Thesis for PhD degree

Molecular regulation and biological function of NRF3 (NFE2L3) in cancer cells

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Abstract

Accumulated evidences suggest physiological relevance between the transcription factor NRF3 (NFE2L3) and cancers. NRF3 modulates gene expression in the nucleus, while it is repressed by endoplasmic (ER) sequestration under physiological conditions. However, the molecular mechanisms underlying the nuclear translocation of NRF3 and its target genes expression in cancer cells remain poorly understood. Here, I found multiple regulation of NRF3 activities promotes cell proliferation. My analyses reveal (1) under physiological conditions, NRF3 is rapidly degraded by the ER-associated degradation (ERAD) ubiquitin ligase HRD1 and valosin-containing protein (VCP) in the cytoplasm; (2) NRF3 is also degraded by β-TRCP, an adaptor for the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase in the nucleus; (3) NRF3 mediates gene expression of the cell cycle regulator U2AF homology motif kinase 1 (UHMK1) for cell proliferation; (4) the nature of the activation mechanism of NRF3 is not the inhibition of its HRD1-VCP-mediated degradation. Collectively, this study provides us many insights into biological function of NRF3 in cancer cells.

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1. Introduction

1.1 - The CNC (cap 'n' collar) family proteins

The transcription factor NRF3 (NF-E2-reated factor 3 or NFE2L3) is a member of CNC (cap 'n' collar) family proteins (1). The CNC proteins are a subgroup of bZIP (basic leucine zipper) transcription factors conserved in worms, insect's, fish, birds and mammals. These proteins are characterized by a highly conserve N- terminally located CNC domain (43 amino acids) important for the DNA binding activity (1). In addition to CNC domain, these transcription factors also contain a bZIP domain essential for heterodimerization and DNA binding (2, 3). CNC family proteins are unable to bind with a target DNA as monomers. They usually exert their biological functions through binding with the small Maf proteins (sMafs) (4).

The CNC family transcription factors play pivotal roles in various cellular processes including antioxidant response, neurodegeneration, respiratory diseases, carcinogenesis, proteasomal homeostasis through the regulation of mammalian genes expression (1). In vertebrate, CNC members are classified into NFE2 (nuclear factor-erythroid derived 2) (5) , NRF1 (nuclear factor erythroid 2-related factor 1) (6) , NRF2 (nuclear factor erythroid 2-related factor-2) (7) NRF3 (nuclear factor, erythroid 2-related factor 3) (8) and more distantly related BACH1 and BACH2 proteins (9) (FIG. 1A).

sMAFs (small MAF proteins) are also bZIP type transcription factors, containing a unique basic region essential for the DNA binding activity (4). In vertebrate, sMAF proteins comprise MAFF, MAFG and MAFK. These sMAF proteins have no functional differences among them in terms of their bZIP structures (4).

1.2 - NRF3 (nuclear factor, erythroid 2-related factor 3)

1.2 (a) - Origin of NRF3

The transcription factor NRF3 is a membrane bound glycoprotein (10) and associated with the endoplasmic reticulum (ER) (11). NRF3 exerts its activity through the antioxidant response element (AREs) or Maf recognition elements (MAREs) by heterodimerizing with sMAF proteins (8). FIG. 2 represents a schematic model of a regulatory mechanism of NRF3 in the cells. The identification of the *NFE2L3* genes were first reported on 1999 (8). The human *NFE2L3* gene is located on the chromosome 7p15-p14(8) and the mouse *Nfe2l3* on chromosome 6B3 (12). The *NFE2L3* gene is located close to the *HOXA* gene cluster and the other CNC genes, *p45 NFE2*, *NRF1*, *and NRF2* are located close to *HOXC*, *HOXB* and *HOXD* genes, respectively (8, 13). Based on these observations, it is assumed that vertebrate CNC family proteins (p45 NFE2, NRF1, NRF2, and NRF3) are derived from a single ancestral gene localized in proximity to the ancestral *HOX* cluster.

1.2 (b) - Expression of *NRF3* mRNA

The *NRF3* mRNA expression has been reported in both human and mouse origins (8, 14). The human *NRF3* shows high expression levels in placenta (8). Its mild expression is observed in heart, brain, lung, kidney, pancreas, colon, thymus and spleen (8). Megakaryocytes and erythrocytes show the very low expression levels of human NRF3 (8). *NRF3* is not expressed in human testis, prostate, skeletal muscle and ovary (8). In mice, *Nrf3* mRNA expression have been reported in thymus, brain, lung, stomach, uterus, placenta, adipose tissue and testis (12, 15). It is not clear whether these tissues actually express NRF3

proteins, because of no available NRF3 antibody that can recognize endogenous NRF3 proteins.

