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Ph.D. Dissertation of Pharmacy

ARD1 Stabilizes NRF2 through Direct Interaction: Implications for Colon Cancer Progression

ARD1 단백질과 산화·환원 민감성 전사인자 NRF2와의 상호작용이 대장암 진행에 미치는 영향 및 기전연구

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College of Pharmacy
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ARD1 Stabilizes NRF2 through Direct Interaction: Implications for Colon Cancer Progression

Under the supervision of Professor Young-Joon Surh

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ABSTRACT

ARD1 Stabilizes NRF2 through Direct Interaction: Implications for Colon Cancer Progression

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Under the supervision of Professor Young-Joon Surh
at the College of Pharmacy, Seoul National University

Aberrant overactivation/overexpression of NRF2 is implicated in tumor progression, which has been largely attributed to its mutation as well as inactivation of the inhibitory protein, KEAP1. However, alternative mechanisms responsible for sustained activation of NRF2 are less understood. Here, I showed that ARD1 with the acetyltransferase activity is a new regulator of NRF2. Elevated levels of ARD1 and NRF2 were detected in human colon tumor tissues as well as human colon cancer cell lines. Knockdown of both ARD1 and NRF2 in human colon cancer HCT-116 cells

suppressed the oncogenicity of these cells. Furthermore, ARD1 knockdown in human colon cancer cells significantly reduced the protein levels of NRF2 without affecting its mRNA expression; however, silencing of NRF2 did not alter ARD1 protein expression. In addition, these two proteins were co-localized and physically interacted with each other both in human colon cancer cells and human colon tumor tissues. Mechanistically, ARD1 overexpression increased the acetylation levels of NRF2. Moreover, the *in vitro* acetylation assay and mass spectrometric analysis demonstrated that ARD1 directly acetylated NRF2. Ectopic expression of mutant forms of ARD1 with defective acetyltransferase activity reduced the half-life of NRF2. In conclusion, ARD1 may potentiate the oncogenic function of NRF2 in human colon cancer by acetylating and stabilizing this transcription factor.

Key words

Colorectal cancer; NRF2; ARD1; Posttranslational modification; Acetylation

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Table of Contents

List of figures

Chapter I

Chapter II

List of figures

List of abbreviations

ARD1	Arrest defective 1
ARE	Antioxidant response element
AMPK	AMP-activated protein kinase
BTB/POZ	Bric-à-brac, tramtrac, broad-complex/proxvirus zinc finger
β-TrCP	β-transducing repeat-containing protein
CRC	Colorectal cancer
CHX	Cycloheximide
CNC	cap'n'collar
CUL	Cullin
CBP	cyclic AMP response element-binding protein
CK2	Casein kinase 2
CDK5	Cyclin-dependent kinase 5
CRIF1	CR6-interacting factor 1
DDB	damaged DNA binding protein
DAPI	4′,6-diamidino-2-phenylindole
DGR	Double glycine repeat
dNTPs	Deoxyribonucleotide triphosphates
dNTPase	dNTP triphosphohydrolase
FBS	Fetal bovine serum
GSK-3	Glycogen synthase 3
H_2O_2	hydrogen peroxide
HCC	hepatocellular carcinoma
HATs	histone acetyltransferases
Hsp	Heat shock protein
HIF-1α	Hypoxia-inducible Factor 1α
ICC	Immunocytochemical analysis
IVIS	In vivo imaging system
IHC	Immunohistochemical analysis

IVR	intervening region
JNK	c-Jun N-terminal kinase
KAT	lysine acetyltransferase
KDAC	lysine deacetylase
KEAP1	Kelch-like ECH associated protein 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	mass spectrometry
MAPKs	Mitogen-activated protein kinases
MOF	Males absent on the first
PTMs	Posttranslational modifications
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PML-NBs	promyelocytic leukemia-nuclear bodies
PRMT1	protein arginine methyltransferase-1
PGK1	phosphoglycerate kinase 1
qPCR	Real-time polymerase chain reaction
ROS	Reactive oxygen species
RXRα	retinoid X receptor alpha
RBX	RING-box protein
RNF4	RING finger protein 4
Runx2	Runt-related transcription factor 2
siRNA	Short interfering RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SD	Standard deviation
SAMHD1	SAM domain and HD domain-containing protein 1
sMAF	small musculoaponeurotic fibrosarcoma
SIRT1	sirtuin 1
SIRT2	sirtuin 2
SUMO	small ubiquitin-like modifier
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	Standard deviation
SAMHD1	SAM domain and HD domain-containing protein 1
sMAF	small musculoaponeurotic fibrosarcoma
SIRT1	sirtuin 1
SIRT2	sirtuin 2
SUMO	small ubiquitin-like modifier
tBHQ	tert-butylhydroquinone
TBST	Tris-buffered saline containing 0.1% tween 20
MSRA	Methionine sulfoxide reductase A
NAT	N-terminal acetyltransferase
NDAC	N-terminal deacetylases
WDR	β-transducin repeats protein

Chapter I

ARD1-mediated Lysine Acetylation as a Novel Post-translational Modification of NRF2

1. Introduction.

Reactive oxygen species (ROS) are several derivatives of molecular oxygen, formed by-products of aerobic metabolism, including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical, and are the driving force of cellular biological activity [1]. A moderate elevation in ROS promotes cell proliferation and diferrentiation [2, 3]; however, aberrant overproduction of ROS induces oxidative damage to lipids, DNA, and proteins [4, 5]. Thus, maintaining the balance of intracellular ROS levels is essential for maintenance of cellular redox homeostasis and effective prevention/treatment of oxidative stress-associated human disorders [1-5].

The transcription factor, nuclear factor erythroid 2–related factor 2 (NRF2) is the central orchestrator in maintaining cellular redox homeostasis and antioxidative defense. In response to ROS-induced oxidative stress and other noxious stimuli, NRF2 is rapidly released from its negative regulator, Kelch-like ECH-associated protein 1 (KEAP1), which increases nuclear translocation of NRF2 and triggers transcription of its target genes encoding mainly antioxidant enzyes and other cytoprotective proteins [6]. Multiple lines of evidence support that the deficiency or reduced expression/activity of NRF2 can contribute to a variety of diseases, including aging [7], cardiovascular diseases [8-11], respiratory diseases [12-14], and neurodegenerative diseases [15-18].

However, abnormally elevated expression/activity of NRF2 has been frequently observed in many different types of human malignancies [19-24] Aberrant NRF2 activity/expression is attributable to various mechanisms, such as KEAP1-dependent [25] or β-transducing repeat-containing protein (β-TrCP)-mediated [26] ubiquitin proteasomal degradation, genomic alterations [27-32], interference of NRF2-KEAP1 interaction by NRF2 binding proteins [33-39], microRNA-mediated regulation [40], and post-translational modifications (PTMs) [41-43].

PTMs play a pivotal role in various cellular processes, and they are involved in modulating almost all cellular biological functions, as well as gene expression, intracellular signal transduction, protein-protein interactions, cell-cell interactions, and communication between intracellular and extracellular surroundings [44]. NRF2 harbors a distinct set of conserved amino acids that can be regulated by various PTMs, including phosphorylation, ubiquitylation, acetylation, methylation, SUMOylation, etc. These PTMs may influence the process of stability and cellular localization of NRF2, its binding to the antioxidant response element (ARE), and target genes transcription. Thus, a thorough understanding of NRF2 PTM is extremely useful to develop precise therapeutic strategies for treating and preventing diseases caused by dysregulated activity/expression of this redox-sensitive transcription factor.

Arrest defective 1 (ARD1), also known as N-α-acetyltransferase 10, is an N-terminal acetyltransferase (NAT) that is a catalytic subunit of N-acetyltransferase complex A; it transfers the acetyl group of acetyl coenzyme A (acetyl-CoA) to the free α-amino group at the N-terminal end of nascent peptides in a co-translational manner (**Figure 1A**) [45-47]. Additionally, ARD1 has lysine acetyltransferase (KAT) activity, which acetylates the ε-(internal)-lysine residues of target proteins (**Figure 1B**) [48]. Through N-terminal and internal lysine acetylation, ARD1 plays crucial roles in various physiological processes, including cell cycle modulation [49], autophagy [50], migration [51], apoptosis [52], cellular homeostasis [53], etc.

ARD1 has also been shown to be linked to pathogenesis of several diseases, such as Ogden syndrome [54-56], Alzheimer's disease [57], and Huntington's disease [58]. Furthermore, increased ARD1 expression has been attributed to various types of human malignancy, such as colon [59], liver [60], and breast cancer [61]. A worse prognosis for cancer patients has been associated with elevated ARD1 expression [62]. On the other hand, it has also been reported that ARD1 inhibits mTOR signaling to reduce carcinogenesis [63].

In this chapter, I summarize the PTMs of NRF2 based on the various modification sites within each structural domain of NRF2 and describe the relevant biological functions. Additionally, I provide a general review of

biological functions of ARD1 in response to oxidative stress and the various cellular processes regulated by its intrinsic acetyltransferase activity.

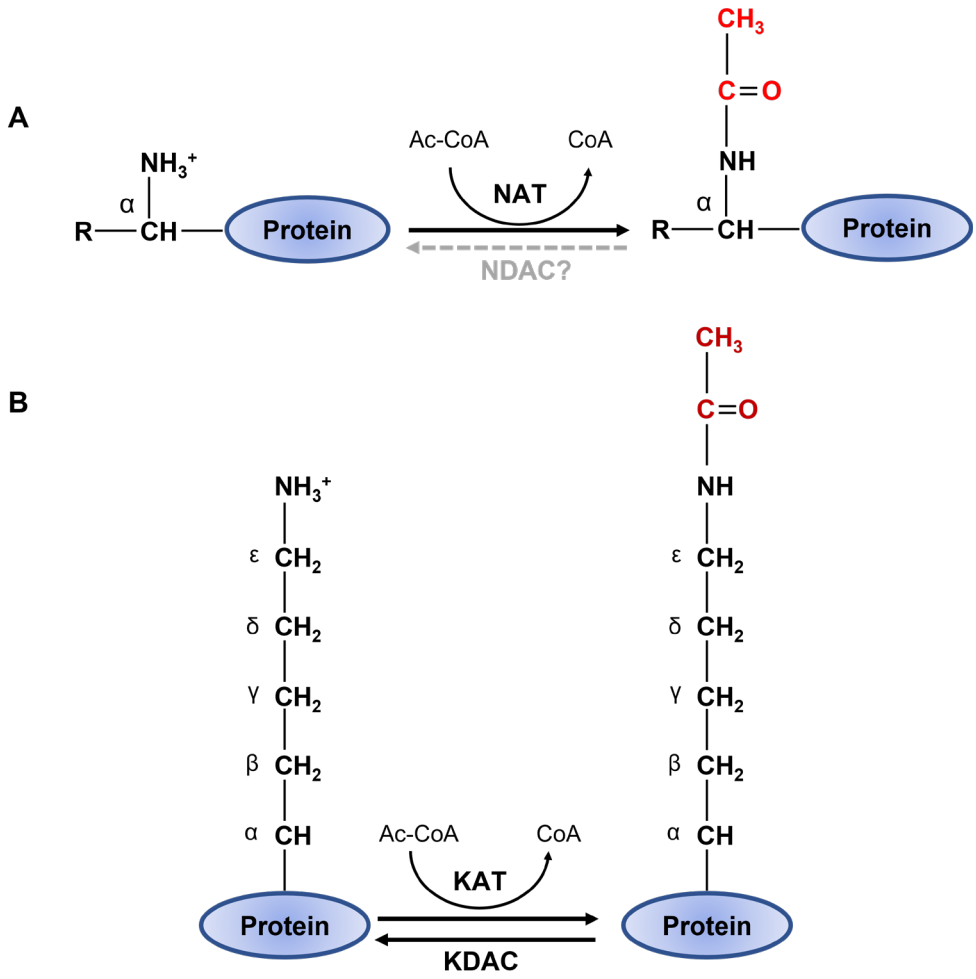

Figure 1. N-Terminal acetylation and internal lysine acetylation by ARD1.

(A) ARD1 has N-terminal acetylation activity. ARD1 transfers the acetyl group of acetyl-CoA (red) to the free α-amino group at the N-terminal end of nascent peptides in a co-translational manner. (B) ARD1 has lysine acetyltransferase activity, which acetylates the ε-(internal)-lysine residues of target proteins in a posttranslational manner. Acetylated lysine can be deacetylated by lysine deacetylase (KDAC), but N-terminal deacetylases

(NDACs) have not yet been reported. NAT, N-terminal acetyltransferase; KAT, lysine acetyltransferase; KDAC, lysine deacetylase

I. NRF2

1. NRF2 protein

NRF2, a member of the human cap'n'collar (CNC) basic-region leucine zipper transcription factor family, was first identified in 1994 [64]. It consists of seven functional NRF2-ECH homology (Neh) structural domains (Neh1 to 7) that have different functions (**Figure 2**). Under unstressed conditions, the basal protein levels of NRF2 are typically low, mostly due to KEAP1-Cullin (CUL) 3-RING-box protein (RBX)1 complex-mediated proteasomal degradation [25]. KEAP1 acts as a substrate adaptor protein that binds to NRF2 as a dimer through its C-terminal Kelch domain with the DLG and ETGE motifs present in the Neh2 domain of NRF2, and recruits the CUL3-E3 ubiquitin ligase, leading to ubiquitin-mediated proteasomal degradation of NRF2 [25, 65]. Moreover, the intracellular protein levels of NRF2 are also regulated by ubiquitylation and proteasomal degradation mediated by other E3 ubiquitin ligase complexes: the β-TrCP-S-phase kinase-associated protein-1 (SKP1)-CUL1-RBX1 [26], the CR6-interacting factor 1 (CRIF1) [66], Hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (HRD1; also known as Synovioloin), an E3 ubiquitin-protein ligase involved in endoplasmic reticulum-associated degradation (ERAD) [67], and the β-transducin repeats protein (WDR) 23-CUL4-damaged DNA binding protein (DDB) 1 complex [68]. Upon exposure to

diverse stresses such as ROS, electrophiles, oncogenic signaling, genetic mutations, inflammatory cytokines, or autophagy dysregulation, NRF2 degradation is rapidly reduced along with a concomitant increase in its nuclear translocation. In the nucleus, NRF2 heterodimerizes with small musculoaponeurotic fibrosarcoma (sMAF) proteins (F, G, and K) through its CNC-bZIP region in the Neh1 domain, and binds to ARE on the promoter region of target genes [69, 70]. The resulting expression of NRF2 target proteins regulate various cellular processes, including antioxidant defense, inflammation, energetic metabolism, iron metabolism, amino acid metabolism, cell growth and differentiation, and detoxification of harmful substances [71, 72].

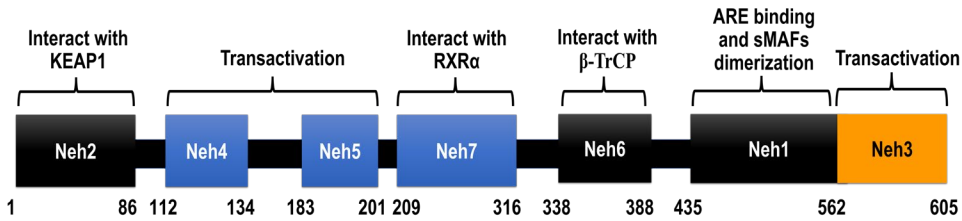

Figure 2. Domain structures of the NRF2 protein.

The Neh2 domain of NRF2 interacts with the KEAP1, which promotes KEAP1-meidated ubiquitylation of NRF2 for proteasomal degradation. The Neh4, Neh5, and Neh3 domains function as transactivation domains, regulating the transcriptional activity of NRF2. The Neh7 domain has been found to interact with the retinoid X receptor alpha (RXRα) [73]. The Neh6 domain interacts with the β-TrCP and promotes β-TrCP-meidated ubiquitylation of NRF2 for proteasomal degradation. The Neh1 domain is essential for the binding of NRF2 to ARE and its ability to form heterodimers with sMAFs.

2. Phosphorylation of NRF2

PMT via phosphorylation is a typical chemical reaction in which a protein is modified by the addition of a phosphate group to the polar group of amino acids [74-77]. This process is carried out by enzymes, collectively called kinases, and can be reversible or irreversible depending on the specific enzyme and reaction conditions. Phosphorylation renders a protein more or less stable, either by preventing or promoting its degradation. NRF2 consists of 605 amino acids (human) or 597 amino acids (mouse), and the presence of multiple serine, threonine, and tyrosine residues allows for its phosphorylation by a variety of protein kinases.

2.1. Protein kinase C (PKC)

NRF2 protein has been found to be phosphorylated by PKC in HepG2 human hepatocellular carcinoma cells [78]. Inactivation of PKC with phorbol 12-myristate 13-acetate (PMA) increased NRF2 activity, but this effect was blocked by the PKC inhibitor staurosporine. Furthermore, treatment with either *tert*-butylhydroquinone (tBHQ) or PMA was found to promote PKC-induced NRF2 phosphorylation and enhance NRF2 nuclear localization [78]. Site-directed mutagenesis was conducted to examine the role of PKC in NRF2 activation [41]. According to this study, PKC directly phosphorylates serine (S) 40 within the Neh2 (amino acids 1-86) structural domain of NRF2, which facilitates the dissociation of NRF2 from its repressor KEAP1 [41]. Like the majority of other proteins, the degradation

of NRF2 occurs through ubiquitin-dependent proteasomal degradation, and phosphorylation of NRF2 increases its stability and its transcriptional activity (**Figure 3B**) [79]. PROSITE search identified seven potential sites within the mouse NRF2 that could be phosphorylated by PKC [80]. In this study, PKC-mediated phosphorylation of NRF2 at the serine 40 residue, could prevent the degradation of NRF2 by KEAP1 in HepG2 cells. However, this phosphorylation did not affect the nuclear translocation of NRF2 or the transcriptional regulation of ARE-mediated NAD(P)H:quinone oxidoreductase 1 expression [80]. The PKC family is composed of serine/threonine kinases with a variety of isoforms including classic isozymes (PKCα, PKCβ, and PKCγ), novel PKC isozymes (PKCδ, PKCε, PKCη, PKCθ), and atypical PKC isozymes (PKCζ, and PKCι) [81]. Numazawa and colleagues reported that the phosphorylation of NRF2 at the S40 residue by atypical PKC-iota (ι) is necessary for the nuclear translocation and activation of NRF2 [82]. Involvement of other PKC isozymes in NRF2 phosphorylation merits further investigation.

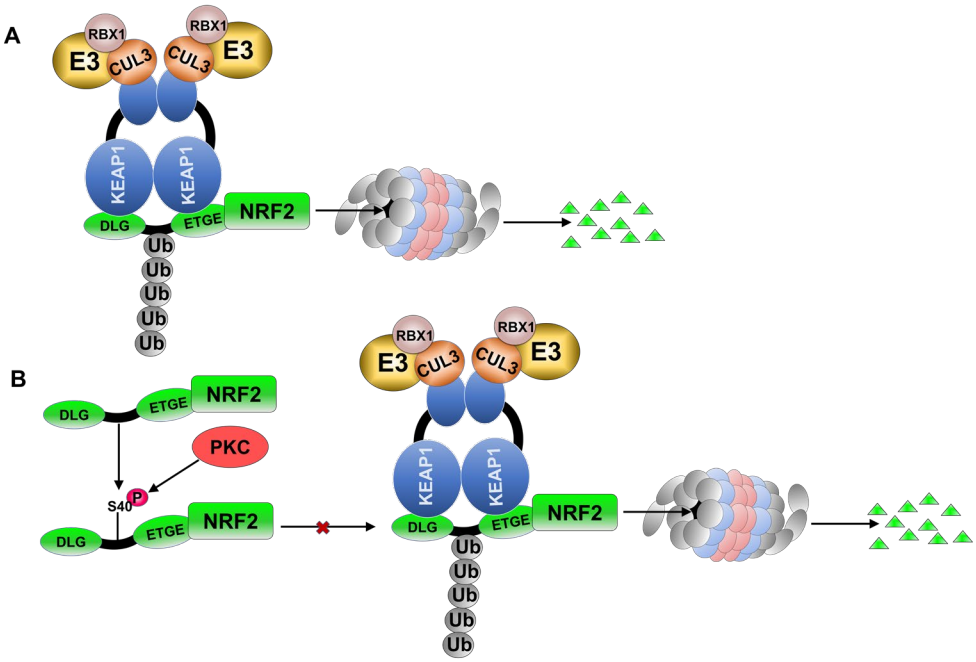

Figure 3. Schematic diagram of NRF2 degradation.

(A) Schematic diagram of the KEAP1-mediated degradation of NRF2. Under normal physiological conditions, NRF2 binds to KEAP1 through its ETGE and DLG motifs, leading to its ubiquitylation and subsequent degradation by the proteasome. (B) Schematic diagram of the PKC-mediated phosphorylation of NRF2 regulates its activity. PKC-mediated phosphorylation of NRF2 at the S40 residue within the Neh2 domain disrupts the interaction between NRF2 and KEAP1, leading to the inhibition of KEAP1-mediated proteasomal degradation of NRF2.

2.2. Casein kinase 2 (CK2)

Pi and colleagues reported that CK2 phosphorylated NRF2 [83]. Later, Apopa and colleagues demonstrated that CK2 phosphorylated NRF2 at multiple sites within its Neh4 (amino acids 112–134) and Neh5 (amino acids 182–200) domains using a PROSITE search and the *in vitro* CK2 kinase assay [42]. They found that treatment with tBHQ increased the CK2-mediated phosphorylation of NRF2, leading to its nuclear accumulation [42]. However, this effect was blocked by CK2 inhibitors such as 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole and 4,5,6,7-tetrabromobenzotriazole [42, 83].

