new insight into the nuclear translocation of NRF1 and Skn1 has led to a major breakthrough. Aspartic proteases DDI2 and DDI1 have been discovered as modulators of their nuclear translocation, respectively.53,54 Similarly, DDI2 is required for NRF3 nuclear translocation.46 Although there is still no experimental evidence that DDI2 directly cleaves NRF3, an AWLVH motif, which is considered a DDI2 cleavage site in the NHB2 domain, is highly conserved among several species and in NRF1. This evidence strongly implies that DDI2 could cleave these sites, resulting in their nuclear entry.

The molecular mechanisms through which DDI2 is activated for processing NRF3 and NRF1 remain obscure. We surmise the presence of certain signals and/or stress promote processing by escape from multiple suppression mechanisms in the ER. This corresponds to oxidative stress in the case of NRF2. Despite artificial stress, proteasome inhibition by MG132 and bortezomib is established as an oxidative stress response, implying the presence of this signal in these cancer cells. Endogenous NRF3 in human colon cancer DLD-1 and HCT116 cells partly enters into the nucleus, indicating the presence of this signal in these cancer cells.46 Delineating this process, as well as identifying a sensor for the signal, is indispensable for a comprehensive understanding of NRF3 function in cancer.

3.3 | Molecular bases underlying functional diversity among NRF1-3

These observations remind us of the functional diversity among NRF1, NRF2, and NRF3: NRF1 and NRF3 are involved in the regulation of proteasome function, whereas NRF2 is involved in the regulation of oxidative stress response. These transcription factors are thought to have evolved from a common ancestral gene CNC in Drosophila and Skn-1 in Caenorhabditis elegans.55 For example, they possess similar CNC-type bZip domains for DNA binding activities, implying that they likely recognize similar DNA sequences and thereby share some target genes, eg, proteasome subunit genes. Nevertheless, the existence of either NHB1/2 domains in NRF1/3 or a Neh2 domain in NRF2 could apparently result in substantial functional differences among these factors because the former is a regulatory interface with the HRD1-DDI2 axis, whereas the latter is related to KEAP1 (Figure 4). Considering that CncC and Skn-1a possess both NHB1/2 and Neh2 domains, we surmise that distributing each domain to NRF1-3 transcription factors in the process of molecular evolution could have led to complexities and diversities in these transcription factors and thereby vertebrates.

3.4 | DDI2 inhibitors might be promising anticancer drugs that suppress NRF3

Identification of the DDI2-mediated NRF3 activation mechanism suggests that DDI2 inhibitors would be promising anticancer drugs to inhibit NRF3 function in tumors. Intriguingly, DDI2 possesses a protease domain similar to that of HIV protease.56 Accordingly, it might be possible to repurpose clinically available HIV protease inhibitors as DDI2 inhibitors. Suppression of DDI2 likely impairs both NRF3 and NRF1 function because DDI2 is required for their nuclear entry.56,57 Alternatively, DDI2 inhibitors might enhance the treatment effect of the anticancer drug bortezomib, which is utilized for multiple myeloma treatment by targeting proteasomes, because both NRF3 and NRF1 appear to reduce bortezomib efficacy through a proteasome bounce-back response.

4 | CONCLUSIONS: PERSPECTIVE ON FUTURE DIRECTIONS OF NRF3 RESEARCH

Human cancer databases indicate that the biological function and regulatory mechanisms of numerous genes in tumors are still not defined. Moreover, these insights shed light on new issues regarding genetic diversity in each tumor, ie, intratumoral and intertumoral heterogeneity.57,58 Thus, continuous investigation of unknown mechanisms underlying tumorigenesis remains indispensable for the development of new cancer therapies and precision medicine.

In this regard, we have succeeded in the identification of new roles of the NRF3-POMP-20S proteasome axis in cancer cells. This axis eliminates the tumor suppressor function of p53 and Rb in a ubiquitin-independent manner, thus promoting tumor growth (Figure 3). Conversely, our findings indicate that inhibition of this axis does not likely reduce the proliferation of p53-deficient tumors, which are approximately half of all human solid tumors.59 Thus, targeting strategies for NRF3 or DDI2 might be suitable for leukemia, in which many blood cells have the intact p53 gene.60 In particular, although the proteasome inhibitor bortezomib is utilized clinically for multiple myeloma therapy, it is assumed that both NRF3 and NRF1 likely confer resistance to this anticancer drug through a “proteasome bounce-back response” in cells. To increase the therapeutic effects of bortezomib, it might be beneficial to cotreat with a DDI2 inhibitor that likely represses both NRF3- and NRF1-mediated bounce-back
responses. We surmise that the advantage of therapies targeting NRF3 is few side-effects because Nrf3 KO mice do not show apparent abnormalities.6,27

We identified that NRF3 promotes 20S proteasome assembly by inducing POMP expression. The biological function of the 20S proteasome itself might be still controversial because there are only some in vitro studies that report its function.37 Alternatively, the 20S proteasome is considered to be an intermediate form that ultimately generates 26S or other forms of proteasomes by accompanying accessory factors such as 19S-RP. Therefore, we do not deny the possibility that our observation reflects the biological effects of the 20S proteasome and other accessory factors. Even so, it remains correct that the NRF3-POMP-20S proteasome axis modulates tumor growth and malignancy in a ubiquitin-independent manner. To establish our findings, we need to undertake the following experiments: compare 20S proteasome activities in many types of cancer cells, comprehensively identify 20S proteasome substrates, and determine the molecular mechanisms of substrate-specific recognition independent of the ubiquitin chain.

One of the remaining important questions in this research field is the physiological role of NRF3 in normal cells. This question is equivalent to another issue regarding the NRF3 activation signals and/or stress inducers under physiological conditions. To answer the essential question, the identification of whole NRF3 target genes would provide us with much insight. Indeed, we have succeeded in the identification of several NRF3 target genes by combining data from ChIP sequencing and transcriptome analyses (these data will be published in the future). These NRF3 target genes are involved in various biological mechanisms, including the mTORC1 signaling pathway and cholesterol metabolism, consistent with NRF3-mediated tumor growth. According to these insights, certain nutrients or metabolites might regulate NRF3 function.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest for this article.

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