1.2 (c) - Structure of NRF3 protein

The human and mouse NRF3 proteins contain 694- and 660-amino-acid, respectively (8, 14). The protein structure of human NRF3 consist of some key domains including the NHB1 (N-terminal homology box 1) domain, the NHB2 (N-terminal homology box 2) domain, the CNC domain, the basic region and the leucine zipper domains (16) (FIG. 1B). Bioinformatics analysis reveals domain structure of NRF3 is fairly conserved among different species (10, 16). This species conservation suggests the significance of these domains in NRF3 functions. The comparison studies further reveal that the NHB1 and NHB2 domains are conserved between mouse Nrf1 and Nrf3 (10, 16) (FIG. 1C). The NHB1 domain, part from the ER signal sequence, is important to the ER targeting for both Nrf1 (17) and Nrf3 (10). On the other hand, biological function of NHB2 domain of NRF3 is still remains unknown, but it is assumed that the NHB2 domain involves in the control of NRF3 function (10).

1.2 (d) - Physiological functions of NRF3

To explore physiological functions of Nrf3, *Nrf3* deficient mice were independently generated in two laboratories (12, 18). *Nrf3* null mice normally develop and grow under physiological conditions. A comparison of phenotypes of CNC family protein knockout mice shown in Table 1. Thus, the physiological functions of NRF3 has been obscure. Recently, the human cancer genome project (the Cancer Genome Atlas (TCGA)) identifies that NRF3 is one of the significantly mutated genes from various types of cancer (19). According to Gene Atlas U133A, gcrma database, *NRF3* is highly expressed in colorectal adenocarcinoma compared to normal colon (FIG. 3), implying the potential physiological significance of NRF3 in colon cancer. In addition, a series of genechip array analyses also demonstrates the

potential involvement of NRF3 in several other cancers including breast cancer (20), lymphoma (21), testicular carcinoma (22). Beside carcinogenesis, NRF3 may also involve in other cellular processes including inflammation (15), smooth muscle cell differentiation (23) and gynecological disease (24).

1.3 - NFE2 (nuclear factor-erythroid derived 2)

NFE2 is a heterodimer complex of two bZIP proteins, consisting of 45-kDa (p45NFE2) and 18-kDa (p18NFE2) subunits (25). p45NFE2 is a tissue specific subunit and its expression is restricted to erythroid cells, megakaryocytes, and mast cells (25). p18NFE2, a member of the Maf oncoprotein family (26), is a small MAF protein. It is widely expressed in various tissues (5). NFE2 is a crucial regulator of megakaryocyte biogenesis and function. *p45Nfe2* deficient mice mostly die at birth, due to arrest in late megakaryocyte maturation, however small fraction of the mice survives and develops primary or secondary phenotypes such as severe megakaryocytosis, splenomegaly, and bone marrow hypercellularity (27). On the other hand, targeted disruption of *p18Nfe2* (*MafK*) in mice resulted in no discernible abnormalities (28).

1.4 - NRF1(nuclear factor erythroid 2-related factor 1)

The transcription factor NRF1 (also referred to as NFE2L1) has a similar protein structure with that of NRF3, indicating a possibility that they share common biological functions. The transcriptional activities of Nrf1 are accomplished by several distinct domains for DNA binding, dimerization, transcriptional activation and subcellular localization. The bZIP domain of Nrf1 is located near the carboxyl terminus of the protein (6). An immediate N-terminal end to the bZIP domain is a highly conserved CNC domain. Nrf1 is localized in the endoplasmic reticulum by the N-terminus transmembrane domain (29) or NHB1 (N-terminal homology box-1) domain (30). The NHB1 domain does not appear to function as a signal peptide but it mediates retention of NRF1 in the ER. After entering the ER, Nrf1 undergoes

N-linked glycosylation at the Asn/Ser/Thr-rich glycodomain (NST). Nrf1 remains in the ER until it is degraded or transported to the nucleus to mediate expression of target genes. In addition, Nrf1 also contains a N-terminal homology box 2 (NHB2) domain (16). Under physiological conditions, Nrf1 is degraded by the ubiquitin-proteasome system. Currently, three different E3 ubiquitin ligases including β-TRCP, FBXW7 and HRD1 have been reported to causes Nrf1 degradation (31, 32).