2.3. Glycogen synthase 3 (GSK-3)

Salazar et al. showed that NRF2 could be directly phosphorylated by GSK-3β [84]. GSK-3β mediated-phosphorylation of NRF2 led to the inhibition of NRF2 target gene expression and decreased cell survival when challenged with ROS, such as H_2O_2 [84]. This study demonstrated that GSK-3β could alter the location of NRF2 within cells: for instance, GSK-3β caused NRF2 to accumulate in the cytoplasm [84]. Moreover, treatment with GSK-3 inhibitors, such as lithium chloride and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione, enhanced NRF2 target genes expression [84]. Rada et al. found that GSK-3-mediated phosphorylation of DSGIS motif (the Neh6 domain; mouse NRF2) linked NRF2 to β-TrCP, which promoted KEAP1-independent proteasomal degradation of NRF2 [85]. In

this research, both the GSK-3α and GSK-3β isoforms participated in the GSK-3-induced degradation of NRF2 [85]. In another study, GSK-3β phosphorylated S343 and S346 of human NRF (equivalent to murine S335 and S338) within the DSGIS motif, allowing the Neh6 domain to interact with the WD40 domain of β-TrCP [86] (**Figure 4**).

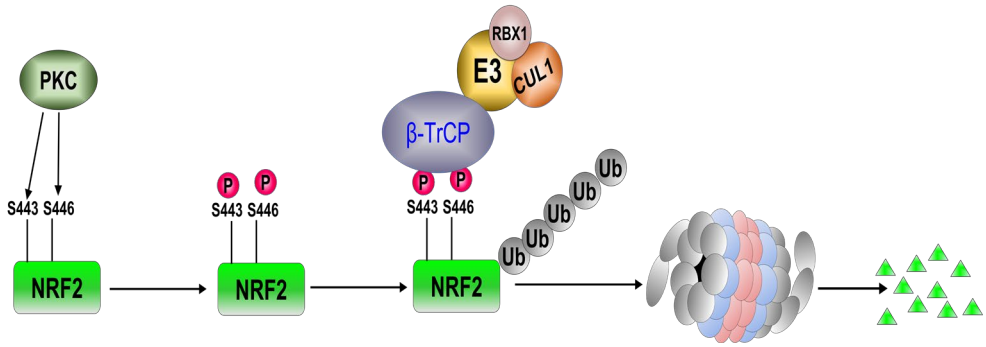

Figure 4. Schematic diagram of the PKC-mediated phosphorylation of NRF2.

The regulation of NRF2 antioxidant activity by GSK-3 is mediated through phosphorylation of NRF2 at serine residues 335 and 338 within the Neh6 domain by GSK-3β. This phosphorylation leads to the formation of a phosphorylated DSGIS motif, which serves as a binding site for the E3 ubiquitin ligase complex consisting of β-TrCP, Skp1, Cul1, and Rbx1. This complex then ubiquitinates and targets NRF2 for proteasomal degradation, thereby suppressing its activity.

2.4. Src family tyrosine kinase Fyn

In 2006, the Src family tyrosine kinase Fyn was reported to phosphorylate tyrosine (Y) 576 of human NRF2 (equivalent to murine Y568) located in the nuclear export sequence within the Neh3 structural domain [87]. This phosphorylation was found to be necessary for the Chromosomal Maintenance 1 to facilitate the nuclear export and subsequent degradation of NRF2 [87]. Further, GSK3β acts as a regulatory factor for Fyn kinase in controlling the nuclear export of NRF2 [88]. Specifically, exposure to H_2O_2 activated GSK3β through the phosphorylation of Y216, which led to the phosphorylation of Fyn kinase at the threonine residue and the subsequent accumulation of Fyn in the nucleus [88]. The Fyn-mediated phosphorylation of Y576 on NRF2 resulted in its nuclear export, ubiquitylation, and degradation [88].

2.5. AMP-activated protein kinase (AMPK)

AMPK has been shown to phosphorylate NRF2 at a specific site, S558 in human NRF2 (S550 in mouse), within the Neh1 domain [89]. This phosphorylation has been shown to play a role in the regulation of NRF2 activity and its nuclear accumulation [89]. Activation of AMPK by use of an AMP analog, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside, increased NRF2 nuclear accumulation in cells expressing wild-type NRF2, but not in cells expressing a mutated version of NRF2 with a serine-to-alanine mutation at this site [89]. It has been reported that AMPK inhibits

the function of GSK-3β [90, 91]. Thus, Joo et al. suggested that AMPK, by inhibiting GSK3β, activated NRF2 through phosphorylation at S550, which promoted its nuclear accumulation and ability to transactivate ARE-driven genes [89]. Additionally, mass spectrometry identified specific serine residues (S374, S408, and S433) on the NRF2 that were hyperphosphorylated in response to activated AMPK [92]. Further experiments with recombinant proteins and co-immunoprecipitation in cells showed that AMPK directly transferred phosphates to these sites on NRF2 [92]. Non-phosphorylatable mutation of these serine residues to alanine did not significantly affect the stability, nuclear accumulation, or transcriptional activity of NRF2 [92]. However, the mechanism by which AMPK-mediated phosphorylation of NRF2 at these three serine residues selectively induces its transcriptional activation of specific target genes remains unclear.

2.6. Cyclin-dependent kinase 5 (CDK5)

Jimenez-Blasco et al. have found that NRF2 is directly phosphorylated by CDK5 in astrocytes [93]. NRF2 is highly stable in astrocytes, which explained their strong antioxidant defense and resistance to oxidative stress [93]. Jimenez-Blasco and colleagues demonstrated that slight, persistent stimulation of *N*-methyl-D-aspartate receptors in astrocytes increased a signal transduction pathway involving the release of calcium from the endoplasmic reticulum (ER) via phospholipase C and activation of PKCδ. Active PKCδ promotes the stabilization of p35, a cofactor for CDK5,

through phosphorylation. The active p35/CDK5 complex in the cytosol then phosphorylates NRF2 at threonine (T) 395, S433, and T439, which is sufficient to promote NRF2 translocation to the nucleus and induce the expression of antioxidant genes [93]. Additionally, this CDK5-NRF2 transduction pathway enhanced glutathione metabolism in astrocytes, effectively protecting nearby neurons from oxidative damage [93]

2.7. Protein kinase RNA-like endoplasmic reticulum kinase (PERK)

In 2003, Cullinan et al. identified NRF2 as a novel PERK substrate [94]. In unstressed cells, NRF2 was located in the cytoplasm due to its interaction with KEPA1 [94]. However, when PERK was activated, it phosphorylated NRF2, leading to the dissociation of the NRF2-KEAP1 complex and NRF2 nuclear accumulation [94].

2.8. Mitogen-activated protein kinases (MAPKs)

The MAPK signaling cascades transmit signals via a series of phosphorylation events, involving the extracellular signal-regulated kinase (ERK) 1 and 2, p38 MAPK, c-Jun N-terminal kinase (JNK), and ERK cascades, in which the upstream kinase phosphorylated and activated the downstream kinase [95]. Xu et al. found that the ERK2 and JNK1 could directly phosphorylate NRF2 *in vitro* [96]. ERK2 and JNK1-induced phosphorylation of NRF2 increased nuclear translocation of NRF2 and its target genes transcription [96]. Moreover, Keum et al. discovered that p38

MAPK isoforms, including p38α, p38β, p38γ, and p38δ, directly phosphorylated NRF2 and stabilized the KEAP-NRF2 interaction, leading to the suppression of NRF2 nuclear translocation [97]. Zheng and colleagues, by using mass spectrometry and site-directed mutation, identified five phosphorylation sites (S215, S408, S558, T559, and S577) of NRF2 targeted by MAPKs and their upstream kinases [98]. However, the substitution of alanine at these phosphorylation residues in NRF2 had only a slight effect on its transcriptional activity [98]. As NRF2 stability was not significantly affected by NRF2 phosphorylation through MAPKs, this direct phosphorylation may have marginal impact, if any, on NRF2 activity [98].

3. Ubiquitylation of NRF2

Ubiquitylation is a PTM process involving the sequential actions of the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 to add ubiquitin to target proteins [99]. This modification can alter various properties of the substrate proteins, including their activity, where they are located in the cell, their interactions with other proteins, and most notably, their lifespan within cells [99, 100]. Therefore, it is essential to thoroughly understand the ubiquitylation process of NRF2 in order to effectively treat diseases that result from abnormal expression of NRF2.

3.1 KEAP1-CUL3-RBX1

KEAP1 is a 625 amino acid protein with 5 structural domains that

contains a zinc metal ion (Figure 2). These domains include the N-terminal region [101], the Bric-à-brac, tramtrac, broad-complex/proxvirus zinc finger (BTB/POZ) domain [25], the intervening region (IVR) [101], the Double glycine repeat/Kelch (DGR/Kelch) domain, and the C-terminal region [25]. The BTB/POZ domain is involved in the homodimerization of KEAP1 and its association with Cul 3 [25], while the DGR/Kelch domain is important in repressing NRF2 [101]. KEAP1 contains several reactive cysteine (C) residues, including C273, C288, and C297 in the BTB/POZ domain [102] and C151 in the IVR domain [103]. These residues can be oxidized or differentially modified by various NRF2 inducers such as ROS and nitrogen species, sulforaphane, tBHQ, and metals like cadmium, arsenic, and selenium. The oxidation or covalent modification of the cysteine residues in KEAP1 leads to a decrease in NRF2 ubiquitylation and an increase in its nuclear translocation and activation [103, 104].

The KEAP1-NRF2 interaction, according the hinge and latch model, involves the ETGE motif (strong affinity binding region) serving as the hinge and the DLG motif (low affinity binding region) serving as the latch [105, 106]. Under unstressed conditions, two KEAP1 proteins bind to NRF2, with one binding the ETGE motif and the other binding the DLG motif, acting as a CUL3-RBX1 E3 ubiquitin ligase substrate recruiting factor [106, 107]. There are seven lysine residues located between the ETGE and DLG in the Neh2 domain that undergo the KEAP1-dependent ubiquitylation [108]. Moreover, this ubiquitylation involves the recruitment of the p97-

UFD1/NPL4-UBXN7 complex, which extracts ubiquitinated NRF2 from the KEAP1-CUL3-RBX1-NRF2 complex and transfers it to the 26S proteasomes for its degradation (**Figure 3A**) [109].

3.2. β-TrCP- SKP1-CUL1-RBX1

The Neh6 domain of the NRF2 contains a redox-insensitive degron, which is a region that can be recognized and targeted for ubiquitylation and degradation by the proteasomes [107]. This degron consists of two motifs, DSGIS and DSAPGS, which can be independently recognized by the F box WD40 substrate adaptor protein β-TrCP [26, 85]. Phosphorylation of the DSGIS motif by GSK3β increases affinity of β-TrCP for NRF2 [84, 85]. β-TrCP then binds to the SKP1-CUL1-RBX1 E3 ubiquitin ligase complex through its F box motif, and this complex induced NRF2 ubiquitylation, marking it for degradation by proteasomes [26, 85]. A model in which KEAP1 and β-TrCP have distinct roles in regulating the stability of NRF2 has been proposed [110]. According to this model, KEAP1 acts as a "restriction valve", while β-TrCP functions as a "regulation valve", both of which control the nuclear flux of NRF2 (**Figure 4**).

3.3. HRD1

HRD1 is ER-transmembrane E3 ubiquitin ligase that negatively regulates NRF2 during cirrhosis [67]. During liver cirrhosis induced by the administration of CCl_4, ER stress led to the repression of NRF2 antioxidant

activity by HRD1 [67]. HRD1 interacts with the Neh4 and Neh5 domains of NRF2 via its C-terminal domain, leading to NRF2 ubiquitylation and degradation. This process is not dependent on KEAP1 or β-TrCP, and prevents NRF2 from activating the antioxidant response, which would otherwise counteract the high levels of ROS produced under ER stress [67].

3.4 WDR23-CUL4-DDB1

WDR23 serves as a receptor for the DDB1-CUL4-RBX1 complex, which is involved in the process of ubiquitylation of proteins [111, 112]. In the nematode *C. elegans*, the ortholog of NRF2 is called Skinhead-1 (SKN-1) [113], and it is regulated by the protein WDR23, which forms a complex with DDB1 and Cul4 and targets SKN-1 for ubiquitylation and degradation [114]. In human cells, WDR23 binds directly to the DIDLID pattern in the Neh2 structural domain of NRF2, facilitating ubiquitylation and proteasomal degradation of NRF2 [68]. However, further investigation is needed to determine the exact mechanism and role of WDR23 in regulating NRF2.

3.5 CRIF1 and SIAH2

CRIF1, a protein previously identified as a regulator of the cell cycle and transcription cofactor, has been found to also negatively regulate the stability of NRF2.

Unlike KEAP1, which only regulates NRF2 stability under normal

conditions, CRIF1 can regulate NRF2 stability and its target gene expression in both normal and oxidative stress conditions. This makes the interaction between CRIF1 and NRF2 and their consequences redox-independent. Additionally, CRIF1 physically interacts with both the N- and C-terminal regions of NRF2, which results in the ubiquitylation and subsequent proteasome-mediated degradation of NRF2 [66].

SIAH2, along with hypoxia-inducible factor 1α (HIF-1α), plays a crucial role in regulating the cellular response to hypoxia during pathological conditions such as ischemia-reperfusion [115-117]. SIAH2 is induced by hypoxia and is responsible for the suppression of NRF2. Inhibition or knockdown of SIAH2 prevented this suppression, while silencing HIF-1α did not have this effect. SIAH2 and NRF2 interacted through a binding motif, which contributed to the degradation of NRF2 through ubiquitylation and proteasome-mediated processes. This degradation occurred regardless of the phosphorylation status of NRF2 [117].

4. Acetylation of NRF2

Protein acetylation is a process in which acetyl groups are added to proteins through the action of acetyltransferase enzymes, using acetyl donors such as acetyl-CoA [118]. Lysine acetylation was first discovered on histones [119]. Since then, numerous other non-histone proteins in eukaryotes have been found to be acetylated, and their acetylation regulated various cellular processes such as metabolism [120, 121], aging [122], cell

cycle [123], and cancer [124]. Mechanistically, lysine acetylation modifies the structure and the function of proteins by altering their positive charges, which can affect stability, enzymatic activity, subcellular localization, and protein-protein interaction [125-127].

4.1. p300/cyclic AMP response element-binding protein (CBP)

The histone acetyltransferases (HATs) p300 and CBP are often referred to together as p300/CBP due to their similarities in sequence, function, and cooperation [128]. In addition to being HATs, p300/CBP can also acetylate nonhistone proteins, such as p53 [129], HIF-1α [130], signal transducer and activator of transcription 3 [131], Snail [132], etc. Sun et al. first reported that p300/CBP acetylated NRF2 in response to stress caused by arsenic [133]. In this study, they identified that multiple lysine residues (K438, K443, K445; K533, K536, K538) within in the NRF2 Neh1 domain were acetylated by p300/CBP. When these lysine residues were mutated to arginine (R), the DNA-binding activity of NRF2 was impaired in a promoter-specific manner, while protein stability was unaffected [133] (**Figure 5**).

It has been reported that acetylation of NRF2 by CBP leads to increased binding of NRF2 to ARE, resulting in increased transcription of its target genes [134]. They also found that Sirtuin 1 (SIRT1), an NAD^+-dependent class III histone deacetylase, decreased acetylation of NRF2 and the transcription of NRF2 target genes [134]. However, this effect could be

blocked by dominant-negative SIRT1-H355A mutation [134]. In addition, SIRT1 inhibitors such as EX-527 and nicotinamide can stimulate the transcription of NRF2 target genes, while resveratrol, a known SIRT1 activator, has the opposite effect [134]. In this study, acetylation of NRF2 by CBP at murine K588 (equivalent to human K596) and murine K591 (equivalent to human K599) increased its nuclear localization and transcriptional activity, while deacetylation of NRF2 blocked this effect [134] (**Figure 5**).

Ganner et al. reported that by physically interacting with NRF2 and disrupting the formation of the NRF2-KEAP1 complex, p300 increased the abundance and stability of NRF2 and promoted its nuclear localization [135]. Importantly, p300-mediated acetylation of NRF2 was necessary for stabilizing NRF2 and increasing cell survival under stressful conditions [135] (**Figure 5**).

However, further studies are needed to identify the residue(s) of NRF2 acetylated by p300/CBP and the mechanism underlying NRF2 stabilization through acetylation.

4.2. Males absent on the first (MOF)

MOF is a HAT of the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) family that acetylates lysine 16 of histone H4 (H4K16) and is positively correlated with gene transcription [136, 137]. Besides as a HAT, hMOF is classified as a KAT capable of acetylating non-histone protein (p54) [138]. It plays

important roles in embryogenesis, oncogenesis, cell proliferation, and DNA damage repair [139-141]. Chen et al. reported that hMOF was overexpressed in human non-small cell lung cancer tissues and associated with poor prognosis [142]. There was a positive correlation between hMOF levels and NRF2 target genes [142]. Importantly, hMOF physically interacted with and murine NRF2 acetylated on K588 (equivalent to human K596) [142]. hMOF-mediated acetylation of NRF2 increased the nuclear accumulation of NRF2 and the transcription of its downstream genes, leading to increased drug resistance and cell proliferation in human lung cancer cells [142].

4.3. ARD1

Recently, I found that ARD1 enhanced the oncogenic function of NRF2 in human colon cancer by direct binding and acetylating this transcription factor [143]. In this study, ARD1 and NRF2 were found to be highly expressed in human colon tumor tissues and human colon cancer cell lines [143]. Silencing of ARD1 in human colon cancer cell lines resulted in a significant decrease in NRF2 protein levels, but failed to affect its mRNA levels [143]. Additionally, these two proteins were also found to physically interact with each other and co-localized in both HCT-116 human colon cancer cells and tumor tissues. Mechanistically, ARD1 interacted with NRF2 within Neh1 and Neh3 domains, and overexpression of ARD1 increased the acetylation levels of NRF2 and its nuclear accumulation.

Through MS analysis, it was speculated that K438 was a specific residue for ARD1-mediated acetylation of NRF2. Moreover, the half-life of NRF2 was reduced in cells that when mutant forms of ARD1 with defective acetyltransferase activity were expressed. Interestingly, ARD1 was associated with KEAP1. Further studies will be necessary to prove that K438 is the specific residue of ARD1-mediated NRF2 acetylation and to explore what effect K438 has on NRF2 properties or functions (**Figure 5**).

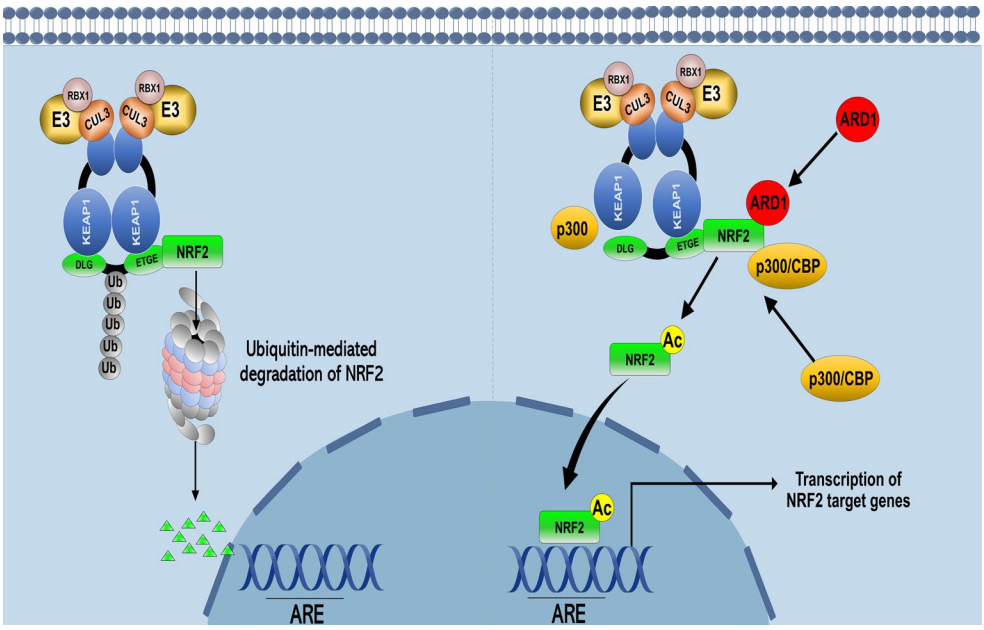

Figure 5. Proposed scheme of acetylation of NRF2.

p300/CBP dicretly binds to and acetylates NRF2, resulting in an increase in the nuclear transcription of NRF2 and the activation of its target genes. ARD1 interacts with NRF2 and can acetylate it, facilitating the nuclear localization and transcriptional activation of NRF2.

5. Deacetylation of NRF2

In contrast to acetylation, deacetylation removes the acetyl groups from proteins [125].

5.1. SIRT1

As mentioned in 4.1, it was found that SIRT1 reduced acetylation of NRF2 and its transcriptional activity [134]. In contrast, SIRT1 stabilized NRF2 by deacetylating it in mouse type II alveolar epithelial cells, resulting in increased transcription of the target genes of NRF2 to counteract the damage caused by paraquat [144]. Xu et al. indicated that NRF2 and SIRT1 physically interacted in cultured cardiomyocytes in both normal and hypoxia/reoxygenation conditions, as well as in myocardial ischemia/reperfusion injury *in vivo* [145]. In this study, SIRT1-mediated deacetylation enhanced the nuclear localization of NRF2 and its target genes transcription [145].

The results of SIRT1-mediated deacetylation of NRF2 may vary depending on the cell type and its environment. As different lysine residues of NRF2 play differential roles in its activity and stability, it is possible that these varying outcomes are also related to the specific lysine residue(s) of NRF2 targeted by SIRT1. Therefore, detailed research is needed to systematically unravel the precise mechanism of SIRT1-mediated deacetylation of NRF2.

5.2 Sirtuin 2 (SIRT2)

SIRT2, one of the least studied members of the SIRT family, is involved in the regulation of metabolism, inflammation, cell-cycle progression, neurodegeneration, and tumorigenesis [146]. Yang et al. demonstrated that SIRT2 enhanced the amount of iron within cells by binding to and deacetylating NRF2 on K506 and K508, resulting in a decrease in total and nuclear accumulation of NRF2 [147]. Moreover, SIRT2-mediated deacetylation of NRF2 promoted its ubiquitylation and reduced the expression of ferroportin 1, which led to an imbalance in cellular iron homeostasis [147].