The human and mouse *NRF1* genes are located on chromosome 17q21.3 and the distal end of chromosome 11, respectively (13, 33). *In situ* hybridization analysis shows that *Nrf1* is widely expressed during development in mouse (34). In addition, analysis of adult rat tissue reveals that *Nrf1* mRNA is expressed in the heart, kidney, fat and brain, liver, pancreas (34) and skeletal muscle tissue (35). Nrf1 has numerous biological functions on various cellular processes. *Nrf1* knockout mice show embryonic lethality due to anemia (36). It has been reported that Nrf1 plays an important role in regulating different cellular functions including oxidative stress response (36, 37), differentiation (38), inflammatory response (39, 40), metabolism (41, 42) and proteasomal homeostasis (43, 44).

1.5 - NRF2 (nuclear factor erythroid 2-related factor 2)

Nuclear factor erythroid 2-related factor 2 (NRF2) is the most studied transcription factor of CNC family proteins (1). Biological function of NRF2 is also regulated through compartmental segregation. Under normal conditions, NRF2 is localized in the cytoplasm and its function is repressed by Kelch-like-ECH-associated protein 1 (KEAP1)-mediated degradation (45, 46). However, under stress condition, the enzymatic activity of the Keap1-Cullin3-Rbx1 E3 ubiquitin ligase complex is inhibited, which promotes activation of Nrf2 by reducing its degradation (45, 46). Activated Nrf2 in the cytoplasm translocates into the nucleus to regulate the gene expression. The physiological functions of Nrf2 are elucidated

by gene targeting experiments. *Nrf2* deficient mice show certain phenotypes including age-related lupus-like syndrome, neurodegeneration and sensitivity to oxidative and ER stress (1). The transcription factor Nrf2 regulates gene expression of many detoxifications and antioxidant enzymes, molecular chaperones, stress response proteins, as well as proteasome subunits (1, 46).

1.6 - The ubiquitin proteasome system

The ubiquitin proteasome system (UPS) involves the degradation mechanisms of vast number of proteins in many biological processes such as cell cycle progression, signal transduction and transcription (47, 48). In the UPS, proteasome usually recognizes a polyubiquitin chain as a degradation signal. The polyubiquitin chain is conjugated to substrate proteins by sequential reactions, catalyzed by three enzymes: the process is starting with activation of ubiquitin by the activating enzymes (E1) and the activated ubiquitin is then transferred to a conjugating enzymes (E2) to form a thioester linkage between the ubiquitin and E2 and the ubiquitin ligases (E3) then transfers ubiquitin to the target proteins (48). A schematic presentation of the UPS mechanism is shown in FIG. 4 (49). The E3 ubiquitin ligases are important factors that determine the substrate specificity in ubiquitination process (47).

1.7 - Endoplasmic reticulum (ER)-associated degradation

Endoplasmic reticulum (ER)-associated protein degradation (ERAD) contributes the removal of misfolded or disassembled proteins from the ER for protein quality control. The ERAD process undergoes three steps: ubiquitination by specific ubiquitin ligases, substrate transportation from the ER to the cytoplasm (dislocation), and proteolysis by proteasome (50). One of the ERAD ubiquitin ligases is HRD1 (also known as synoviolin) responsible for proteasomal degradation of misfolded/unfolded proteins in the ER (51). HRD1 was initially identified as a human homologue of yeast Hrd1p/Der3p (52). HRD1 forms a complex with its

stabilizing factor SEL1L and this interaction is important to HRD1 mediated degradation of ER substrates (53). The HRD1-mediated degradation of ER substrates are facilitated by a cofactor called VCP (vasolin containing protein, p97 or CDC48 (yeast)) (54). VCP is an ubiquitously expressed protein that involves in numerous cellular processes such as proteasomal protein degradation, membrane fusion, immune signaling, autophagosome maturation and so on (55). In the ERAD process, VCP recognizes ER substrates ubiquitinated by HRD1-SEL1L complex and then transported it to the proteasome for degradation (53, 56) (FIG. 5A).

1.8 - The β-transducin repeat-containing protein

β-TRCP (the β-transducin repeat-containing protein) is one of the F-box proteins of SCF (SKP1-Cullin 1-F-box protein) E3 ligase complexes (57). F-box proteins are a subunit employing element of the SCF complex and play crucial roles in various cellular processes including cell proliferation, apoptosis, invasion and metastasis (58). As an adaptor of the SCF complex, β-TRCP determines the target specificity and recruits the target substrate into the scaffold protein Cullin1 (Cul1) along with SKP1 (S phase kinase associated protein 1) (FIG. 5B). Mammals express two distinct paralogs of β-TRCP with biochemically indistinguishable characters and these are termed as β-TRCP1 and β-TRCP2. β-TRCP is involved in the regulation of numerous cellular processes by mediating the degradation of target proteins including cell cycle regulators, pro-apoptotic regulators and transcription factors (57).

1.9 - *UHMK1* (U2AF Homology Motif Kinase 1)

UHMK1 (U2AF Homology Motif Kinase 1) is a gene that encodes the protein KIS (Kinase-interacting stathmin). *UHMK1* is localized in the nucleus and has RNA recognition motif (59). Involvement in the control of RNA trafficking or splicing through phosphorylation might be the functional attributes of *UHMK1* (59). It has been reported that *UHMK1*

phosphorylates the tumor suppressor gene p27Kip1 (cyclin-dependent kinase (CDK) inhibitor) and regulates cell cycle progression (60, 61) (FIG. 6).

1.10 - Deubiquitinating enzymes

Deubiquitinating enzymes (DUBs) are important regulators of the ubiquitin system. They process inactive ubiquitin precursors, proof-read ubiquitin–protein conjugates, remove ubiquitin from cellular adducts, and keep the 26S proteasome free of inhibitory ubiquitin chains (62). DUBs are specific for both protein substrates and ubiquitin chains and thereby enable multiple ways to regulate protein ubiquitination (63, 64). Ubiquitin-specific protease 15 (USP15), which is a ubiquitously expressed DUB, regulates the biological function of many substrate proteins for various cellular processes (65, 66).

1.11 – A research objective and general overview

To understand the activation mechanism and biological functions of NRF3 in cancer, my objective in this research was to elucidate the molecular regulation of NRF3 in the cytoplasm and nucleus. NRF3 exerts the transcriptional activity through liberating from the ER and then translocating into the nucleus. Thus, elucidation of NRF3 regulatory mechanisms in the ER will provide us insights into the issue as to how this transcription factor translocates into the nucleus for activating target gene expression. Given the previous report that the proteasome inhibitor MG132 treatment stabilizes NRF3 (11), the UPS should be involved in NRF3 regulation. To understand the activation mechanism of NRF3, identification of specific UPS components involve in NRF3 regulation is crucial. On this basis, my investigation uncovers that two-distinct proteasomal degradation mechanisms regulate the turnover of transcription factor NRF3. In the cytoplasm, NRF3 is rapidly degraded by HRD1 and its cofactor VCP. In the nucleus, NRF3 is also degraded by β-TRCP. Regarding the physiological function of NRF3 in cancer cells, my research discovers that *UHMK1* is the target gene of NRF3 and

NRF3 promotes colon cancer cell proliferation by inducing the *UHMK1* gene expression. This research also reveals that the nature of the NRF3 activation mechanism is not the inhibition of the HRD1-mediated degradation of NRF3. In addition, I also investigated the effects of deubiquitinating enzyme USP15 on the regulation of CNC family transcription factors p45, Nrf2 and Nrf3. Under my experimental conditions, I found that USP 15 stabilizes p45 but not Nrf2 and Nrf3. Overall this research provides us substantial insights into molecular regulation and physiological function of NRF3 in cancer cells.

2. Materials and Methods

2.1 - Antibodies

The antibodies utilized in the current immunoblot analysis were anti-NRF3 (#9408) (1/10 dilution), anti-FLAG (M2; Sigma) (1/4,000 dilution), anti-α-Tubulin (DM1A; Sigma) (1/20,000 dilution), anti-Lamin B (Invitrogen) (1/2,000 dilution), anti-Nrf1 (D5B10; Cell Signaling Technology) (1/1,000 dilution), anti-HRD1 (D302A; Cell Signaling Technology) (1/1,000 dilution), anti-VCP (H-120; Santa Cruz) (1/1,000 dilution), anti-HA (Y-11; Santa Cruz) (1/1,000 dilution), anti-HA (clone 3F10 ; Roche) (1/1,000 dilution), anti-Myc (A-14 ; Santa Cruz) (1/100 dilution), and anti-KIS1 (UHMK1) (a kind gift from Alexandre Maucuer, Universite Pierre et Marie Curie) (1/30 dilution) and anti-Nrf2 (H300; Santa Cruz) (1/1,000 dilution) antibodies.

2.2 – Plasmids

Myc-hNRF3, HA–β-TRCP2 and HA-ubiquitin were kindly provided by Yiguo Zhang (Chongqing University), Keiko Nakayama (Tohoku University) and Dirk Bohmann (University of Rochester), respectively. Flag-Nrf2 and Flag-p45 were kindly gifts from Ken Itoh (Hirosaki University). Myc-tagged β-TRCP2 and ΔF-box β-TRCP2, 3xFlag-mNrf3 and 3xFlag-Nrf1 were generated by my laboratory (32).

2.3 - Cell culture and transfection

DLD-1, HCT116, HeLa, COS7 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% fetal bovine serum (FBS) (Nichirei), 40 μg/ml streptomycin, and 40 units/ml penicillin (Life Technology). The transfection of plasmid DNA and short interfering RNA (siRNA) was performed using Lipofectamine 2000 and RNAiMAX (Invitrogen), respectively.