6. SUMOylation of NRF2

SUMOylation is one of PTMs in which small ubiquitin-like modifier (SUMO) proteins (SUMO1–SUMO4) are covalently and reversibly attached to lysine residues on target proteins [148]. SUMOylation can affect stability, subcellular localization, activity of target proteins, their interaction with other proteins, transcription, DNA repair, and cell cycle regulation [148-150].

Ramani et al. reported that SUMO-1-mediated SUMOylation of NRF2 and MafG increased heterodimerization and transcription of an NRF2 target protein glutamate-cysteine ligase catalytic subunit, thereby maintaining the quiescent state of hepatic stellate cells [151]. In this study, it was found that K525 and K595 of murine NRF2 (equivalent to human K532 and K603,

respectively) were SUMOylated [151]. Later, He et al. further demonstrated that NRF2 was also a substrate for ubiquitin-conjugating enzyme 9 (UBC9)-mediated SUMOylation in murine pancreatic beta cells, and confirmed K525 and K595 (equivalent to human K532 and K603, respectively) as two SUMOylation sites for NRF2 [152]. SUMOylation of these two lysine residues promoted NRF2 transcriptional activity and its stability [152].

More recently, it was revealed that the K110 of NRF2 was the conserved acceptor site for SUMO1 and when this SUMOylation process was deficient, it inhibited the progression of hepatocellular carcinoma (HCC) [153]. Of note, SUMOylation of NRF2 promoted the production of serine by increasing the removal of intracellular ROS and activating the enzyme phosphoglycerate dehydrogenase [153]. Additionally, serine starvation induced NRF2 SUMOylation, which could contribute to the continued growth of HCC [153].

Contrary to the aforementioned studies demonstrating that SUMOylation was required for cellular antioxidant defense, SUMOylation of NRF2 by SUMO-1 and SUMO-2 induced the degradation of this transcription factor in HepG2 cells [154]. In this study, NRF2 was found in promyelocytic leukemia-nuclear bodies (PML-NBs) and modified by RING finger protein 4 (RNF4), a poly-SUMO-specific E3 ubiquitin ligase [154]. Overexpression of wild-type RNF4 decreased the steady-state levels of NRF2 in PML-NB-enriched cell fractions, but this effect could be blocked by the proteasome inhibitor MG-132, resulting in elevated levels of polySUMOylated and

ubiquitylated NRF2 [154]. Respiratory syncytial virus infection led to the degradation of NRF2 in a manner dependent on the E3 ubiquitin ligase RNF4 and SUMOylation [155]. Blocking SUMOylation or silencing of RNF4 expression could rescue both NRF2 nuclear levels and transcriptional activity [155].

In summary, the SUMOylation of NRF2 has two opposite effects on its stability and activity. Detailed studies systematically untangling the SUMOylation of NRF2 are warranted.

7. Glycation and de-glycation of NRF2

Glycation is a non-enzymatic chemical reaction in which proteins are modified by the attachment of sugar molecules, which can vary in their susceptibility and susceptibility to removal depending on their spatial and chemical context and the activity of de-glycation enzymes [156, 157]. Sanghvi et al. discovered that glycation of NRF2 reduced the stability, binding ability to sMAF proteins, and transcriptional activity of this oncogenic transcription factor [43]. However, fructosamine-3-kinase (FN3K)-mediated de-glycation of NRF2 blocked this effect and enhanced the pro-oncogenic and drug resistance effects of NRF2 [43].

8. Methylation of NRF2

Protein methylation, a type of PTM that can occur on various amino acid residues such as arginine, lysine, histidine, proline, and carboxyl groups, has

been widely studied for its role in regulating transcription through the methylation of histone proteins and non-histone proteins [158-160]. The methylation of lysine and arginine residues on non-histone proteins, which is a prevalent PMT, plays important regulatory roles in various cellular processes beyond histone methylation [161, 162]. It has been reported that the protein arginine methyltransferase-1 (PRMT1) directly binds to NRF2 and methylates at R437 of NRF2 [163]. They found that PRMT1-mediated methylation of NRF2 leads to a moderate increase of its transactivation and DNA-binding activity [163]. This, in turn, protects cells from damage caused by depletion of the antioxidant glutathione and cell death triggered by *tert*-butyl hydroperoxide [163].

9. Concluding remarks

Dysregulation of NRF2 has differential effects on the pathogenesis of aging, cardiovascular diseases, respiratory diseases, neurodegenerative diseases, and cancer. The intricate nature of NRF2 biology is a result of its multifaceted roles and numerous interactions with various cellular components. The expression, stability, and activity of NRF2 depend on the type of a molecule it binds to and the signaling pathway that ensues. NRF2 and its binding partners are known to undergo a variety of PTMs that can greatly influence the cellular response to external stimuli. PTMs of NRF2 such as phosphorylation, acetylation, ubiquitylation, SUMOylation, glycosylation, and methylation play a crucial role in its stability and its

interaction with other proteins (**Table 1**). These modifications can ultimately determine the fate of a cell and even an entire organism. However, how PTMs finally affect NRF2 activity seems to remain unpredictable and is likely to be highly context-dependent (e.g., cell type, stressor, etc.) and PTMs site-dependent. Systematic unraveling of the NRF2 PTMs network in detail is warranted.

The following are a few considerations for studying PTMs of NRF2.

1. Selection of proper cell models: different cell types respond differently to NRF2, and the selection of an appropriate cell model has a great impact on the accuracy of the study results.

2. Cellular different stress states: PTMs of NRF2 are associated with the environment surrounding the cells, and one should ensure that the cells are studied under the appropriate stress state.

3. Types of post-translational modifications: There are multiple PTMs of NRF2 and one should make sure that the specific type of PTM is being studied.

Table 1. Post-translational modifications of NRF2.

Modification	Protein(s)	Domain(s)/residue(s)	Molecular out come	References
Phosphorylation	PKC (PKCι)	S40	Disuruption of the KEAP1-NRF2 complex , stabalization of NRF2 and enhancement of its transcriptional activity	[39,75-77,79]
	CK2	Neh4 and Neh5	Increased nuclear translocation and transcriptional activity of NRF2	[40,80]
	GSK-3β	S343, S346	Degradation of NRF2	[81-83]
	Fyn	Y576	Increased nuclear export and degradation of NRF2	[85]
	AMPK	S378, S408, S433, S558	Increased nuclear translocation and transcriptional activity of NRF2	[86,89]
	CDK5	T395, S433, T439	Increased stability, nuclear translocation, and transcriptional activity of NRF2	[90]
	PERK		Increased stability and nuclear translocation of NRF2	[91]
	ERK2 and JNK1		Increased nuclear translocation and transcriptional activity of NRF2	[93]
	p38α, p38β,		Degradation of NRF2 through stabilizing KEAP1-	[94]

	p38γ, and p38δ,		NRF2 interaction	
	MAPKs	S215, S408, S558, T559, S577	Not significantly affected stability and activity of NRF2	[95]
Ubiqutylation	KEAP1	Neh2 (ETGE and DLG motifs)	Degradation of NRF2	[103-106]
	β-TrCP	Neh6 (DSGIS and DSAPGS motifs)	Degradation of NRF2	[24,81,82]
	HRD1	Neh4 and Neh5	Degradation of NRF2	[65]
	WDR23	Neh2 (DIDLID pattern)	Degradation of NRF2	[66]
	CRIF1	N- and C-terminal regions	Degradation of NRF2	[64]
	SIAH2		Degradation of NRF2	[114]
Acetylation	p300/CBP	K596,K598	Increased stability, nuclear translocation, and transcriptional activity of NRF2	[130-132]
	hMOF	K596	Increased nuclear translocation, and transcriptional activity of NRF2	[139]
	ARD1	Neh1 and Neh3	Increased stability, nuclear translocation, and	[140]

			transcriptional activity of NRF2	
Deacetylation	SIRT1		Decreased transcriptional activity of NRF2/Increased stability, nuclear translocation, and transcriptional activity of NRF2	[131,141,142]
	SIRT2	K506, K508	Degradation of NRF2	[144]
SUMOylation of NRF2	SUMO-1	K110,K532,K603	Increased transcriptional activity of NRF2	[148]
	UBC9	K532,K603	Increased stability, nuclear translocation, and transcriptional activity of NRF2	[149]
Glycation			Decreased stability and transcriptional activity of NRF2	[41]
De-glycation	FN3K		Increased stability, nuclear translocation, transcriptional activity, pro-oncogenic, and drug resistance effects of NRF2	[41]
Methylation	PRMT1	R437	Moderately increased transcriptional activity of NRF2	[160]

II. ARD1

1. Discovery of ARD1

Whiteway and Szostak discovered ARD1 for the first time in *Saccharomyces cerevisiae* in 1985 [45]. The yeast protein yeast ARD1 was thought to have a significant role in the cell cycle and cell survival, as suggested by its full name "Arrest-Defective 1". ARD1 is involved in regulating the switch between mitosis and alternative cell fates because mutations of yeast ARD1 are linked to defects in entering the stationary phase and sporulating under nutrient-limited conditions [45, 47, 164]. From yeast to mammals, ARD1 is extremely conserved across all species. On chromosome Xq28, mammalian ARD1 is present and controls a wide range of cellular processes, including cell division, migration, apoptosis, autophagy, differentiation, development, and illness [57, 165-169].

2. ARD1 isoforms

In mammals, several different isoforms of ARD1 have been discovered, including mouse (m) $ARD1^{198}$, $mARD1^{225}$, $mARD1^{235}$, human (h) $ARD1^{131}$, and $hARD1^{235}$ [170, 171]. The ARD1 isoforms are named based on the number of amino acids they contain, such as $ARD1^{235}$ (containing 235 amino acids), $ARD1^{225}$ (containing 225 amino acids), $ARD1^{198}$ (containing 198 amino acids), and $ARD1^{131}$ (containing 131 amino acids). Among these, $mARD1^{225}$, $mARD1^{235}$, and $hARD1^{235}$ have been most extensively studied

and characterized.

hARD1[131] and mARD1[198] only contain incomplete N-acetyltransferase domains, while mARD1[225], mARD1[235], and hARD1[235] isoforms have a well-conserved N-acetyltransferase domain between amino acids 45-130. The N-acetyltransferase domain is considered a core region of ARD1 because it contains an acetyl-CoA-binding site located at amino acids 82–87 that is critical for the acetyltransferase enzyme activity of ARD1. The N-acetyltransferase domain of ARD1 is crucial for its catalytic activity as it comprises an acetyl-CoA-binding site situated at amino acids 82-87. Additionally, this domain also comprises a nuclear localization signal located at amino acids 78-83, indicating that ARD1 has the ability to shuttle between the cytosol and the nucleus [165, 170].

3. ARD1 as a KAT

ARD1, originally identified as a NAT, has been found to have additional functions due to its subcellular localization in non-ribosomal forms and the nucleus [46]. Additionally, mammalian ARD1 has both NAT and KAT activities. In this section, the various cellular functions controlled by lysine acetylation mediated by ARD1 are summarized.

3.1 Cellular stress response

Oxidative stress impairs peptide synthesis and many methylation processes by decreasing the intracellular level of free methionine through

the oxidation of methionine to methionine sulfoxide [172]. Methionine sulfoxide reductase A (MSRA) protects proteins from oxidative damage by recovering sulfoxidized methionine and restoring the activity of antioxidant enzymes that have been inactivated due to oxidation [172-175]. Shin et al. reported that ARD1 increased intracellular ROS by acetylating MSRA in lung carcinoma cells [176]. In this study, ARD1 has been shown to directly bind to and inhibit the enzymatic function of MSRA by acetylating it at K49. This led to an increase in cellular levels of ROS, carbonylated proteins, and DNA breaks, ultimately promoting cell death [176]. Additionally, the livers and kidneys of ARD1 transgenic mice have been found to be more susceptible to oxygen toxicity [176]. Based on these findings, it has been suggested that ARD1 plays a significant role in the cellular response to oxidative stress by controlling MSRA, and that ARD1 can be a target to reduce damage caused by oxidative stress or to enhance the effectiveness of anticancer treatments that rely on the production of ROS [176].

Conversely, Seo et al. found that Heat shock protein (Hsp) 70, which protects cellular proteins under stress conditions, can quickly undergo acetylation at the K77 residue by ARD1 and then deacetylation by histone deacetylase 4 [53]. Initially, Hsp70 facilitates protein refolding through acetylation by ARD1 at K77 and binding to the co-chaperone Hop [53]. However, as the stress response progresses, Hsp70 is deacetylated and binds to the ubiquitin ligase protein CHIP to complete protein degradation [53]. This switch is crucial for preventing cell death and maintaining protein

homeostasis under oxidative stress [53]. Moreover, Fang et al. reported that ARD1-mediated acetylation of NRF2 increased the stability and nuclear accumulation of NRF2, which is a key regulator of the cellular antioxidant response [143].

It is possible that the protein ARD1 has conflicting roles in the response to oxidative stress, which may depend on the intensity or duration of the stress. These opposing functions may be mediated by acetylating different substrate proteins. Given that ARD1 plays a crucial role in maintaining cellular redox balance, targeting ARD1 in the management of diseases related to oxidative stress requires consideration of its opposing functions and a thorough examination of the downstream proteins that it acetylates in relation to the specific disease.

3.2 Autophagy

Autophagy is essential for maintaining cellular homeostasis [177]. It has been reported that ARD1 promotes cell survival under harsh conditions by initiating autophagy through its KAT activity [50]. Specifically, under conditions of glutamine deprivation and hypoxia, ARD1 acetylates K388 on phosphoglycerate kinase 1 (PGK1). ARD1-mediated acetylation of PGK1 induces this phosphoglycerate kinase to then phosphorylate Beclin1 at S30 [50]. Autophagy and brain tumorigenesis are dependent on the acetylation of PGK1 by ARD1 and the phosphorylation of Beclin1 at S30 by PGK1 during conditions of glutamine deprivation and hypoxia [50]. Additionally, the

levels of PGK1 K388 acetylation are found to be linked to the levels of Beclin1 S30 phosphorylation and to poor prognosis in patients with glioblastoma [50]. This study revealed the crucial role of ARD1 in the regulation of autophagy and maintaining cellular homeostasis through acetylation of PGK1 at K388.

3.3 Cell cycle

Yeast ARD1 has been reported to be associated with the processes of G_0 entry, cell growth, and sporulation in yeast cells [45, 164, 178]. Lim et al. revealed that inhibition of hARD1 in H1299 and A549 lung cancer cells decreased cell proliferation and caused G_1 arrest [179]. This was accompanied by a decrease in cyclin D1 expression, which can be rescued by re-introducing cyclin D1 [179]. hARD1 knockdown repressed cyclin D1 promoter activity by reducing the binding of β-catenin/TCF4 transcription factor to its promoter and decreasing its transcriptional activity [179]. Conversely, hARD1 overexpression increased β-catenin transcriptional activity [179]. hARD1 directly binds and acetylates β-catenin without altering its expression or degradation [179]. In 2010, Seo et al. discovered that the autoacetylation of hARD1 at K136 plays a crucial role in activating β-catenin and activator protein-1, resulting in an increase in the expression of cyclin D1, and promoting cancer cell proliferation and tumor growth [180].

Deoxyribonucleotide triphosphates (dNTPs) are essential building blocks

for DNA synthesis and maintaining proper balance of dNTPs is crucial for accurate DNA replication and repair in cells [181]. Cells regulate the supply of dNTPs to maintain genomic stability during proliferation by using diverse mechanisms to ensure that the availability of dNTPs remains within an optimal range, with the largest pools during S phase and the smallest in G_0.[182, 183]. SAM domain and HD domain-containing protein 1 (SAMHD1) is a dNTP triphosphohydrolase (dNTPase) that has been found to cleave dNTPs [184]. Most studies on SAMHD1 have focused on its role in inhibiting retroviruses, such as human immunodeficiency virus type 1, in immune cells by depleting cellular dNTPs to block retroviral replication [185, 186]. Furthermore, SAMHD1 promotes cell growth in both lung fibroblasts and THP-1 cells, as its expression levels vary during the progression of the cell cycle [187, 188]. Despite being linked to the regulation of dNTPs and cell cycle progression, the role of SAMHD1 in cancer proliferation remains largely unexplored. Lee et al. discovered that the activity of the dNTPase enzyme within SAMHD1 is controlled by acetylation [189]. Specifically, ARD1 acetylates SAMHD1 at K405, which enhances its dNTPase activity in cancer cells [189]. In vitro studies have shown that ARD1-mediated acetylation at K405 of SAMHD1 increases its dNTPase activity, while non-acetylated arginine substituted mutants (K405R) do not exhibit this activity [189]. Additionally, cancer cells expressing the K405R mutant have slower proliferation and a reduced transition from G1 to S phase compared to wild-type cells [189].

Furthermore, SAMHD1 acetylation levels are highest in the G1 phase, suggesting a role for this modification during this stage of the cell cycle [189]. Thus, these findings indicate that controlling the levels of cellular dNTPs may be beneficial in preventing cancer growth, and that adjusting the acetylation level of SMAHD1 or the KAT activity of ARD1 could be a viable strategy for managing the cellular dNTP pool.

3.4 Osteogenesis

During bone development and regeneration, the transcription factor Runt-related transcription factor 2 (Runx2) plays a crucial role in promoting osteoblast differentiation in the early phase [190, 191]. Yoon et al. have reported that ARD1 is stabilized by Runx2 and controls differentiation by inhibiting Runx2 [192]. They showed that ARD1 delays the healing of bone in a rat calvarial defect model and also delays bone development in neonatal mice [192]. Mechanistically, ARD1 acetylates the K225 residue of Runx2, which interferes with Core-binding factor β binding to Runx2, ultimately inhibiting Runx2-driven transcription [192]. Thus, ARD1 may play a pivotal role in promoting bone formation by fine-tuning Runx2 signaling in a balanced manner [192].

4. Concluding remarks

ARD1 is a member of the KAT protein family, known for its vital role in regulating cellular activity through its enzymatic activity on specific

substrates. Its regulation is complex and subject to multiple modifications, such as overexpression, dysregulation, or depletion. These changes can disrupt cellular homeostasis and contribute to the development of diseases such as oncogenesis and neurodegeneration. Despite its importance, the specific mechanisms by which ARD1 acetylates its partners and how it is affected by other modifications, such as autoacetylation and phosphorylation, remains poorly understood. Additionally, the structure of human ARD1 has not been fully validated. To better understand the role of ARD1 in disease development, further research is needed, including the development of inhibitors that specifically target ARD1. Such inhibitors would provide new insights into ARD1 and potential treatment options for ARD1-related diseases.

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STATEMENT OF PURPOSE

NRF2 plays a central role in the cellular response to ROS and oxidative stress. While originally recognized as a target for chemopreventive compounds that help prevent cancer and other illnesses, accumulating evidence has established the NRF2 pathway as a driver of cancer progression, metastasis, and resistance to therapy. Therefore, the aberrant overactivation/overexpression of NRF2 is implicated as a driving event in tumor progression, which has been attributed to its mutation or phosphorylation as well as inactivation of the inhibitory protein, KEAP1. However, alternative mechanisms responsible for sustained activation of NRF2 are less understood. ARD1, an N-terminal acetyltransferase that catalyzes N-terminal acetylation of target proteins, is involved in mediating various (patho)physiological processes, such as proliferation, apoptosis, autophagy, and differentiation. Aberrant overexpression of ARD1 is correlated with metastasis and poor prognosis in several types of cancer, suggesting that it may act as a tumor promoter. Conversely, ARD1 has also been reported to suppress tumorigenesis through inhibition of mTOR signaling. Further, several studies have pointed out that ARD1 does not possess the ability to be a lysine acetyltransferase. Due to these different results, the role of ARD1 in tumorigenesis and whether it has the ability to KAT still needs further investigation. The present study is aimed to investigate the role of NRF2 and ARD1 in CRC and to explore the possible

interaction between them, as well as the significance of this interaction in the progression of CRC.

Chapter II

ARD1 stabilizes NRF2 through direct interaction and promotes colon cancer progression

1. Introduction

Nuclear factor erythroid 2-related factor 2 (NRF2) is regarded as a central hub that maintains cellular redox homeostasis by regulating antioxidant gene expression [1]. Under normal conditions, Kelch-like ECH-associated protein 1 (KEAP1) in the cytoplasm induces ubiquitination of NRF2 for proteasomal degradation. Degradation of NRF2 reduces rapidly upon oxidative stress with a concomitant increase in its nuclear translocation, leading to transcription of its target genes. Although NRF2-KEAP1 defines a physiologically important stress response mechanism to maintain cellular homeostasis [1], many recent studies have revealed that hyperactivation of the NRF2-mediated stress response can promote the growth and progression of cancer cells by creating an optimal environment for their survival. Thus, NRF2 protects cancer cells from oxidative stress often caused by chemotherapy and radiotherapy, and also promotes metabolic reprogramming towards anabolic pathways [2, 3]. Therefore, it is imperative to unravel the regulatory network of NRF2 to develop proper therapeutic strategies against antioxidant-addicted cancer.

Abnormally elevated expression or activation of NRF2 has been attributed to mutations, especially in the sequences involved in interaction with its negative regulator, KEAP1 [4, 5]. Besides mutation of NRF2, some NRF2-competitive-binding proteins can impair NRF2 ubiquitylation by blocking the association of KEAP1 with the DLG or ETGE motif of NRF2 [6-9]. Post-translational modifications (PTMs) play a key role in the

regulation of various signaling pathways in cells. These include phosphorylation, acetylation, methylation, hydroxylation, and ubiquitination that can change the characteristics of a protein by chemically modifying its amino acid residues [10]. PTMs have also been reported to regulate the stability of NRF2 [11-13] and to inhibit the function of KEAP1 [14-16]. Nevertheless, most studies have focused on phosphorylation of NRF2, and few have explored other types of NRF2 PTM.

Arrest defective 1 (ARD1; also known as N-α-acetyltransferase 10), an N-terminal acetyltransferase, was first identified in *Saccharomyces cerevisiae* [17]. It catalyzes the alpha (N-terminal) acetylation of nascent peptides as a co-translational modification [18-20]. In addition to catalyzing the N-terminal acetylation of target proteins, ARD1 can also acetylate the ε-(internal)-lysine residues of mature proteins, such as hypoxia-inducible factor 1-alpha [21], β-catenin [22], myosin light chain kinase [23], ARD1 itself (autoacetylation) [24], androgen receptor [25], methionine sulfoxide reductase A [26], runt-related transcription factor 2 [27], heat shock protein 70 [28], sterile alpha motif domain and histidine-aspartic domain containing protein 1 [29], and phosphoglycerate kinase 1 [30].