2.4 - Immunoblot analysis

To prepare whole cell extracts, the cells were lysed with SDS sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol and 1% SDS). The protein quantities in cell extracts were measured with a bicinchoninic acid (BCA) kit (Thermo). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Immobilon-P transfer membrane, EMD Millipore corporation, Billerica, USA). After blocking the membranes with Blocking one (Nacalal Tesque) at 4°C for overnight, the membranes were incubated for 1 hr with a primary antibody at room temperature, washed with TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween20) and were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hr. The blots were then washed with TBS-T and developed with enhanced chemiluminescence (ECL; GE Healthcare).

2.5 - Cycloheximide chase experiments

DLD-1 cells were transfected with indicated siRNA. At 48 hr after transfection, the cells were treated with 20 ug/ml cycloheximide (CHX), and the whole cell extracts were prepared at the indicated time points. Immunoblot analysis was conducted with the indicated antibodies. In the case of β-TRCP-related experiment, the cells were treated with 10 μM MG132 for 6 hr at 48 hr after siRNA transfection for promoting nuclear translocation of NRF3, then washed two times with phosphate-buffered saline (PBS), and treated with 20 μg/ml cycloheximide.

2.6 - Cell fractionation

At 48 hr after transfection of indicated siRNA into DLD-1 cells, the cells were suspended in buffer A (20 mM HEPES-KOH [pH 8.0], 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Nacalai Tesque) and 10 μM MG132), followed by lysis with the addition of NP-40 (a final concentration of 2.5%). After flash centrifugation at 10,000 rpm, the supernatants and precipitates were separated. The supernatants were further subjected to centrifugation at 20,000 x g for 10 min, and resultant supernatants were utilized for cytoplasmic extracts. For preparation of nuclear extracts, the precipitates were washed two times with buffer A and lysed with the SDS sample buffer under mild sonication to shear genomic DNA. After centrifugation, the supernatants were collected as nuclear extracts. The protein quantities in cytoplasmic and nuclear extracts were measured with a BCA kit (Thermo).

2.7 - Immunocytochemical staining

COS7 cells were transfected with 3xFlag-tagged Nrf3 and/or HA–β-TRCP2 plasmids. At 24 hr after transfection, the cells were treated with MG132 (10μM) for 6 hr. The cells were fixed with formaldehyde for 15 min at room temperature, washed three times with 0.1% PBS-T (0.1% Triton X-100 in PBS), permeabilized with 0.5% Triton-X for 10 min at room temperature and washed twice with PBS-T. After treatment with blocking solution (1% skim milk in PBS-T) for 1 hr at room temperature, the cells were incubated with anti-Flag or anti-HA antibodies for 1 hr at room temperature, washed three times with PBS-T and then were incubated with Alexa 488- or Alexa546-conjugated secondary antibodies along with 4´,6´-diamidino-2-phenylindole (DAPI) for 2hr at room temperature. Finally, the samples were washed twice with PBS-T. The cells were again washed with PBS and were placed on the glass slides containing a drop of fluorescent mounting medium (Dako). Fluorescence images were captured by an Olympus IX71 microscope.

2.8 - The ubiquitination assay

HCT116 cells were transfected with 3xFlag-Nrf3 and HA-ubiquitin, along with the wild-type Myc-β-TRCP2 or the ΔF-box β-TRCP2 mutant. At 24 hr after transfection, the cells were treated with MG132 (10 μM) for 6 hr, and the whole cell extracts were prepared with lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% SDS, 1x protease inhibitor cocktail (Nacalal Tesque), 10 μM MG132, and 10 mM N-ethylmaleimide (NEM)). The cell extracts were then boiled and sonicated. After centrifugation at 18,000 x g for 15 min at 8 °C, the supernatants were diluted with dilution buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 x protease inhibitor cocktail, 10 μM MG132, and 10 mM NEM) to reduce the SDS concentration to less than 0.03%, at which point they were incubated with anti-Flag antibody and protein G Sepharose beads (GE Healthcare) at 4 °C overnight. The immunocomplexes were washed three times with dilution buffer and eluted by boiling in SDS sample buffer. The ubiquitinated Nrf3 was visualized by immunoblot analysis using the anti-HA antibody.

2.9 - siRNA knockdown experiment

DLD-1 and HCT116 cells were cultured for 16 hr in the medium without antibiotics. The cells were then transfected with 40 nM siRNA using RNAiMAX. At 48 hr after transfection, the cells were utilized for each experiment except cell counting. For FACS and cell count experiments, the cells were cultured in the medium without antibiotics and transected with 40 nM siRNA using RNAiMAX without prior incubation. HeLa cells were cultured for 16 hr in the medium without antibiotics. The cells were transfected twice with 40 nM siRNA (at 16 and 40 h after plating) using RNAiMAX. At 24 hr after the last transfection, the cells were transected with indicated plasmids and incubated for 24 hr. The sequences of the sense strand of the siRNA duplexes employed in the present study were as follows: Control, 5´-UUCUCCGAACGUGUCACGUdTdT-3´;

β-TRCP1/2, 5´- GUGGAAUUUGUGGAACAUCdTdT-3´;

VCP, 5´-GUAGGGUAUGAUGACAUUGdTdT-3´;

HRD1, 5´- GGUGUUCUUUGGGCAACUGdTdT-3´;

NRF3, 5´- CGCAAAUUGGACAUAAUUUdTdT-3´;

NRF3(A), 5´- GCAAAGAAGGAAACUCUUAdTdT-3´;

GP78, 5´-CAUGCAGAAUGUCUCUUAAdTdT-3´;

TEB4, 5´-UUAAGAGUGUGCUGCCUAAdTdT-3´;

2.10 - RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was prepared using the ISOGEN Ⅱ (Nippon Gene). One microgram of total RNA was utilized for cDNA synthesis using random hexamer primers (Takara Bio) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Real-time quantitative PCR was conducted using the SYBR Premix Ex Taq II (Takara Bio) and the Thermal Cycler Dice Real Time System Ⅱ (Takara Bio). The sequences of the primers are listed in Table 2.

2.11 - Cell cycle analysis using FACS

The cell cycle analysis was conducted using Click-iT® EdU Flow Cytometry Assay Kits (Invitrogen) according to the manufacturer's protocol. DLD-1 cells were transfected with the indicated siRNA. At 48 hr after transfection, the cells were treated with Edu (10 μM) for 2 hr at 37°C and washed two times with 1% BSA in PBS. The cells were fixed by using Click-IT fixative (containing paraformaldehyde) for 15 min at room temperature, followed by washing them twice with 1% BSA and then permeabilizing with P/W (1x Click-iT saponin-based Permeabilization and Wash Reagent) for 15 min at room temperature. After their treatment with Click-iT Reaction Mixtures for 30 min at room temperature in a dark place, the cells were then washed with P/W and stained with Propidium Iodide (PI) buffer for 20 min at 37°C in a dark place. Finally, the cells were washed twice with P/W and subjected to the cell cycle analysis using FACS.

2.12 - Cell counting

DLD-1 cells were plated onto 6-well dishes (1 x 105 cells per well), transfected with indicated siRNA and cultured for 72 hr. The cells were detached from plates with 0.05% trypsin and gently resuspended with ice-cold PBS. Cell counting was performed using a hemocytometer.

2.13 - Immunoprecipitation

COS7 cells were transfected with 3xFlag-tagged Nrf3 or HA–β-TRCP2 plasmid. At 24 hr after transfection, the cells were treated with MG132 (10μM) for 6 hr. After MG132 treatment the cells were lysed with lysis buffer (50 mM Tris-HCl [pH 8.0], 10% glycerol, 100 mM NaF, 50 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.1% NP-40, and 1x protease inhibitor cocktail [Nacalal Tesque, Inc. Kyoto, Japan], to prepare whole-cell extracts. The whole-cell extracts were then subjected to immunoprecipitation with anti-Flag antibody and protein G Sepharose beads (GE Healthcare) at 4°C overnight. After incubation the immunocomplexes were washed three times with wash buffer (50mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1% NP-40). The immunocomplexes were then eluted by boiling in SDS sample buffer and subjected to immunoblot analysis using the antibodies indicated on the figures.

Statistical analysis

Statistical significance was evaluated with Student's t-test for repeated measurements.

3. Results

3.1 - HRD1 and VCP regulate the cytoplasmic degradation of NRF3.

To elucidate the mechanisms underlying molecular regulation of NRF3, proteome analysis was conducted in my laboratory to identify NRF3-associated proteins as described in (32). NRF3 complexes were immunopurified from the cell extract of HEK293 cells transiently expressing NRF3-Flag using anti-flag antibody. The resultant NRF3 complexes were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Consequently, this experiment succeeded in identification of several NRF3 associated factors including proteasome subunits and a transcriptional mediator (data not shown). Among them, I focused on the ubiquitin ligase related factors VCP and SKP1, because in my laboratory it has been previously reported that the NRF3-related factor NRF1 is degraded by two distinct E3 ubiquitin ligase VCP-HRD1 and SKP1-β-TRCP complexes, in the cytoplasm and nucleus, respectively (32). This observation allowed me to make the hypothesis that the stability of NRF3 is also regulated by these E3 ubiquitin ligases, similar with that of NRF1.