Through N-terminal or internal acetylation of above proteins, ARD1 mediates a series of cellular functions involved in cell homeostasis, migration, differentiation, proliferation, and tumorigenesis. Notably, elevated ARD1 expression has been reported to be associated with a variety of human malignancies, including breast [31], liver [32], and colon cancer

[33]. The increased ARD1 expression has been linked to poor prognosis for cancer patients [33, 34]. Conversely, ARD1 has also been reported to suppress tumorigenesis by inhibiting mTOR signaling [35]. Here, I report that ARD1 could modulate NRF2 by acetylating and stabilizing this transcription factor, thereby stimulating the growth and progression of human colon cancer cells.

2. Materials and methods

Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL), RPMI 1640 medium (Gibco-BRL), fetal bovine serum (FBS) (Gibco-BRL), MG-132 (BML-PI102–0025, Enzo), cycloheximide (CHX) (C7698, Sigma-Aldrich), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (M2003, Sigma-Aldrich), D-luciferin (LUCK-1G, Goldbio), recombinant NFE2L2 (human) protein (H00004780-P01, Abnova), recombinant ARD1 (human) protein (H00008260-P01, Abnova), Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (354230, Corning), anti-actin (sc-47778, Santa Cruz Biotechnology), anti-ARD1 (sc-373920, Santa Cruz Biotechnology), anti-lamin B1 (sc-374015, Santa Cruz Biotechnology), anti-α-tubulin (sc-5286, Santa Cruz Biotechnology), anti-NRF2 (ab137550, Abcam), anti-Myc-tag (2278, Cell Signaling Technology), anti-acetylated-lysine (9441, Cell Signaling Technology), anti-Flag-tag (F1804, Sigma-Aldrich), and anti-Ubiquitin (13–1600, Invitrogen) were purchased from the indicated manufacturers.

Cell culture

Human colon cancer cell lines (HCT-116, HCT-15, DLD-1, HT-29, and SW480) were obtained from the Korean Cell Line Bank (KCLB). HCT-116-Luc2 and normal human colon epithelial CCD841CoN cells were purchased

from American Type Culture Collection (ATCC). HCT-116, HCT-116-Luc2, SW480, and HT-29 cells were routinely maintained in DMEM. HCT-15 and DLD-1 cells were grown in RPMI 1640, and CCD841CoN cells were cultured in MEM containing 10% FBS and a 100 ng/mL antibiotics mixture. All cell lines were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO_2.

Transient transfection of small interfering RNA (siRNA) and plasmid

Transient transfection of cells with siRNA (20 nM) targeting NRF2 or ARD1 was conducted with lipofectamine RNAiMAX, and transfection with NRF2 or ARD1 plasmid was performed using lipofectamine 2000 (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA) in accordance with the manufacturer's procedure. Scrambled siRNA was used as a negative control. The target sequences for human NRF2 siRNA #1 were sense 5′-GAGACUACCAUGGUUCCAA-3′ and antisense 5′-UUGGAACCAUGGUAGUCUC-3′, for human NRF2 siRNA #2 were sense 5′-CAGCUAUGGAGACACACUA-3′ and antisense 5′-UAGUGUGUCUCCAUAGCUG-3′, for human ARD1 siRNA #1 were sense 5′-CCAGCUCUCUUACAUUGGU-3’ and antisense 5′-AGCAAUGUAAGAGAGCUGG-3′. siRNA oligonucleotides targeting NRF2 and ARD1 were supplied by Bioneer (Seoul, South Korea). Human ARD1 siRNA #2 (sc-44713) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). After 48 h of transfection, cells were harvested. pcDNA-

Myc-NRF2 plasmid was obtained from Addgene (plasmid # 21555). Myc-tagged NRF2 deletion constructs were generated by PCR and cloned into the BamHI and NbaI sites of pcDNA-Myc3 (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA); △d1, 5′-CTGGAATTCAGGATCCTCATGATGGACTTGGAGC-3′ (forward) and 5′-ATAGGGCCCTCTAGATAGACACTTCCAGGGGCACT-3′ (reverse); △d2, 5′-ATAGGGCCCTCTAGATAATTAAGACACTGTAACTC-3′ (reverse); △d3, 5′-ATAGGGCCCTCTAGATAAATTGGGAGAAATTCACC-3′ (reverse) [36]. NRF2 amplification was performed using high-fidelity i-StarMAX^{TM} GH Taq polymerase (iNtRON Biotechnology, Seongnam, South Korea) following the manufacturer's instructions. pCMV-tag2b (Flag-ARD1) was kindly provided by Prof. Kyu-Won Kim (College of Pharmacy, Seoul National University, Seoul, South Korea) [29].

Lentivirus-mediated stable transfections

Subconfluent HCT-116-Luc2 cells were transfected with control shRNA Lentiviral Particles-A (sc-108080, Santa Cruz Biotechnology), ARD1 shRNA Lentiviral Particles (sc-44713-V, Santa Cruz Biotechnology), and NRF2 shRNA Lentiviral Particles (sc-37030-V, Santa Cruz Biotechnology) in accordance with the manufacturer's procedure. After lentivirus transduction, cells were incubated with 3 μg/mL of puromycin for a

selection. The selected cells were maintained in culture media containing puromycin at 2 μg/mL until further use. The knockdown efficiency in HCT-116-Luc2 cells was determined by Western blot analysis.

Flow cytometric analysis

Cell apoptosis and necrosis were assessed by the FITC Annexin V Apoptosis Detection Kit with PI (640914, BioLegend) according to the manufacturer's procedure. After silencing of NRF2 or ARD1, HCT-116 cells were collected and washed with BioLegend's Cell Staining Buffer. Cells were then counted and d then resuspend cells in Annexin V Binding Buffer. Cells were stained with 5 μL of FITC Annexin V and 10 μL Propidium Iodide for 15 min at room temperature in the dark, and then cells were analyzed using Flow Cytometer (BD, FACSLyric). FlowJo software was used to analyze the data.

ROS measurement

HCT-116 cells were transiently transfected with scrambled or ARD1 siRNA for 24 h. Cells were treated with or without H_20_2 (0.5 mM) for 3 h. Whole cells were stained by 2′,7′-Dichlorofluorescin diacetate (DCF-DA) solution for 0.5 h, and the total intensity of DCF fluorescence in each group was measured by flow cytometry (BD, FACSAria II) or fluorescent microscopy (Nikon; Tokyo, Japan).

Western blot analysis

Lysates from cells were separated by running through 6-10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to the polyvinylidene difluoride membranes as described previously [37]. The membranes were then incubated with indicated primary antibodies. Blots were washed with Tris-buffered saline with 0.1% Tween-20 and were then probed with horseradish peroxidase-conjugated secondary antibodies (1:5000) (Pierce Biotechnology; Rockford, IL, USA). The transferred proteins were detected by using a Western blotting detection reagent (AbClon; Seoul, South Korea).

Real-time polymerase chain reaction (qPCR)

Total RNA was isolated from cells by using the TRIzol® reagent (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA), and RT-PCR was carried out in accordance with the standard protocol. To synthesize cDNA, 1 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega; Madison, WI, USA). qPCR was performed on a 7500 Real-Time PCR instrument (Applied Biosystems; Foster City, CA, USA) using the RealHelix™ SYBR Green I qPCR kit (NanoHelix Co., Ltd.; Daejeon, South Korea). The PCR cycling conditions included initial denaturation of 95 °C for 5 min followed by 40 cycles of 20 s 95 °C and 60 °C for 40 s. The primers used for the qPCR are as follows: for NRF2, 5′-TCCAGTCAGAAACCAGTGGAT-3′, and 5′-

GAATGTCTGCGCCAAAAGCTG-3′. For ARD1, 5′-ATGAAGCGGGACCTCACTCA-3′ and 5′-GCTCTCCACCTTGTTCTCGATG-3′. For GAPDH, 5′-ACAACTTTGGTATCGTGGAAGG-3′ and 5′-GCCATCACGCCACAGTTTC-3. Data were calculated using the comparative cycle threshold (ΔΔCt) method.

Immunoprecipitation

Cell lysates were prepared as described previously [37] and were immunoprecipitated with an appropriate antibody at 4 °C overnight by the addition of Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology) and additional shaking for 4 h at 4 °C. After resolving the samples by SDS polyacrylamide gel electrophoresis (PAGE), membranes were incubated with the indicated primary antibodies.

Preparation of cytosolic and nuclear extracts

Pellets were washed by cold phosphate-buffered saline (PBS) and suspended in ice-cold hypotonic lysis buffer to obtain cytosolic extract. The remaining pellets were suspended in ice-cold hypertonic lysis buffer to collect the nuclear extract according to the previously reported procedure [37]. Each separately obtained supernatant containing cytosolic or nuclear proteins was kept at −70 °C until use.

Transient transfection and the luciferase reporter assay

After silencing of ARD1, HCT-116 cells were co-transfected with 2 μg of ARE-luciferase reporter plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the co-transfection was carried out according to the instructions supplied by the manufacturer. The cells were then washed with PBS and lysed in 1× reporter lysis buffer (Promega). One hundred μL of the luciferase assay reagent was added to 20 μL lysed cell extract, and the luciferase activity was analyzed by a luminometer (AutoLumat LB 953, EG&G Berthold).

In situ proximity ligation assay (PLA)

PLA was performed using the Duolink® *In Situ* Red Starter Kit Mouse/Rabbit (Sigma-Aldrich) according to manufacturer's instructions. In brief, HCT-116 cells on glass coverslips were fixed with 4% formaldehyde for 20 min at room temperature and then stained cell membrane with Alexa Fluor 488 conjugated wheat germ agglutinin (Thermo Fisher Scientific) for 5 min at dark. Cells were permeabilized, and blocked with Duolink® Blocking Solution and incubated with anti-NRF2 and anti-ARD1 antibodies in a heated humidity chamber for 1 h at 37 °C. PLA plus and minus affinity probes were then added, and the mixtures were incubated for additional 1 h at 37 °C. After ligation and amplification, the images were visualized by fluorescence microscopy (Nikon; Tokyo, Japan).

Wound healing assay

Cells were plated into culture-insert (Ibidi; Martinsried, Germany) to create a 500 μm gap uniformly. After 24-hour incubation, Ibidi Culture-Inserts were gently removed and then photographed with a microscope (Nikon; Tokyo, Japan).

Orthotopic mouse model of colorectal cancer

Eight-week-old male BALB/c nude mice were supplied by Orient Bio Inc. (Seongnam-si, South Korea). The mice were housed in the specific pathogen-free facility with a 12-h light/12-h dark cycle and acclimated for 1 week before use. Mice were anesthetized with isoflurane (2%–3% isoflurane in oxygen flow rate of 1.5 L/min) and rinsed with 70% ethanol. After making a 1.0–1.5 cm incision along the linea alba, the cecum was exteriorized by using a pair of straight blunt forceps. Cell suspension of viable tumor cells (1×10^6 scrambled shRNA NRF2 shRNA or ARD1 shRNA HCT-116-Luc2 cells) in equal volumes of PBS and matrigel (total volume of 10 μL) were injected to the subserosal layer of the cecum. After surgery, the peritoneum of the abdominal wall was closed using PERMA-HAND 5.0 gut sutures, and then the skin was closed using surgical silk suture. Luciferase signals from the primary and metastatic tumors were measured weekly using an IVIS *in vivo* animal imaging system (PerkinElmer) following intraperitoneal injection of D-luciferin (LUCK-1G, Goldbio). After administering D-Luciferin (150 mg/kg) intraperitoneally, the

bioluminescence images were obtained using an IVIS *in vivo* animal imaging system and Living Image (ver. 4.5) software (PerkinElmer). All procedures and protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (authorization number: SNU-200702-4-5).

Collection of human specimens

Colorectal tumor and matched adjacent normal colon tissues were obtained from patients with stage I-III CRC aged 19–89 years who were registered in an Institute's tissue bank and received curative surgical resection at Gachon University Gil Medical Center (Incheon, South Korea) between January 2017 and December 2019. Patients with hereditary colorectal cancer and numerous primary cancers were not included. Specimens were frozen in liquid nitrogen and stored in a −70 °C freezer. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association, and the study was approved by Institutional Review Board (IRB) of Gachon University Gil Medical Center (authorization number: GDIRB2020-040).

Immunofluorescent analysis

Human paraffin-embedded colon cancer tissue array slides with matched adjacent normal colon tissues (US Biomax, Inc., cat. no. CO703; Rockville, MD, USA) were subjected to three times deparaffinization with xylene and

rehydration through graded alcohol bath. Following antigen retrieval by heated citrate buffer (pH 6.0), sections were permeabilized and blocked as described previously [37]. Tissue sections were incubated with anti-NRF2 and anti-ARD1 antibodies for dual staining overnight at 4 °C. The tissue sections were washed with PBS and then labeled with FITC-conjugated secondary antibody in combination with TRITC-conjugated secondary antibody for NRF2 and ARD1 detection for 1 h at room temperature. Nuclei were stained with 0.1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The slides were then analyzed under a fluorescent microscope (Nikon; Tokyo, Japan).

Immunocytochemical analysis

Cells were seeded on coverslips and cultured for 48 h. After fixation with 100% methanol for 10 min at −20 °C, cells were blocked with 5% bovine serum albumin and probed with anti-NRF2 together with anti-ARD1 antibodies overnight at 4 °C. Cells were then washed with PBS and were labeled with FITC-conjugated secondary antibody in combination with TRITC-conjugated secondary antibody for 1 h at room temperature. For visualization of the nuclei, cells were further stained with DAPI. The slides were then analyzed under a fluorescent microscope (Nikon; Tokyo, Japan).

Immunohistochemical analysis (IHC)

The colon cancer tissues were prepared for immunohistochemical

analysis with primary antibodies, NRF2 and ARD1. Four-micrometer sections of 10% formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated in a series of xylene and ethanol buffer. The sections were heated in a microwave and boiled twice for 6 min in a 10 mM citrate buffer (pH 6.0) for antigen retrieval. To reduce nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. The slides were incubated with a diluted primary antibody at room temperature for 40 min in Tris-HCl-buffered saline containing 0.05% Tween-20, then incubated with a horseradish peroxidase-conjugated secondary antibody (Dako; Glostrup, Denmark). The tissues were treated with 3,3′-diaminobenzidine tetrahydrochloride to detect the peroxidase binding sites. Finally, counterstaining was performed with Mayer's hematoxylin. The slides were then analyzed by the Automated Multimodal Tissue Analysis System (PerkinElmer, Vectra). The average optical density (AOD) of IHC staining strength of NRF2 and ARD1 was quantitatively analyzed by ImageJ software.

MTT assay

For the MTT assay, 0.5×10^4 cells were seeded in 48-well plates. After incubation for the indicated periods of time, cells were added with the MTT solution (final concentration; 1 mg/mL) for additional 2 h. After removal of the supernatant, 400 μL of DMSO was added to each well. After the formazan crystals had dissolved, 200 μL of resultant solution was

transferred to a 96-well plate. The absorbance at 570 nm was read using a microplate reader.

In vitro acetylation assay

An *in vitro* acetylation assay was performed as described elsewhere [28]. Briefly, 0.5 μg of NFE2L2 (Human) recombinant protein in the absence or presence of ARD1 (Human) recombinant protein was incubated in a total 20 μL of reaction mixture containing 50 mM Tris–HCl (pH 8), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 2 mM acetyl-CoA at 37 °C for 1 h. Reaction products were then subjected to SDS-PAGE and immunoblotted with anti-acetyl lysine antibody. After stripping membrane, input proteins were immunoblotted with indicated antibodies.

Mass spectrometric analysis

Recombinant NRF2 (human) in a final volume of 20 μL 50 mM Tris–HCl (pH 8), containing 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 2 mM acetyl-CoA at 37 °C was incubated for 1 h at 37 °C in the presence or absence of recombinant ARD1 (human) protein. The products of in-gel digestion of recombinant NRF2 pre-incubated with or without recombinant ARD1 were processed according to the procedure as previously described [37]. A Thermo Scientific Quadrupole-Orbitrap instrument (Thermo Fisher Scientific Inc.; Waltham, MA, USA) equipped with Dionex U 3000 RSLCnano HPLC system was used. Mass spectrometric analyses were

performed using a Thermo Scientific Orbitrap Exploris 240 mass spectrometer. Fractions were reconstituted in solvent A [water/acetonitrile (98:2 v/v) containing 0.1% formic acid] and then injected into LC-nano ESI-MS/MS system. Samples were first trapped on an Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper C18, 5 μm, 100 Å, Thermo Scientific, part number 164564) and washed for 6 min with 98% solvent A at a flow rate of 4 μL/min, and then separated on a PepMap RSLC C18 column (75 μm × 15 cm, nanoViper C18, 3 μm, 100 Å, Thermo Scientific, part number ES900) at a flow rate of 300 nL/min. The LC gradient was run at 2% to 8% solvent B over 10 min, then from 8% to 30% over 55 min, followed by 90% solvent B (100% acetonitrile containing 0.1% formic acid) for 4 min, and finally 2% solvent B for 20 min. Xcaliber software version 4.4 was used to collect MS data. The Orbitrap analyzer scanned precursor ions with a mass range of 350–1800 m/z with 60,000 resolutions at m/z 200. Mass data were acquired automatically using proteome discoverer 2.5 (Thermo Fisher Scientific Inc.; Waltham, MA, USA).

Statistical analysis

Comparison of differences between two experimental conditions analyzed using the student's *t*-test. Differences among more than two groups were assessed by one-way ANOVA. Differences among more than three conditions were analyzed using two-way ANOVA. The data are presented as the mean ± standard deviation (S.D.) and a value of $P < 0.05$ was considered

statistically significant. All the statistical analyses were applied using GraphPad Prism 8.0 (GraphPad Software; San Diego, CA, USA).

3. Results

3.1. Correlation of NRF2 and ARD1 with CRC

As an initial approach to explore how NRF2 and ARD1 are related to each other in the progression of CRC, their expression was analyzed on human CRC tissue microarray by immunofluorescence staining. As illustrated in **Figure 6**, the expression of NRF2 and ARD1 was upregulated in the CRC tumor tissues compared with that in the normal tissues. Pearson correlation analysis indicated that ARD1 was positively correlated with NRF2 (**Figure 7A**). To further confirm these results, we examined the protein expression levels of NRF2 and ARD1 in colorectal cancer specimens with their matched adjacent normal colorectal tissues. Immunohistochemical analysis revealed that NRF2 and ARD1 were elevated in tumor tissues compared to adjacent normal tissues, and these two proteins were colocalized in both cytoplasm and nucleus in tumor tissues (**Figure 7B**). Western blot analysis also showed that both NRF2 and ARD1 were upregulated in tumor tissues in most cases (**Figure 8**). Moreover, the NRF2 and ARD1 were coordinately overexpressed in different human CRC cell lines (HCT-116, HCT-15, and DLD1) compared with the normal human colon epithelial (CCD841CoN) cells (**Figure 9**). Thus, elevated levels of NRF2 and ARD1 are likely to be associated with CRC development and progression.

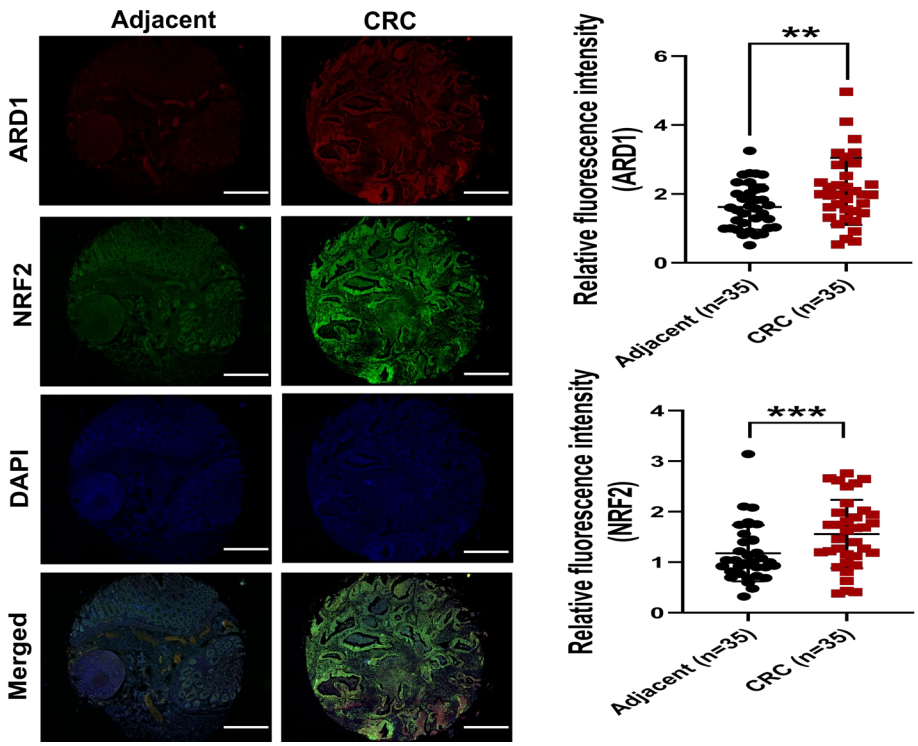


Figure 6. Immunofluorescence staining of tissue microarray.

Immunofluorescence staining of tissue microarray containing thirty-five pairs of colon adenocarcinoma and matched adjacent normal colon tissues was measured using anti-NRF2 and anti-ARD1 antibodies. Nuclei were stained with DAPI. Each scale bar represents 500 μm. The fluorescence intensity was analyzed by the image processing program Image J. Results are analyzed by two-tailed paired Student's *t*-test, expressed as means ± S.D. ($n = 35$ each). $^{**}p < 0.01$; $^{***}p < 0.001$.