To confirm the hypothesis, I investigated effects of *HRD1* or *VCP* knockdown on the stability of endogenous NRF3 in human colon adenocarcinoma DLD-1 cells. *HRD1* or *VCP* siRNA was transfected into the cells, and whole cell extracts were prepared and subjected to immunoblot analysis. *HRD1* or *VCP* knockdown markedly stabilized endogenous NRF3 as well as NRF1 in DLD-1 cells (FIG. 7A). I also verified by real time quantitative PCR analysis (FIG. 7C) and immunoblot analysis (FIG. 7B) that each siRNA significantly reduces mRNA and protein expression levels of HRD1 and VCP, respectively. HRD1 and VCP-mediated degradation of NRF3 was also observed in HCT116 cells by similar experiments (FIG. 8A and B). Furthermore, cycloheximide chase (CHX) experiment also clearly indicated that the *HRD1* or *VCP* knockdown significantly stabilizes NRF3 (FIG. 9). Alternatively,

knockdown of other ERAD-related ubiquitin ligase *GP78* or *TEB4* did not stabilize NRF3 in DLD-1 cells (FIG. 10A and B). These results indicate that NRF3 undergoes cytoplasmic degradation via HRD1 and VCP under physiological conditions.

3.2 - β-TRCP promotes nuclear degradation of NRF3.

Next, I focused to examine whether β-TRCP as a cofactor of SKP1 exerts NRF3 degradation in the nucleus, because β-TRCP promotes proteasomal degradation of NRF3-related factor NRF1 (32). Considering the functional redundancy between β-TRCP1 and β-TRCP2, I simultaneously knocked down both factors by siRNA in all subsequent experiments. I first investigated the effects of *β-TRCP* siRNA on transiently overexpressed NRF3 in HeLa cells. *β-TRCP* knockdown markedly stabilizes Myc-tagged human NRF3 (Myc-hNRF3) and 3xFlag-tagged mouse Nrf3 (3xFlag-mNrf3) into HeLa cells as well as 3xFlag-tagged mouse Nrf1 (3xFlag-mNrf1) as a positive control (FIG. 11A). Significant knockdown of *β-TRCP1* and *β-TRCP2* mRNA in HeLa cells was confirmed by qRT-PCR analysis (FIG. 11B).

Next, I examined the effects of β-TRCP on endogenous NRF3 stability in DLD-1 cells (FIG. 12A and B). Unexpectedly, *β-TRCP1/2* siRNA did not promote accumulation of NRF3 in the cells, although it significantly repressed *β-TRCP* expression. I assumed that it is due to different cellular localization between NRF3 and β-TRCP. Because NRF3 is mainly localized in the endoplasmic reticulum (ER) under physiological conditions (10), while β-TRCP mediates proteasomal degradation of NRF3-related factor NRF1 in the nucleus (32). In this regard, I found that the proteasome inhibitor MG132 promotes the nuclear entry of endogenous NRF3 (FIG. 13). Then I performed a similar experiment of FIG. 12A under the MG132 treatment (FIG. 12A, MG132 +). Consequently, *β-TRCP* siRNA stabilized endogenous NRF3 in the nucleus. Similar results were observed using HCT116 cells (FIG. 14A and B). Further validation by cycloheximide chase (CHX) experiment revealed that β-

TRCP knockdown stabilizes endogenous NRF3 (FIG. 15). Overall, these data clearly demonstrate that β-TRCP mediates NRF3 degradation in the nucleus.

3.3 - Colocalization and physical interaction of Nrf3 and β-TRCP.

I further investigated the subcellular colocalization of Nrf3 and β-TRCP under MG132 treatment by coimmunostaining. The 3xFlag tagged Nrf3 and HA-tagged β-TRCP2 expressed in COS7 cells were visualized with anti-Flag and anti-HA antibodies, respectively. Nrf3 predominantly colocalized with β-TRCP 2 (FIG. 16A) in the nucleus. Next, I examined the physical interaction between Nrf3 and β-TRCP2 by immunoprecipitation analysis. COS7 cells were transfected with the 3xFlag tagged Nrf3 and HA-tagged β-TRCP2 plasmids. After transfection, whole cell extracts were subjected to immunoprecipitation with anti-Flag antibody and protein G sepharose beads. Immunoblot analysis with anti-HA (3F10) antibody revealed that β-TRCP2 was coimmunoprecipitated with Nrf3 (FIG. 16B). Collectively, these results suggest physical interaction between Nrf3 and β-TRCP in the cells

3.4 - β-TRCP mediates the degradation of Nrf3 through polyubiquitination.