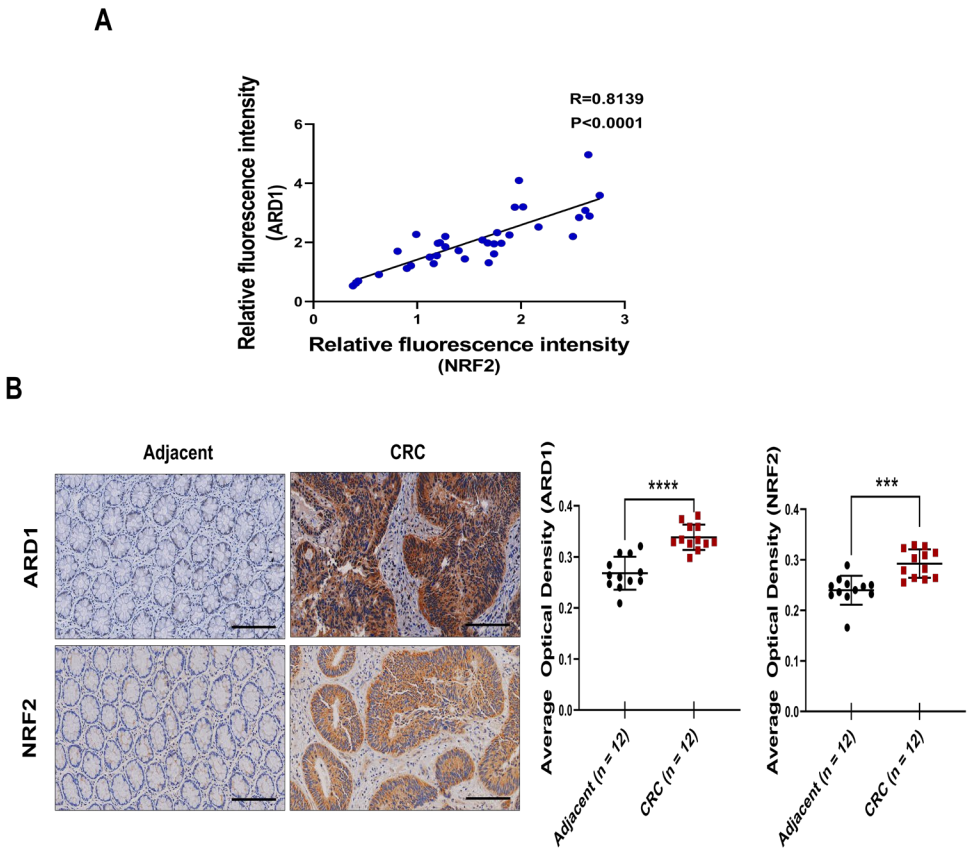


Figure 7. Correlation between ARD1 and NRF2 and their expression in CRC patient's samples.

(A) Pearson correlation analysis of the fluorescence intensity score showed a positive correlation of ARD1 with NRF2 (r = 0.8139). (B) Immunohistochemical analysis of ARD1 and NRF2 was performed on sections from CRC tissues using anti-NRF2 and anti-ARD1 antibodies. Each scale bar represents 200 μm. The average optical density (AOD) of IHC staining intensity of NRF2 and ARD1 was quantitatively analyzed by

the ImageJ software. DATA are revealed as the mean ± S.D. of twelve pairs of human tissue specimens, analyzed by two-tailed paired Student's *t*-test. ***$p < 0.001$; ****$p < 0.0001$.

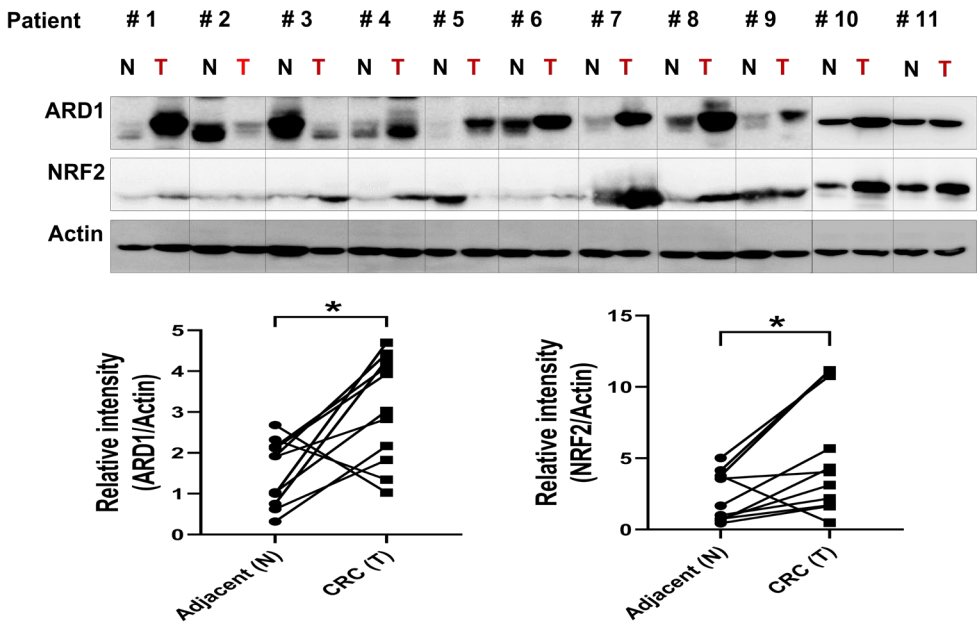


Figure 8. The expression of ARD1 and NRF2 in CRC patients' tissues.

Whole lysates of CRC tissues were measured by Western blot analysis using an anti-ARD1 or anti-NRF2 antibodies. The quantitative data are presented as the mean ± S.D. of eleven pairs of human tissue specimens, analyzed by two-tailed paired Student's *t*-test. *$p < 0.05$.

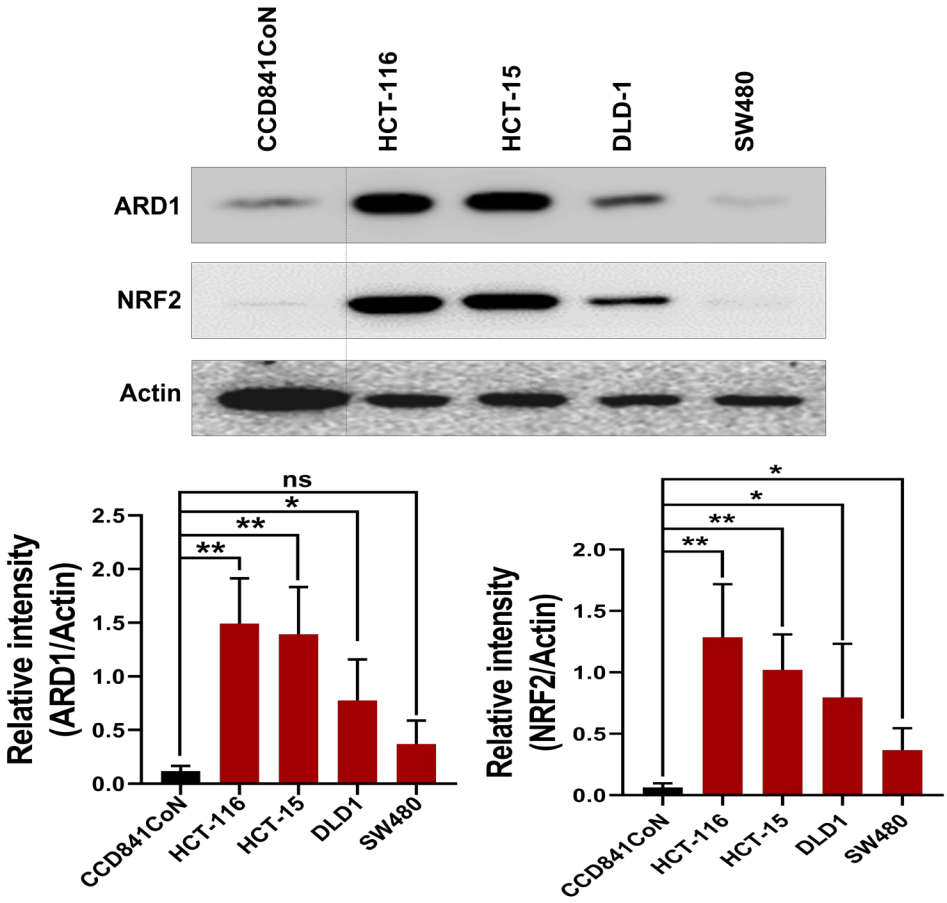


Figure 9. The expression of ARD1 and NRF2 in CRC cell lines.

The protein levels of ARD1 and NRF2 in different human CRC cell lines (HCT-116, HCT-15, and DLD-1) as well as normal human colon epithelial CCD841CoN cells were assessed by immunoblot using anti-NRF2 and anti-ARD1 antibodies. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-tailed unpaired Student's t -test. *$p < 0.05$; **$p < 0.01$; ns, not significant.

3.2 Knockdown of NRF2 and ARD1 attenuates oncogenicity of CRC cells

To further investigate the potential involvement of NRF2 and ARD1 in human CRC progression, both proteins were individually silenced in human CRC cells (HCT-116) by transfection with their specific siRNA. I noticed that silencing of NRF2 or ARD1 significantly reduced the cell viability (**Figure 10A**) with a concomitant increase in apoptotic cell death (**Figure 10B**). Moreover, the clonogenic (**Figure 11A**) and migrative (**Figure 11B**) capacities were also reduced in NRF2 and ARD1 knockdown cells. To confirm these results *in vivo*, scrambled shRNA, NRF2 shRNA, or ARD1 shRNA HCT-116-Luc2 cells were orthotopically transplanted to the cecum wall of BALB/c nude mice. I found that stable knockdown of NRF2 or ARD1 markedly retarded the tumor growth (**Figure 12**) as compared with that observed in mice inoculated with HCT-116-Luc scrambled shRNA cells.

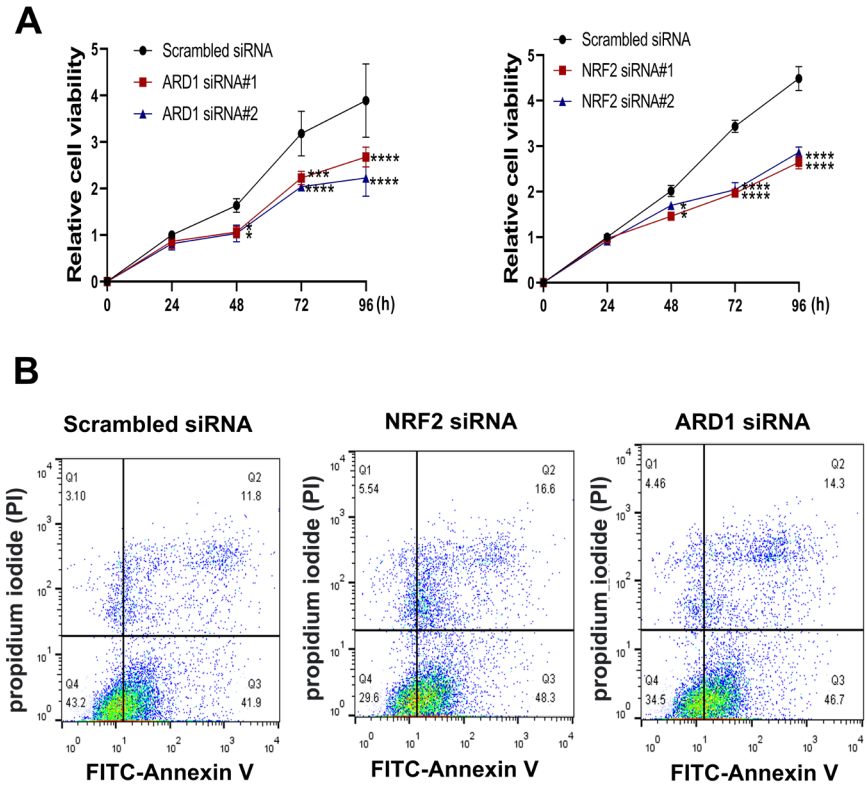


Figure 10. Effects of NRF2 and ARD1 depletion on the viability of HCT-116 cells.

(A) HCT-116 cells were transiently transfected with scrambled, ARD1 or NRF2 siRNA for the indicated period of time. The cell viability was measured by the MTT assay as described in Materials and Methods. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-way ANOVA. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. (B) HCT-116 cells were transiently transfected with scrambled, ARD1 or NRF2 siRNA for 24 h. The early, late apoptosis, and

necrotic cell death were detected by flow cytometric analysis.

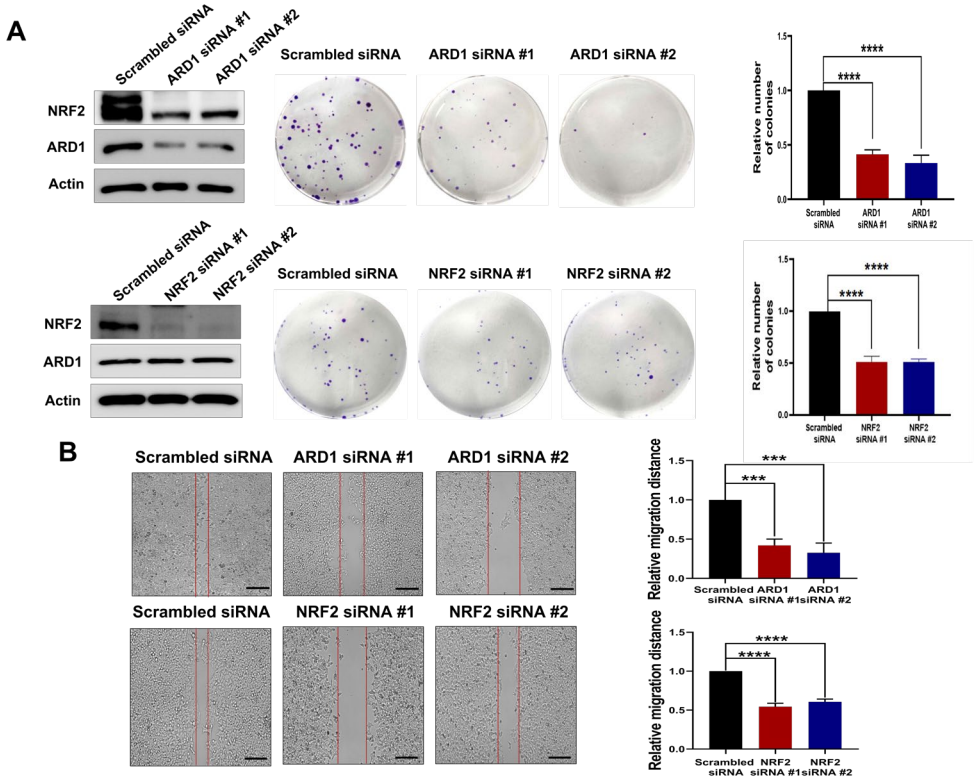


Figure 11. Effects of NRF2 and ARD1 suppression on the clonogenic and migration capacities of HCT-116 cells.

(A) HCT-116 cells were transiently transfected scrambled, ARD1 or NRF2 siRNA for 24 h, and clonogenic efficiency of cells was measured as described in Materials and methods. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. ****$p < 0.0001$. (B) For wound healing assays, HCT-116 cells were transiently transfected scrambled, ARD1 or NRF2 siRNA for 24 h and were then plated into culture-insert (Ibidi). After 24-h incubation, cell migration was measured. Each scale bar represents 500 μm. Data are shown

as the mean ± S.D. of three independent experiments, analyzed by one- way ANOVA. ***$p < 0.001$; ****$p < 0.0001$.

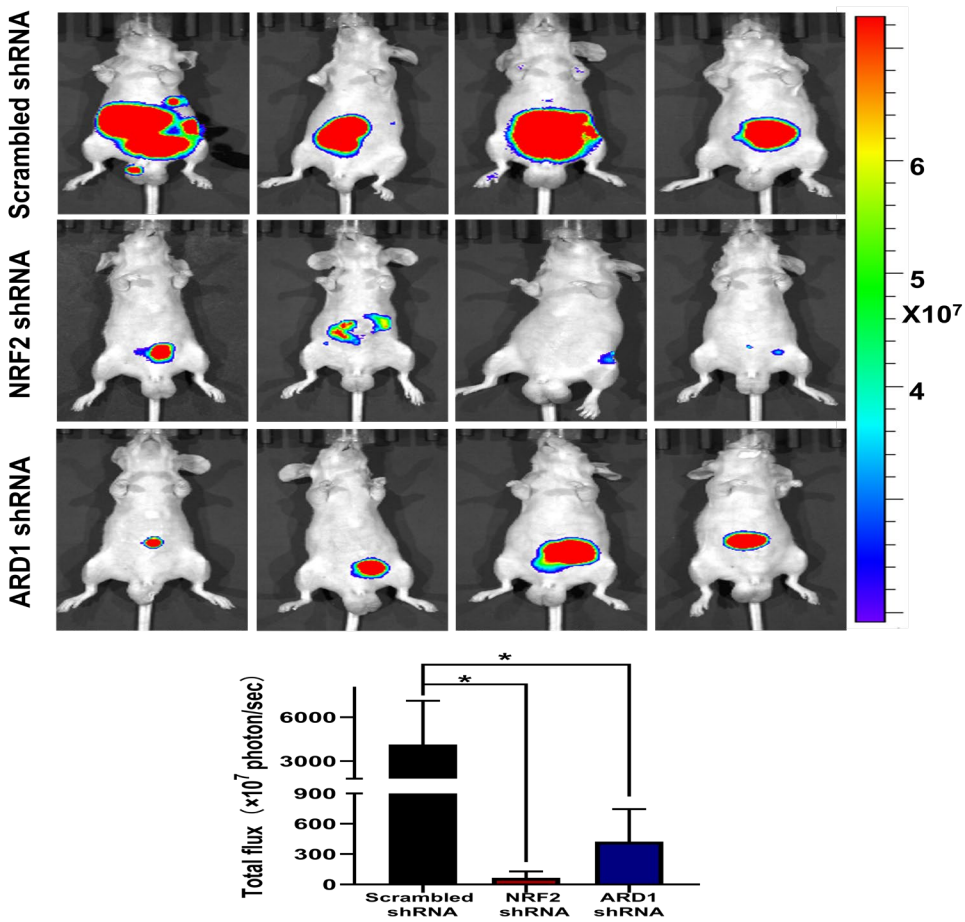


Figure 12. Effects of NRF2 and ARD1 knockdown on the tumor growth *in vivo*.

Scrambled shRNA, NRF2 shRNA, or ARD1 shRNA transfected HCT-116-Luc2 cells were injected to the cecum subserosal layer of BALB/c nude mice. IVIS images for visualizing primary tumors and quantification. The quantitative data are shown as the mean ± S.D. (n = 4 per group), analyzed by one-way ANOVA. *$p < 0.05$.

3.3 ARD1 knockdown reduces the stability of NRF2 in human colon cancer cells

To verify the correlation between NRF2 and ARD1 in CRC cells, I suppressed their expression individually in different CRC cell lines. Genetic inhibition of ARD1 by siRNA abolished the NRF2 expression in HCT-116 cells (**Figure 13A**) and other CRC cell lines (**Figure 14**). In contrast, there was no change in the protein level of ARD1 by silencing of NRF2 (**Figure 13B**). Similar observations were made by and fluorescence-activated cell sorting (**Figure 13C**) and immunocytochemical analyses (**Figure 15**). To investigate the subcellular localization of NRF2 and ARD1, I isolated cytoplasmic and nuclear fractions in ARD1 knockdown HCT-116 cells. Silencing of ARD1 did not influence the expression levels of cytoplasmic NRF2 protein, but the nuclear accumulation of NRF2 was significantly attenuated in the ARD1 silenced cells with or without hydrogen peroxide (H_2O_2) stimulation (**Figure 16**).

To determine whether the observed changes in the NRF2 protein level in response to ARD1 interference was due to suppression of NRF2 transcription, the NRF2 mRNA level was measured by qPCR. As shown in **Figure 17A**, the NRF2 mRNA level remained unchanged in the presence or absence of ARD1. Furthermore, to test that silencing of ARD1 could decrease the NRF2 protein stability, I then measured preexisting NRF2 protein levels in the presence of CHX, a protein synthesis inhibitor. Notably,

silencing of ARD1 led to a marked decrease in the half-life of NRF2 (**Figure 17B**).

Like the majority of other proteins, the central mechanism regulating intracellular levels of NRF2 is ubiquitination and subsequent proteasome-mediated degradation [3]. Treatment with a proteasome-specific inhibitor (MG-132) abolished the degradation of NRF2 stimulated by knockdown of ARD1 (**Figure 18A**). These data implicate that ARD1 may prevent proteasome-mediated degradation of NRF2. Furthermore, the level of ubiquitinated NRF2 (Ub-NRF2) was significantly elevated in cells with transient (**Figure 18B**) or stable knockdown (**Figure 18C**) of ARD1. This effect was more prominent by ectopic overexpression of ubiquitin in ARD1 stable knockdown cells (**Figure 18D**). Taken together, these data lend support to the speculation that ARD1 may stabilize the NRF2 protein.

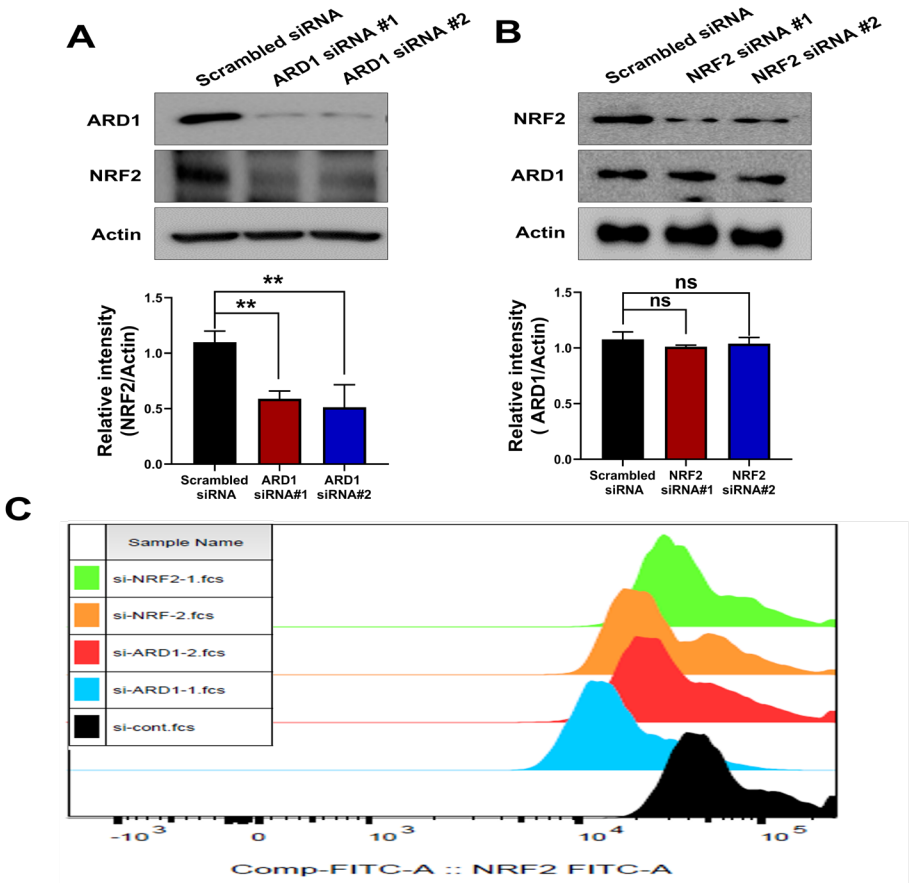


Figure 13. Effects of ARD1 knockdown on the NRF2 expression in HCT-116 cells.

HCT-116 cells were transiently transfected with scrambled, two different ARD1 or two different NRF2 siRNAs for 24 h. The expression of ARD1 and NRF2 was measured by Western blot (A and B) or fluorescence-activated cell sorting analyses (C). The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. $^{**}p < 0.01$; ns, not significant.

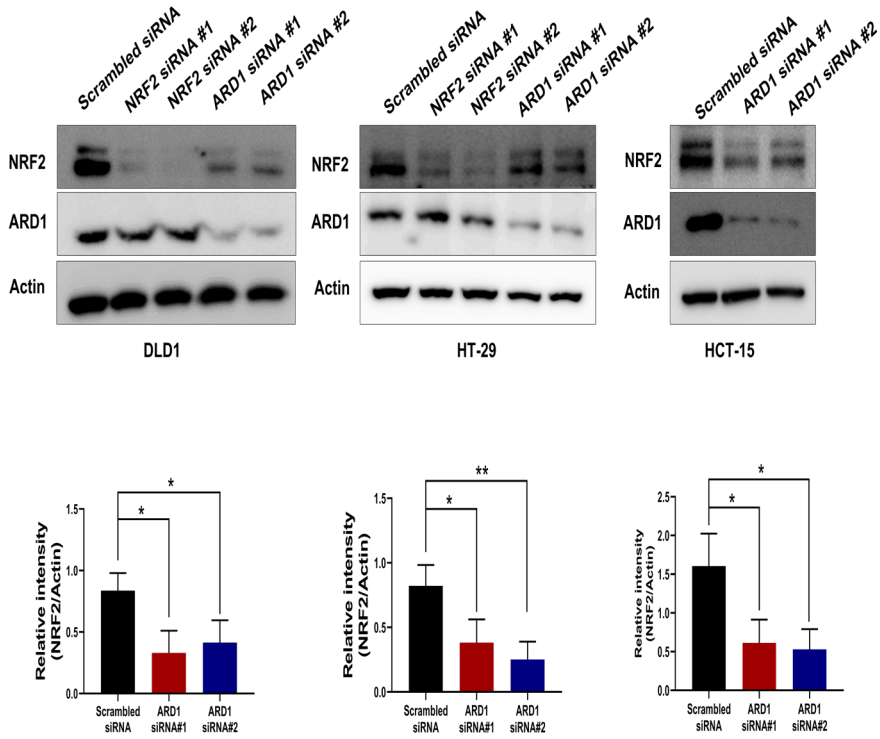


Figure 14. Attenuation of NRF2 expression in ARD1 silenced CRC cell lines.

Following transient transfection of ARD1 siRNA or NRF2 siRNA in CRC cell lines, whole-cell lysates were subjected to immunoblot with an anti-ARD1 or anti-NRF2 antibodies. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. *$p < 0.05$; **$p < 0.01$.

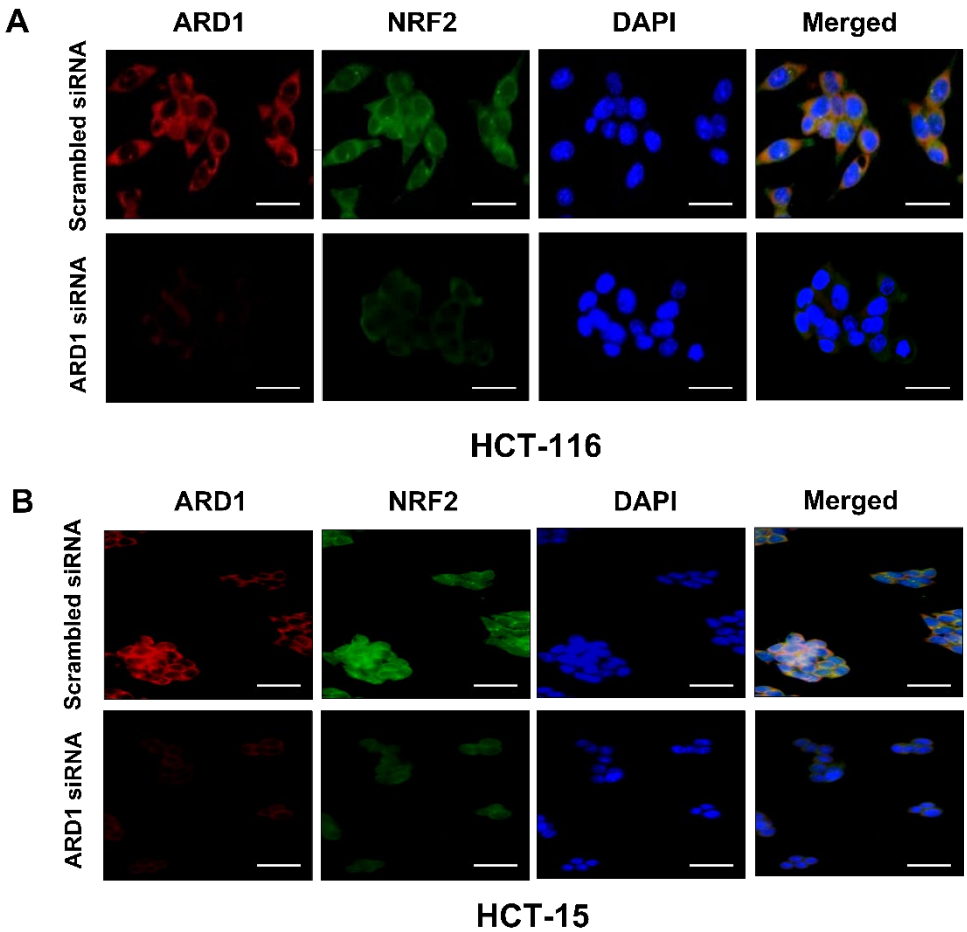


Figure 15. Reduced NRF2 expression in ARD1-silenced CRC cell lines. Immunocytochemical analysis was performed using anti-NRF2 together with anti-ARD1 antibodies in ARD1 knockdown HCT-116 (A) or HCT-15 (B) cells. Nuclei were stained with DAPI. Scale bar represents 200 μm.

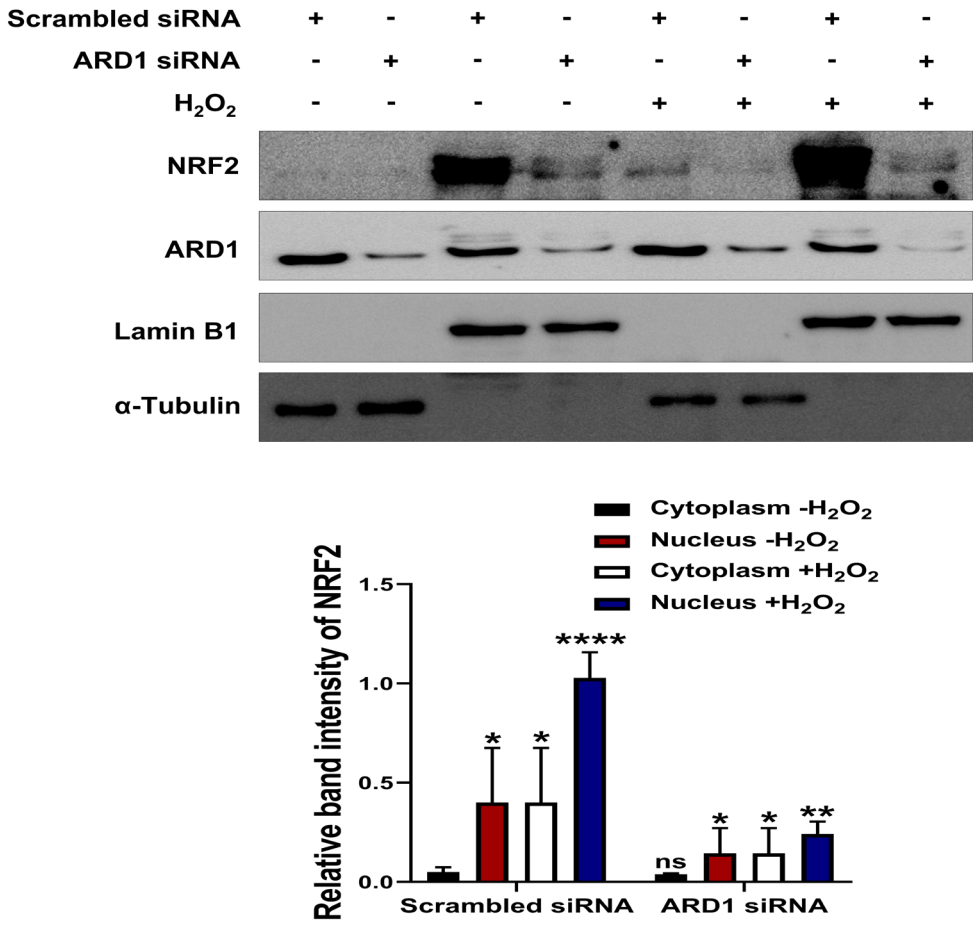


Figure 16. Distribution of NRF2 and ARD1 in HCT-116 cells.

HCT-116 cells were treated with or without H_2O_2 (0.5 mM) for 3 h in the absence or presence of ARD1. Cells were harvested and separated into nuclear and cytosolic fractions. The protein lysates were subjected to immunoprecipitation followed by Western blot analysis. The fractions were immunoblotted for lamin B1 as a nuclear marker and α-tubulin as a cytosolic marker. The relative intensity of NRF2 in each group was measured and presented after normalization to the corresponding control

(scrambled siRNA without H_2O_2 treatment). The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-way ANOVA. *$p < 0.05$; ****$p < 0.0001$; ns, not significant.

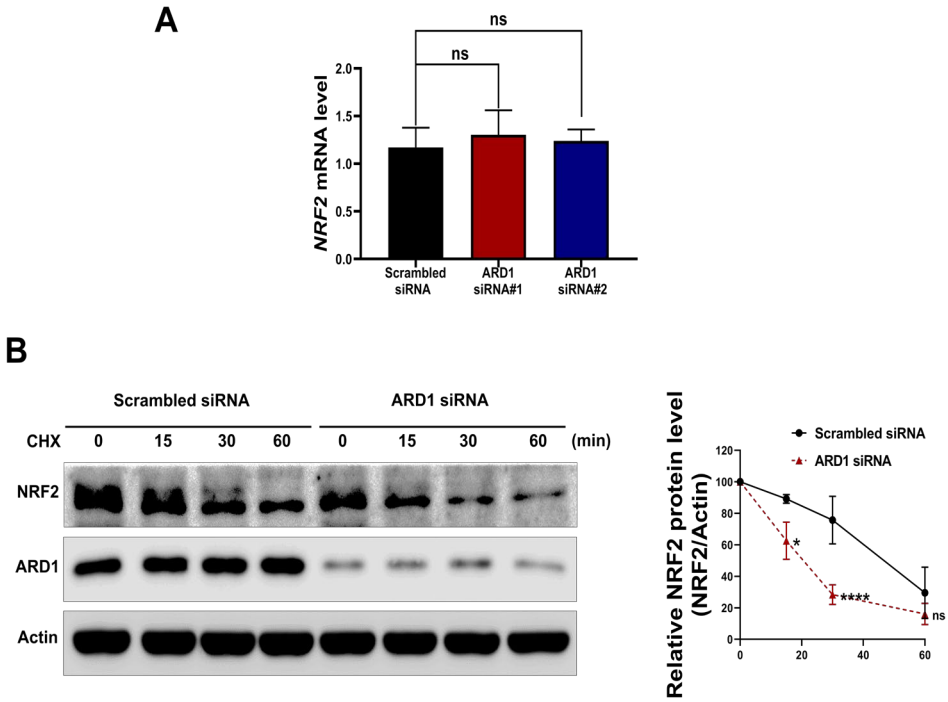


Figure 17. Shortened half-life of NRF2 in ARD1 knockdown HCT-116 cells.

(A) The mRNA levels of NRF2 were carried out by qPCR in the presence or absence of ARD1. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. (B) HCT-116 cells were treated with CHX (177.71 μM) for indicated periods in the absence or presence of ARD1. The relative protein levels of NRF2 remaining at different time points were assessed. Actin was used as the internal control. NRF2 protein levels were quantified using Image J, and band intensities were normalized to those of actin (band intensity at t_0 was defined as 100%). The quantitative data are shown as the mean ± S.D. of

three independent experiments, analyzed by two-way ANOVA. *$p < 0.05$; ****$p < 0.0001$; ns, not significant.

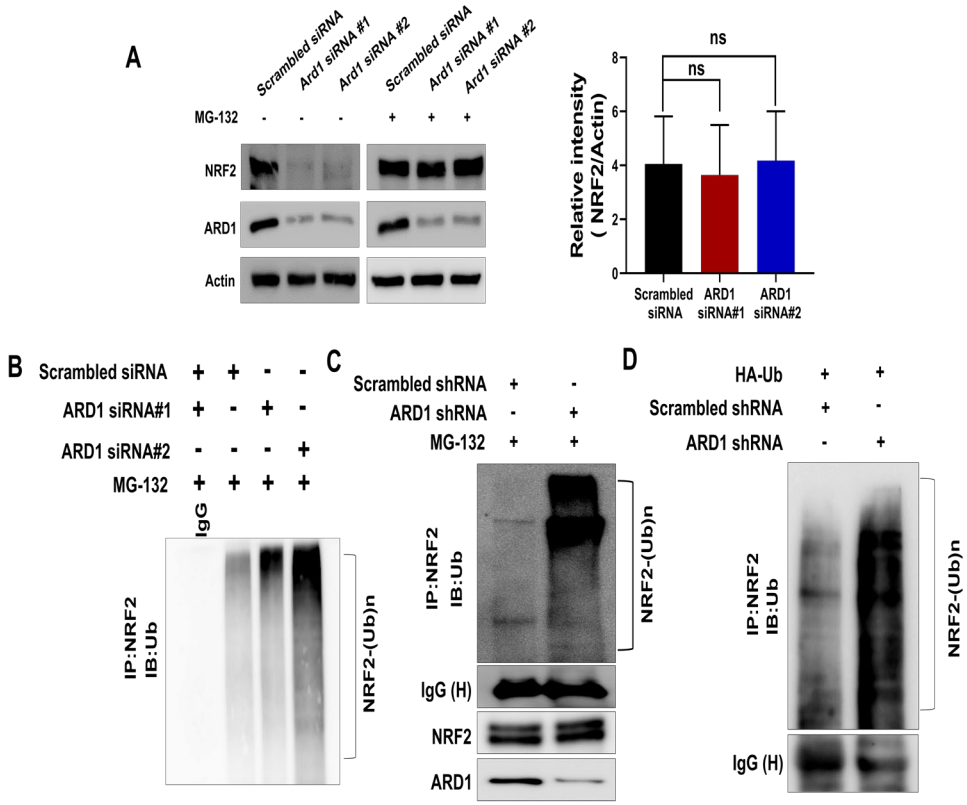


Figure 18. The effect of ARD1 on NRF2 ubiquitination.

(A) HCT-116 cells were transiently transfected with scrambled or ARD1 siRNA for 24h, followed by MG-132 (20 μM) treatment for 6 h. The protein levels of NRF2 were measured by Western blot analysis. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. (B) After silencing of ARD1, followed by MG-132 (20 μM) treatment for 6 h. Whole-cell lysates were subjected to immunoprecipitation with an anti-NRF2 antibody, and Western blot analysis was performed using an antibody against ubiquitin. (C) Following treatment of scrambled or ARD1 stable knockdown HCT-116 cells with MG-132 (20

μM) for 6 h, the ubiquitination of endogenous NRF2 was immunoprecipitated and immunoblotted using an antibody against ubiquitin. (D) ARD1 stable knockdown HCT-116 cells were transiently transfected HA-ubiquitin for 48 h, followed by MG-132 (20 μM) treatment for 6 h. Whole-cell lysates were subjected to immunoprecipitation with an anti-NRF2 antibody. The ubiquitination of NRF2 was measured by Western blot analysis using an antibody against ubiquitin.

3.4 ARD1 physically interacts with NRF2

As ARD1 co-localizes with NRF2 in human CRC cells (**Figure 15 and Figure 16**), I examined whether these proteins could physically interact with each other. I found that there was a strong endogenous (**Figure 19A)** and exogenous (**Figure 19B**) interaction between ARD1 and NRF2 in HCT-116 cells. The interaction was verified by the PLA (**Figure 20A**) which detects enhanced fluorescent signal when two proteins are localized in proximity. Furthermore, a similar result was obtained with CRC patients' tissue samples by immunoprecipitation analysis (**Figure 20B**).

NRF2 is composed of seven functional NRF2-ECH homology (Neh) structural domains (Neh1 to 7) (**Figure 21A**). In order to better understand which NRF2 structural domain(s) is/are involved in the interaction with ARD1, full-length NRF2 (NRF2 WT) and three truncated NRF2 mutants (NRF2 ΔD1, ΔD2, and ΔD3) were expressed ectopically in HCT-116 cells together with ARD1 (**Figure 21A**). Notably, ARD1 was prominently present in immunoprecipitates of full-length NRF2 containing the Neh1 and Neh3 structural domains (**Figure 21B**) that play important roles in NRF2 binding to DNA and transactivation, respectively [3].

Next, to monitor the transcriptional activity of NRF2, I transfected ARE reporter plasmid (ARE-luc) or an empty vector (pTi-luciferase) in HCT-116 cells. Under ARD1 knockdown conditions, the transcriptional activity of NRF2 was significantly dampened (**Figure 22A**). In another experiment, ectopic expression of ARD1 promoted nuclear translocation of NRF2

(**Figure 22B**). Taken together, these results suggest that the direct interaction of ARD1 with NRF2 facilitates nuclear localization and transcriptional activation of the latter protein.

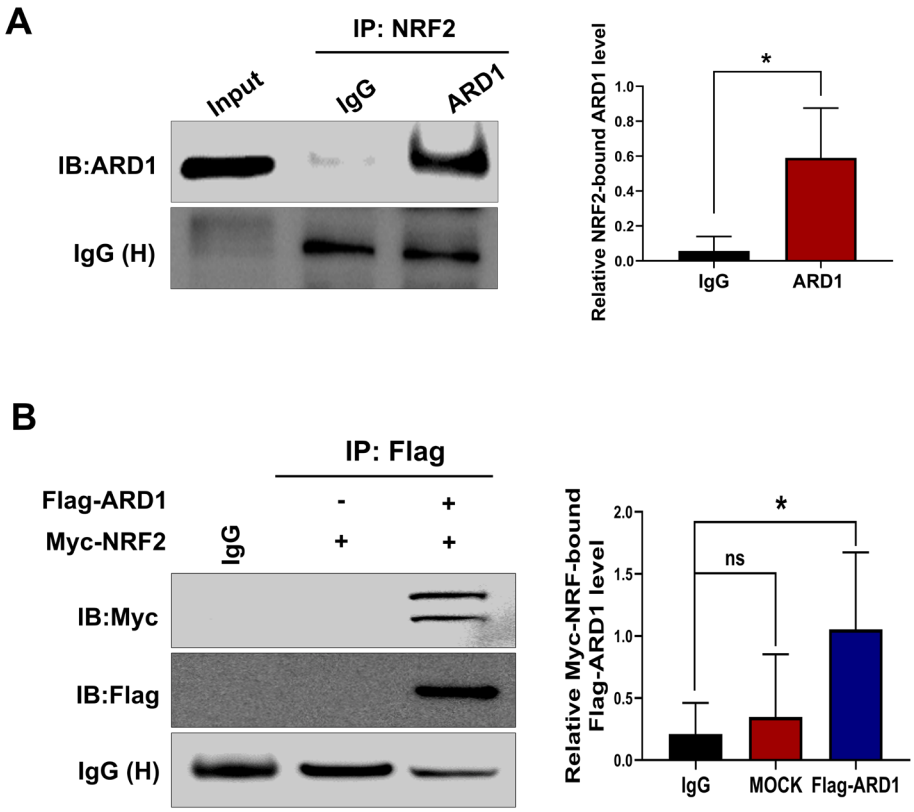


Figure 19. Interaction between ARD1 and NRF2.

(A) Whole-cell lysates from HCT-116 cells were subjected to immunoprecipitation with anti-ARD1 antibody, and Western Blot analysis was performed using anti-NRF2 antibody. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-tailed unpaired Student's t-test. *$p < 0.05$. (B) Following co-transfection of HCT-116 cells with mock, Flag-ARD1 or Myc-NRF2, whole-cell lysates were subjected to immunoprecipitation with an anti-Flag antibody, and

Western blot analysis was performed using an anti-Myc and anti-Flag antibodies. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-tailed unpaired Student's t-test. *$p < 0.05$; ns, not significant.

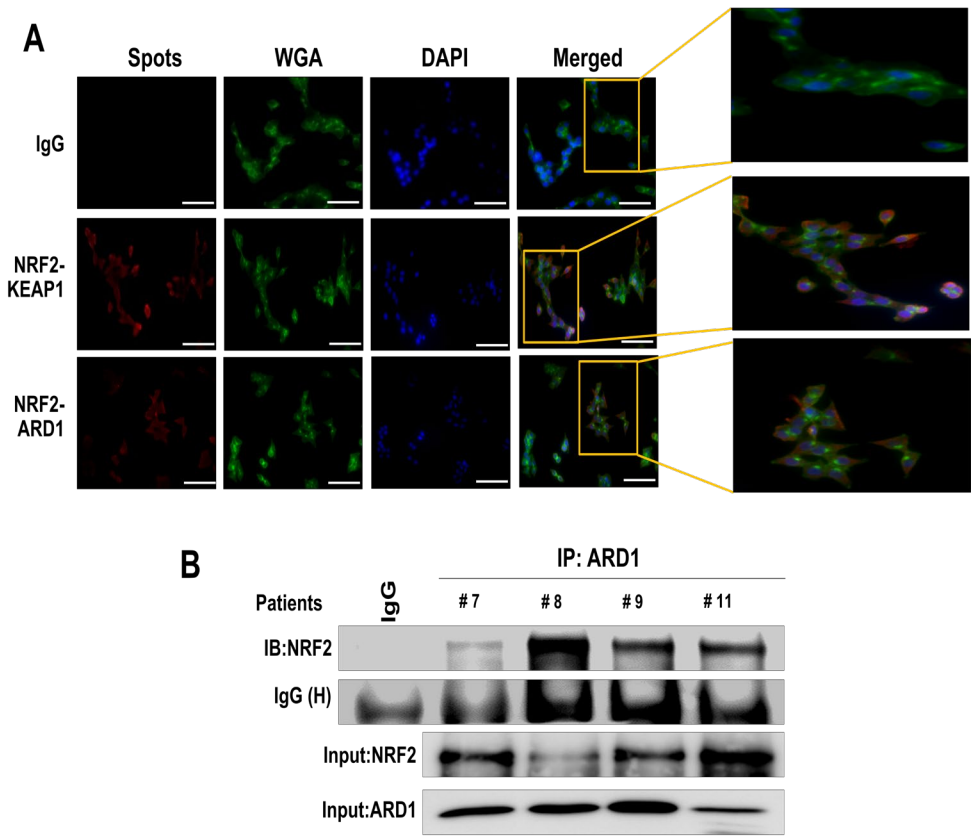


Figure 20. ARD1 directly interacts with NRF2.

(A) Detection of ARD1-NRF2 interaction *in situ*. The interaction of ARD1 with NRF2 was visualized by Duolink analysis (PLA). ARD1, NRF2, and KEAP1 were co-labeled with corresponding antibodies. The interaction of NRF2 with KEAP1 was used as a positive control group. Nuclei were counterstained with DAPI. Cell membranes were stained with wheat germ agglutinin. Each scale bar represents 200 μm. (B) Whole-cell lysates from CRC patients' tumor tissues were subjected to immunoprecipitation with

anti-ARD1 antibody, and Western Blot analysis was performed using anti-NRF2 antibody.

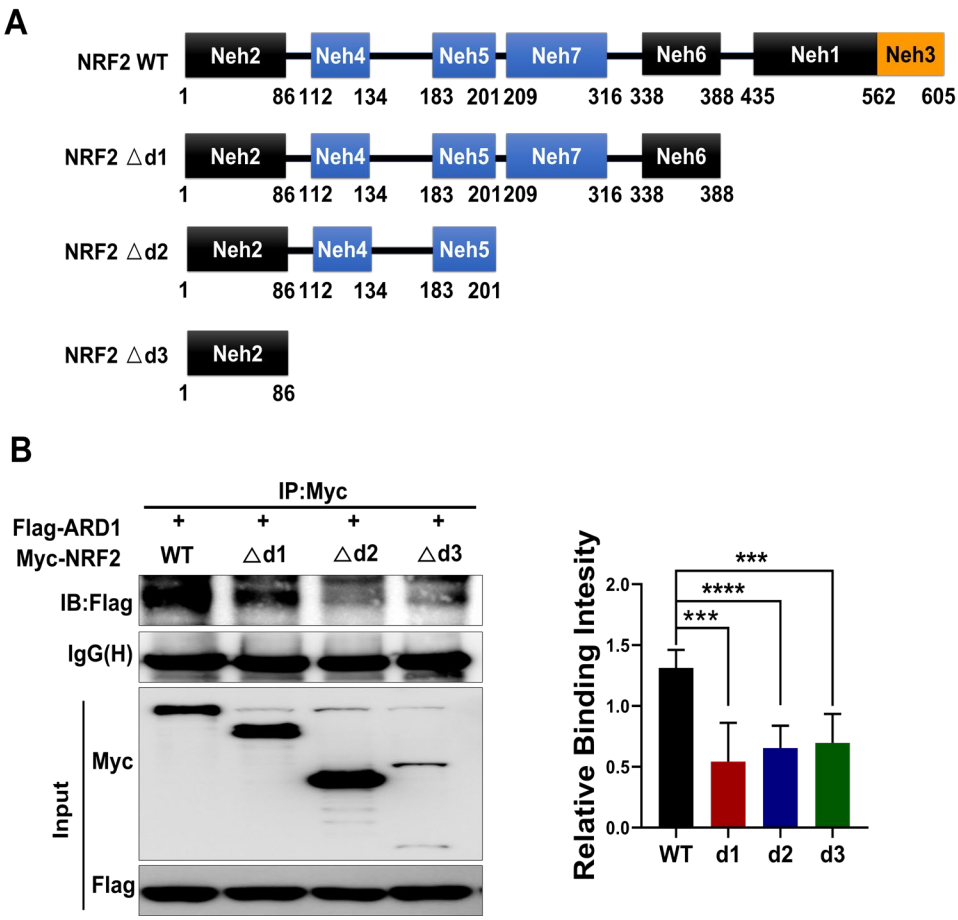


Figure 21. ARD1 interacts with NRF2 structural domains.

(A) Schematic depiction of NRF2 WT and deletion mutants of Myc-tagged NRF2. (B) Following transfection with truncated Myc-NRF2 and Flag-ARD1 in HCT-116 cells, whole-cell lysates were immunoprecipitated with an anti-Myc antibody, and immunoblotted with antibody for Flag. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one way ANOVA. ***$p < 0.001$; ****$p < 0.0001$.

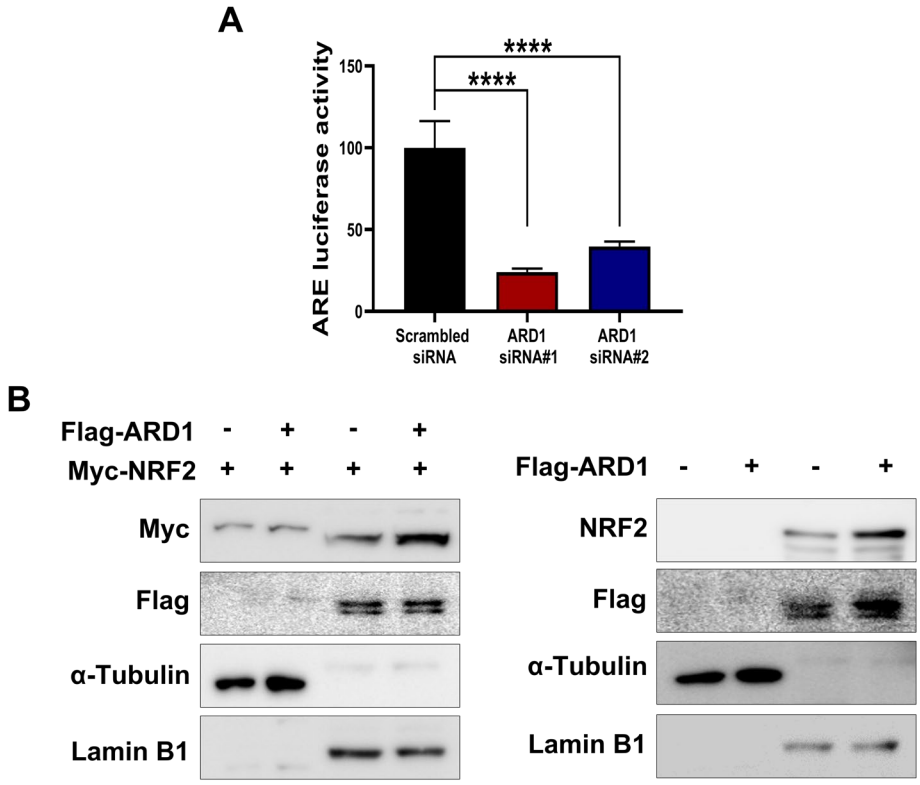


Figure 22. ARD1 promotes nuclear translocation of NRF2

(A) HCT-116 cells were transiently transfected with scrambled or ARD1 siRNA. After 24 h, the cells were transfected with the ARE luciferase reporter gene. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by one-way ANOVA. ****$p < 0.0001$. (B) Following transfection with mock, Flag-ARD1 or Myc-NRF2 in HCT-116 cells, both cytosolic and nuclear extracts were prepared and subjected to immunoblot analysis using anti-Myc (left panel) or anti-NRF2 (right panel)

antibodies. α-Tubulin and lamin B1 were used as cytosolic and nuclear markers, respectively.

3.5 ARD1 decreases cellular responses to oxidative stress in HCT-116 cells.

NRF2 is a major regulator of cellular responses to oxidative stress [1]. Conversely, Shin et al. reported that ARD1 may increase intracellular ROS by repressing the enzymatic function of methionine sulfoxide reductase A via acetylating its K49 residue in human lung carcinoma cells [26]. Since these two proteins are inconsistent in regulating ROS, prompting me to investigate the role of ARD1 in oxidative stress in CRC cells. I measured the levels of ROS in ARD1 knockdown HCT-116 cells with or without hydrogen peroxide (H_2O_2) incubation. As shown in **Figure 23A and 23B**, the population of DCF fluorescence-positive HCT-116 cells was significantly increased by ARD1 knockdown in both unstimulated and H_2O_2-stimulated conditions. Thus, it seems likely that ARD1 may regulate intracellular ROS by stabilizing NRF2 in HCT-116 colon cancer cells.

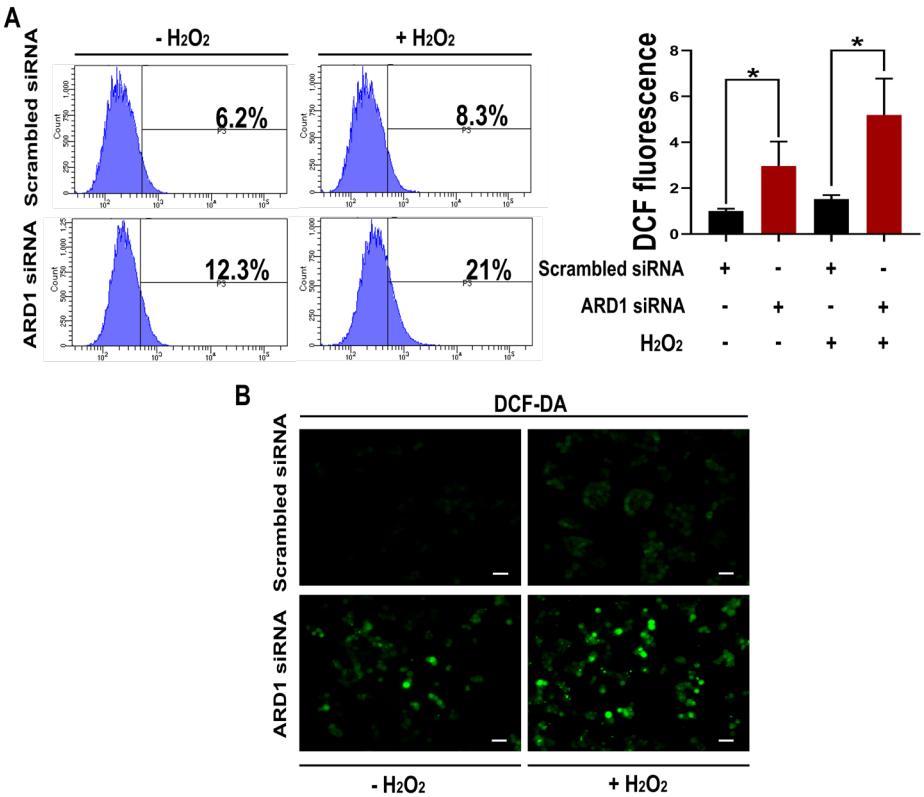


Figure 23. ARD1 reduces oxidative stress in cells through stabilizing NRF2

HCT-116 cells were transiently transfected with scrambled or ARD1 siRNA for 24 h. Cells were treated with or without H_20_2 (0.5 mM) for 3 h. Whole cells were stained by 2′,7′-Dichlorofluorescin diacetate (DCF-DA) solution for 0.5 h, and the total intensity of DCF fluorescence in each group was measured by flow cytometry (A) or fluorescent microscopy (B). The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-tailed unpaired Student's t-test. *$p < 0.05$.

3.6 NRF2 is acetylated by ARD1

The stability of NRF2 has been reported to be regulated by PTMs. As ARD1 has lysine acetyltransferase activity, this prompted our interest in whether ARD1 could acetylate NRF2. As depicted in **Figure 24A**, ectopic expression of ARD1 induced the acetylation of NRF2. Consequently, I performed *in vitro* acetylation assays in order to determine whether NRF2 could be a putative substrate of ARD1. I noticed that recombinant GST-NRF2 was directly acetylated by recombinant GST-ARD1 (**Figure 24B**). However, even in the absence of recombinant GST-ARD1, recombinant GST-NRF2 was still acetylated. This finding is consistent with the previous studies demonstrating that the target protein undergoes the non-enzymatic acetylation in the *in vitro* acetylation experiments [38].

It has been reported that autoacetylation of ARD1 at K136 and active sites of R82/Y122 are essential for the catalytic activity of ARD1[24, 28]. To ensure that lysine acetyltransferase activity of ARD1 contributes to the NRF2 stability, a low acetyltransferase activity mutant (R82A/Y122F) [39, 40] and a non-acetylatable mutant in which lysine is replaced by arginine (K136R) were utilized. The cells transfected with acetylation defective ARD1 mutants exhibited reduced stability of NRF2 compared with those expressing functional ARD1 (**Figure 25**).

In order to identify the acetylated site(s) of NRF2, recombinant GST-NRF2 reacted with recombinant ARD1 in the presence of acetyl-CoA was subjected to nano liquid chromatography-tandem mass spectrometry (nano-

LC-ESI MS/MS), which revealed four lysine residues (K438, K462, K596, and K598) as putative acetylation sites on NRF2 (**Figure 26 and Figure 27**). As the control group (without recombinant ARD1) failed to generate the acetylation at the K438 (**Figure 28**), I speculated that this particular lysine residue may be the acetylation site of NRF2 (**Figure 27**).

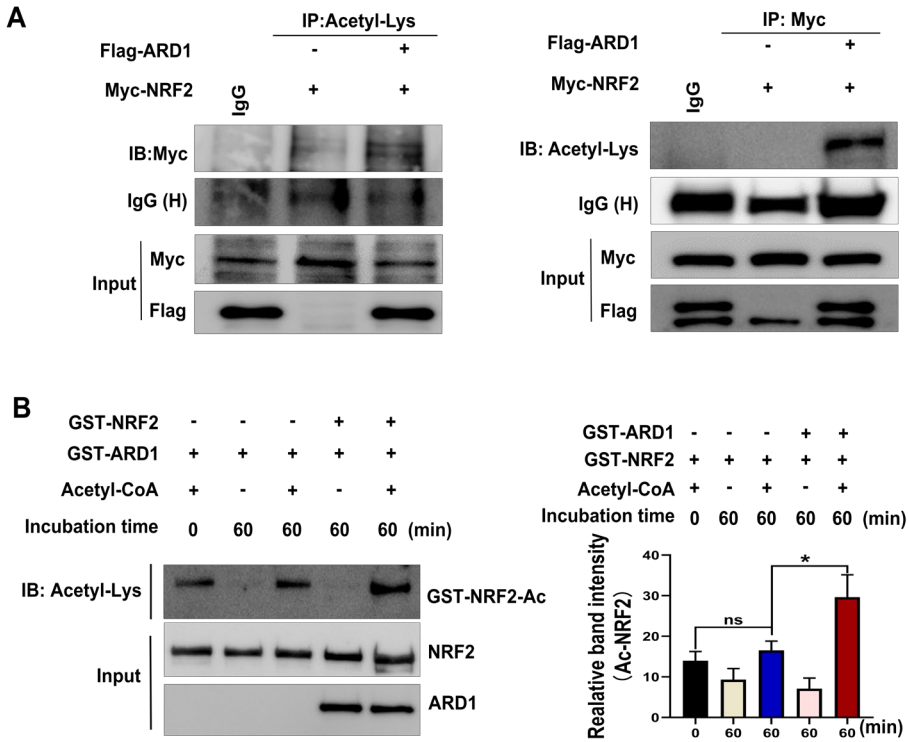


Figure 24. ARD1 acetylates NRF2.

(A) Following transfection of HCT-116 cells with Myc-NRF2 in the absence or presence of Flag-ARD1, whole-cell lysates were subjected to immunoprecipitation with an anti-acetylated lysine or anti-Myc antibodies, and Western blot analysis was performed using anti-Myc or anti-acetylated lysine antibodies. (B) Recombinant GST-NRF2 was subjected to *in vitro* acetylation assays with or without recombinant GST-ARD1. Acetylated GST-NRF2 was detected by immunoblot analysis using anti-acetyl lysine antibody. The quantitative data are shown as the mean ± S.D. of three

independent experiments, analyzed by two-way ANOVA. *$p < 0.05$; ns, not significant.

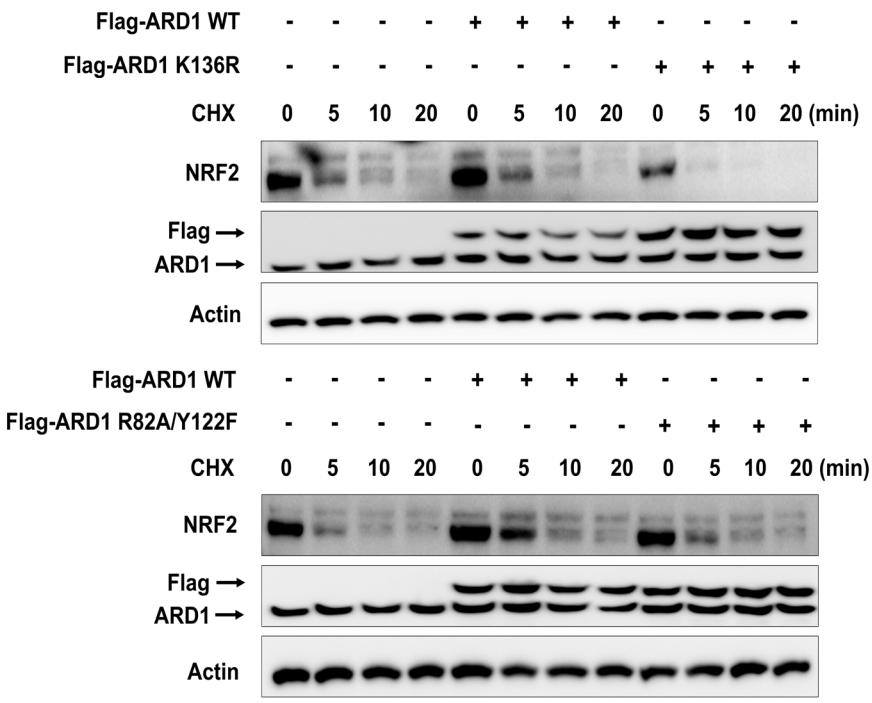


Figure 25. ARD1 mutants shorten the half-life of NRF2.

HCT-116 cells were transiently transfected with Flag-ARD1-WT, Flag-ARD1-K136R, or Flag-ARD1-R82A/Y122F, followed by CHX treatment for indicated time periods. The protein levels of NRF2 were measured by Western blot analysis.

GST-NRF2 + GST-ARD1 :

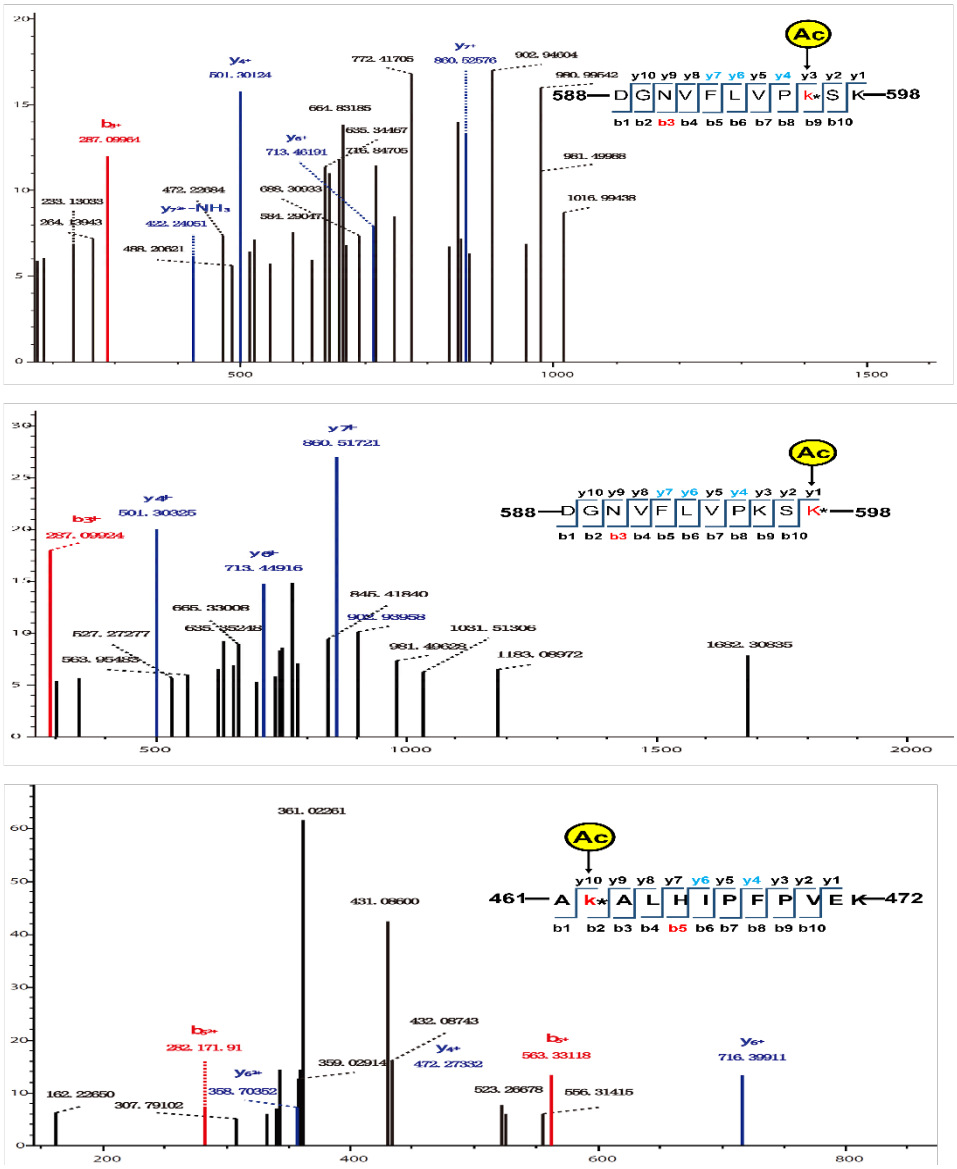


Figure 26. The putative acetylation sites of recombinant GST-NRF2 were identified by nano-LC-ESI MS/MS (with recombinant GST-ARD1).

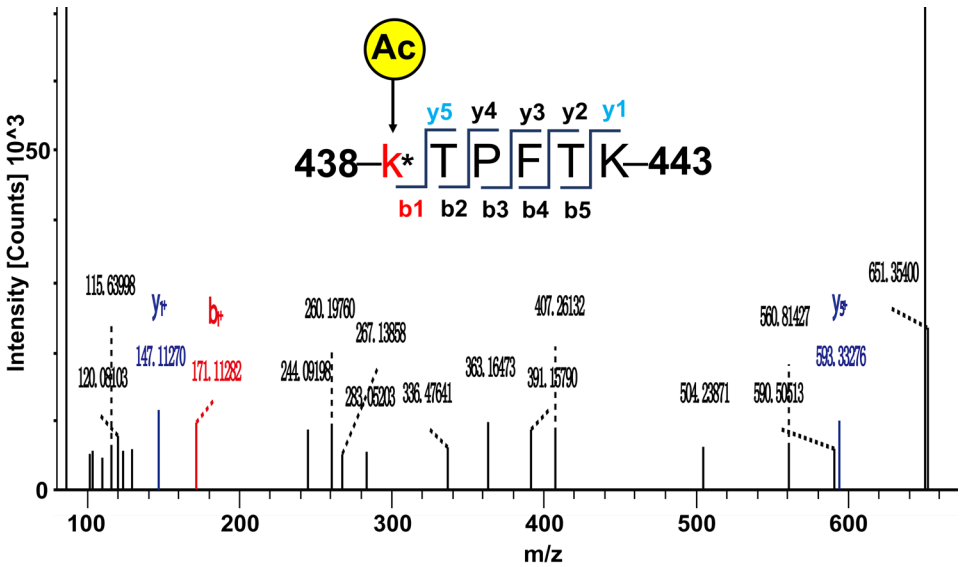
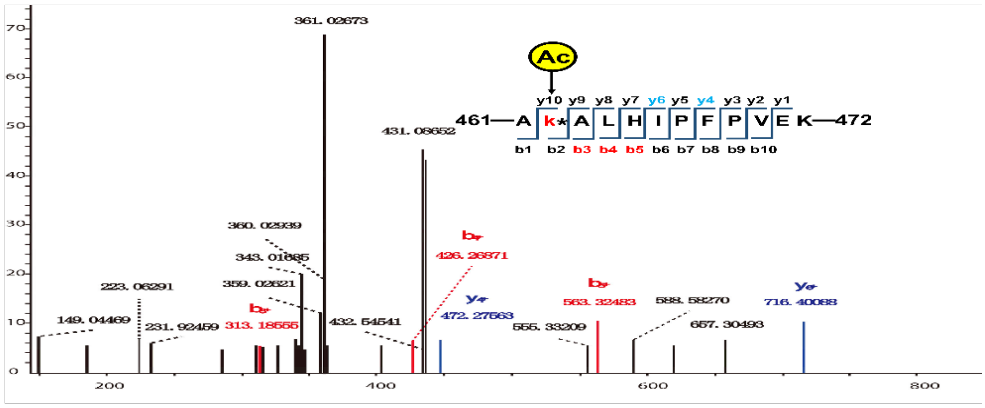
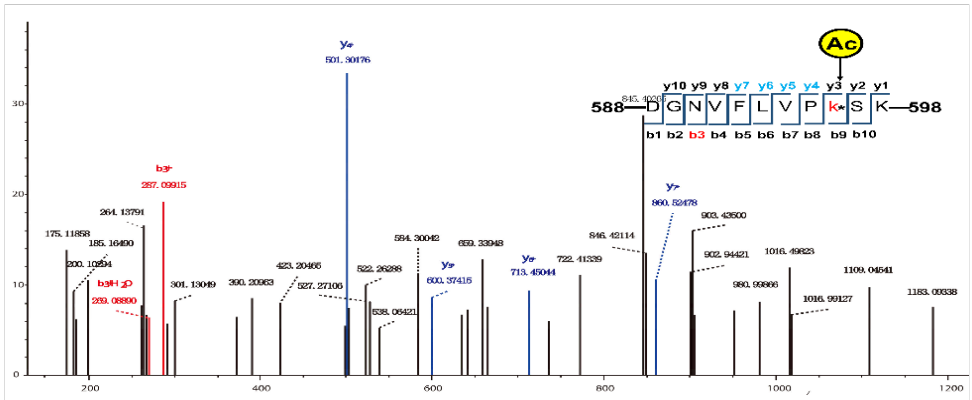


Figure 27. Recombinant GST-ARD1 acetylates recombinant GST-NRF2 at lysine 438 residue.

GST-NRF2 :





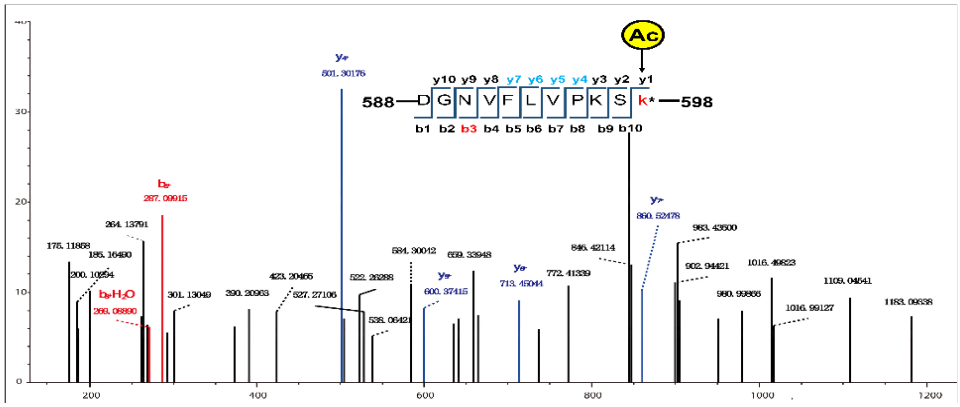


Figure 28. The acetylation site of recombinant GST-NRF2 were identified by nano-LC-ESI MS/MS (without recombinant GST-ARD1).

4. Discussion

Dysregulation of the NRF2-KEAP1 axis, including overexpression of NRF2 or loss of KEAP1 function, is frequently observed in many human tumors, but the underlying molecular mechanisms remain largely unexplored. Besides mutagenic activation, PTMs can also contribute to regulation of the aberrant overexpression/overactivation of NRF2 [41, 42]. Compared with phosphorylation of NRF2 by distinct kinases in the context of its functional activation/inactivation, there is a paucity of data on other types of NRF2 protein modification.

Lysine acetylation of non-histones plays a pivotal role in the regulation of fundamental biological processes, including gene transcription, DNA damage repair, cell division, metabolism, autophagy, protein function, etc. [43]. Thus, the acetylation of NRF2 merits systematic investigation in better understanding the oncogenic function of this transcription factor. Here, I demonstrate that ARD1-mediated acetylation of NRF2 affects its stability and nuclear localization, which in turn can influence its oncogenic activity (**Figure 29**).

In the present study, I found that human CRC tissues and cell lines with higher levels of ARD1 expression tended to also express elevated levels of NRF2, suggesting that ARD1 may contribute to elevated activation of NRF2 in CRC. In support of this speculation, silencing of ARD1 in cultured CRC cell lines resulted in a dramatic reduction of NRF2 protein without changes in its mRNA levels. Only two acetyltransferases have been reported so far to

acetylate NRF2 [44-46]. My cell-based and *in vitro* data strongly suggest that ARD1 binds directly to NRF2 and acetylates it. Then, the question is what kind of effects the ARD1-mediated acetylation of NRF2 would have on it.

It was reported that the co-activator, p300/CBP acetylated multiple lysine residues within the Neh1 structural domain of NRF2, which enhanced the binding of NRF2 to the ARE and improved the transcription of NRF2 target genes [44]. Another study showed that cyclic AMP response element-binding protein (CBP)-mediated acetylation of NRF2 at murine K588 (equivalent to human K596) and murine K591 (equivalent to human K599) also increases its nuclear localization and transcriptional activity [45]. Moreover, the histone acetyltransferase MOF (males absent on the first) has been described to acetylate NRF2 at murine K588 (equivalent to human K596), resulting in increased nuclear retention of NRF2 and elevated transcription of its downstream genes [46].

Notably, acetylation of NRF2 occurred mainly at lysine residues of the Neh1 and Neh3 structural domains containing nuclear localization signals and nuclear export signals, leading to its enhanced nuclear accumulation [44-46]. In line with this notion, my structural domain binding and protein mass spectrometry data suggest that ARD1 interacts and acetylates NRF2 primarily in these domains. Meanwhile, I showed that the overexpression or silencing of ARD1 altered the cellular localization as well as the stability of NRF2. SIRT2, a cytoplasmic sirtuin (class III HDAC), was found to

deacetylate NRF2 at K506 and K508, which decreased the stability and nuclear localization of NRF2 [47]. p300-mediated acetylation of NRF2 leads to disruption of the NRF2-KEAP1 complex, which increased NRF2 nuclear translocation and target gene transcription [48]. My present study revealed that ARD1-mediated NRF2 acetylation enhanced the stability and nuclear translocation of NRF2. However, further studies on histone deacetylase-mediated NRF2 deacetylation corresponding to ARD1 are warranted to better understand the biological significance of ARD1-mediated NRF2 acetylation.

ARD1 regulates the biological activity of some proteins including heat shock protein 70 [28] and methionine sulfoxide reductase A [26] through acetylation. Here, I propose NRF2 as a novel substrate for ARD1. It has been reported that lysine acetyltransferase activity of recombinant ARD1 is affected by changes in time, the extent of oligomerization after purification, and ARD1 autoacetylation [24, 49]. In the present study, I performed an *in vitro* acetylation assay with commercially available purified recombinant ARD1, but encountered some technical problems in precisely assessing the intrinsic lysine acetyltransferase activity affected by the oligomerization of purified ARD1. As an alternative approach, I used ARD1 mutants that lost catalytic activity and demonstrated that its acetyltransferase activity could affect the NRF2 stability. Thus, these results suggest that ARD1-mediated NRF2 acetylation is an enzymatic reaction rather than a nonspecific chemical reaction.

KEAP1 is the master inhibitor of NRF2. The KEAP1 homodimers directly binds to NRF2 through DLG and ETGE motifs within the NRF2 Neh2 structural domain, and this facilitates the ubiquitin-mediated proteasomal degradation of NRF2 [50, 51]. Previous studies have shown that ARD1, in addition to regulating proteins by acetylation, also enhances or inhibits the activity of its partner proteins by interacting directly with them in an acetylation-independent manner [39, 40, 52, 53]. Interestingly, I found that ARD1 was also associated with KEAP1 (**Figure 30A and 30B**). Additionally, several proteins; such as DPP3 [54], p62/SQSTM1 [55], iASPP [56], and HBXIP [57], have been reported to bind and inactive KEAP1, leading to NRF2 accumulation and target gene transcription. In this context, ARD1 may not only acetylate NRF2, but may also be a new inactivator of KEAP1.

It is difficult to obtain NRF2-specific transcriptional inhibitors because NRF2 belongs to a large family of basic leucine zipper transcription factors, which are extensively involved in the regulation of key biological functions. In contrast, acetylation-mediated PTM of NRF2 is targetable. Thus, elucidating the mechanism of NRF2 regulation mediated by acetylation would provide practicable ways to suppress the oncogenic NRF2 activity in a highly precise manner.

5. Conclusion

In conclusion, ARD1 stabilizes NRF2 through acetylation, thereby affecting its nuclear localization and transcriptional activity in CRC. Accordingly, targeting the ARD1-NRF2 axis may offer a promising therapeutic strategy for inhibiting colon cancer progression.

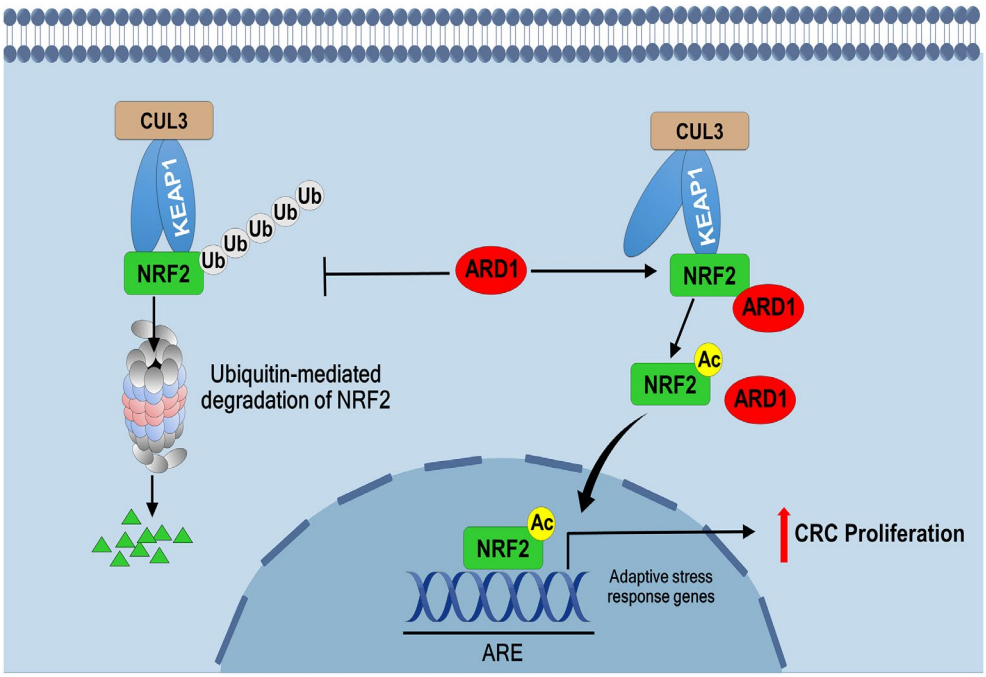


Figure 29. A proposed mechanism underlying ARD1-mediated NRF2 acetylation in the progression of colon cancer.

ARD1 inhibits proteasome-mediated degradation of NRF2 by directly binding and acetylating this transcription factor. This allows nuclear translocation and subsequent transactivation of NRF2, thereby promoting proliferation of human colon cancer cells.

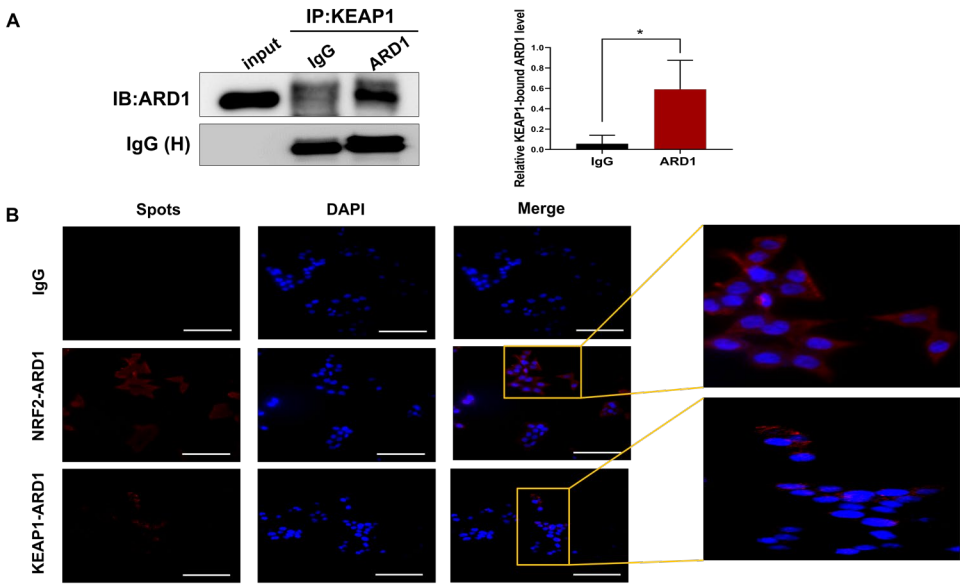


Figure 30. Interaction between KEAP1 and ARD1.

(A) Whole-cell lysates from HCT-116 cells were subjected to immunoprecipitation with anti-KEAP1 antibody, and Western Blot analysis was performed using anti-ARD1 antibody. The quantitative data are shown as the mean ± S.D. of three independent experiments, analyzed by two-tailed unpaired Student's *t*-test. *$p < 0.05$. (B) Detection of ARD1-KEAP1 interaction in situ. The interaction of ARD1 with KEAP1 was visualized by Duolink analysis (PLA). ARD1, NRF2, and KEAP1 were co-labeled with corresponding antibodies. The interaction of NRF2 with ARD1 was used as a positive control group. Nuclei were counterstained with DAPI. Each scale bar represents 200 μm.

6. References

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국문초록

Nuclear factor erythroid-2-related factor 2 (NRF2)는 산화적 또는 친전자적 스트레스에 대항하는 항산화 효소들의 발현을 조절하는 전사인자로, 염증, 노화 및 암의 발생과 같은 다양한 병리적 현상으로부터 세포를 보호하는 것으로 알려져 있다. 그러나, 최근 여러 암세포에서 NRF2는 종양 증식 및 진행에 관여하며 항암제와 같은 외부 스트레스에 대한 보호기전으로 작용한다. 암세포에서 비정상적인 NRF2의 과발현은 NRF2의 대표적 음성조절자인 KEAP1의 비활성화나 NRF2의 자체의 체세포 변이를 통해 발생하는 것으로 알려져 있지만 NRF2의 지속적인 활성화를 담당하는 대안적인 (alternative) 기전에 관해서는 명확히 규명된 바가 없다.

N-아세틸화 효소로 알려진 arrest defective1 protein (ARD1)은 세포분열, 증식 및 발암기전에 관여하며 산화적 스트레스에 대한 세포내 보호작용에서 중요한 역할을 하는 것으로 알려져 있다. ARD1은 유방암, 전립선암, 폐암, 간암, 자궁경부암, 방광암, 대장암에서 높게 발현되어 있으며 ARD1의 발현이 높을수록 암 환자들의 낮은 생존율이 보고된 바 있다.

번역 후 변형 형태 중 하나인 아세틸화는 단백질의 안정화에 관여하며, NRF2 아미노산 염기서열 내에 아세틸화가 가능한 라이신 잔기가 있음에도 불구하고 아세틸화에 의한 NRF2의 안정화 기전 관한 연구는 크게 이루어진 바가 없다. 본 연구에서는 ARD1이 NRF2의 아세틸화를 유도함으로서 대장암의 진행과정에 관여하는 분자 기전에 관하여 알아보고자 하였다.

면역형광염색 기법을 통하여 인체대장암 조직을 염색해 보았을 때 ARD1과 NRF2의 발현이 서로 positive한 상관 관계를 보였으며,

ARD1 유전자 발현을 선택적으로 억제할 수 있는 siRNA를 인체 대장암 세포주에 주입하였을 때 NRF2의 mRNA에는 영향을 미치지 못하였으나 NRF2의 단백질이 유의적으로 감소되었다. 이는 ARD1이 NRF2의 신생 합성에 관여하는 것이 아닌 단백질 번역 후 변형에 관여함을 시사하였다. 또한, 이 두 단백질은 인간 대장암 세포인 HCT-116와 인간 대장 종양 조직에서 물리적으로 상호작용함을 관찰하였으며 NRF2의 serial deletion construct를 통하여 NRF2의 Neh1와 Neh3 domain이 두 단백질의 결합에 직접 관여함을 알 수 있었다. ARD1의 과발현시 NRF2의 아세틸화가 증가되었으며 *in vitro* acetylation assay와 질량분석법을 통해 ARD1이 NRF2를 직접 아세틸화시킬 수 있음을 증명하였다. ARD1의 아세틸화 효소활성이 NRF2의 단백질 안정화에 관여하는지 확인하고자 아세틸화 효소활성 기능이 손상된 ARD1 돌연변이를 통하여 NRF2 단백질의 반감기를 측정한 결과 ARD1을 통한 NRF2의 아세틸화가 단백질 안정화에 관여함을 확인할 수 있었다. 결론적으로, ARD1은 NRF2의 아세틸화를 통하여 단백질 안정화에 관여하며 인간 대장암 세포의 이동 및 증식과 같은 암의 진행과정 참여한다.

주요어 (Key words)

Colorectal cancer; NRF2; ARD1; Posttranslational modification; Acetylation

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