To understand the molecular basis underlying the β-TRCP-mediated NRF3 degradation, I performed a ubiquitination assay in cultured cells. Whole cell extracts from HCT116 cells expressing the 3x Flag-tagged Nrf3, Myc-tagged β-TRCP2 and HA-tagged ubiquitin were subjected to immunoprecipitation with anti-Flag antibody-conjugated beads. Ubiquitination of Nrf3 was detected by immunoblotting with anti-HA antibody. This result revealed that the ubiquitination of Nrf3 is markedly increased by coexpression of wild-type β-TRCP2 (FIG. 17, WT). On the other hand, coexpression of the ΔF-box mutant of β-TRCP2 decreased ubiquitination of Nrf3 (FIG. 17, ΔF). Therefore, I conclude that β-TRCP promotes proteasome-mediated degradation of Nrf3 through polyubiquitination.

3.5 - NRF3 modulates the gene expression of the cell cycle regulator *UHMK1*.

Next, I explored on NRF3 target gene(s) in cancer cells to elucidate its physiological function. Previously, microarray analyses were performed in my laboratory by Mr. Hiroki Kato (Graduated in March, 2017) to identify the genes of which expression was reduced upon siRNA-mediated *NRF3* knockdown (FIG. 18). In addition, he listed up the genes possessing species-conserved ARE sequences in the 3-kbp upstream region from their transcriptional start sites. Through computational combination of these data, he highlighted 10 genes as putative NRF3 target genes (FIG. 18). Among these candidate genes, I focused on *UHMK1* (KIS) gene, because *UHMK1* has been reported to regulate cell cycle progression by phosphorylating the tumor suppressor p27Kip1 (cyclin-dependent kinase (CDK) inhibitor) (60, 61). qRT-PCR analysis and immunoblot analysis confirmed that *NRF3* knockdown reduces expression of the *UHMK1* gene in mRNA and protein levels, respectively (FIG. 19A and B). The consistent results were observed in DLD-1 cells and HCT116 cells using additional NRF3 siRNA (NRF3(A)) (FIG. 20 and 21).

To exploit whether NRF3 directly mediates the *UHMK1* expression through ARE sequence in its promoter, I performed chromatin immunoprecipitation (ChIP) analysis. However I could not succeed this experiment due to unknown reasons (data not shown). Alternatively, I performed a time-course study to examine the *UHMK1* expression upon the siRNA-mediated *NRF3* knockdown (FIG. 22). After transfection of NRF3 siRNA into DLD-1 cells, expression of *NRF3* and *UHMK1* mRNA was monitored over time as indicated time points by qRT-PCR. I found that the progressive reduction in *UHMK1* mRNA expression was slightly delayed compared to that in *NRF3* mRNA, suggesting that NRF3 is an upstream regulator of the *UHMK1* gene expression. Collectively, these results clearly demonstrate that NRF3 regulates *UHMK1* expression in colon cancer cells.

3.6 - NRF3 promotes the proliferation of colon cancer cells.

Given that *UHMK1* induces proliferation and cell cycle progression of cancer cell (60, 61), my finding gave rise to the next important question whether NRF3 knockdown reduces cell proliferation. To address this question, I examined the effects of NRF3 siRNA on proliferation of DLD-1 cells (FIG. 23). After the siRNA transfection, numbers of DLD-1 cells were counted. Expectedly, *NRF3* knockdown significantly reduced the cell proliferation (FIG. 23). Cell cycle analysis using flow cytometer (FACS) further demonstrated that *NRF3* knockdown significantly causes the cell cycle arrest (G0/G1) and reduction of the G2/M and S population of DLD-1 cells (FIG. 24). Altogether, these results demonstrate that NRF3 promotes colon cancer cell proliferation by activating the *UHMK1* gene expression.

3.7 - Inhibition of HRD1 does not activate NRF3.

The involvement of HRD1 in the molecular regulation of NRF3 prompted me to investigate the effects of HRD1 inhibition on NRF3 activation, *i.e.* its nuclear translocation. The ubiquitin–proteasome pathway has been reported to involve in the transcriptional regulation of several transcription factors (67, 68). To find out an involvement of HRD1 on NRF3 activation, I investigated effects of *HRD1* knockdown on the expression levels of *UHMK1* mRNA and protein in DLD-1 cells by qRT-PCR and immunoblot analyses. Interestingly *HRD1* knockdown increased the *UHMK1* mRNA and protein expression levels (FIG. 25A and 26), while *HRD1-NRF3* double knockdown restored it to the control levels (FIG. 26). This finding indicates a possible mechanism that HRD1 inhibition induces the *UHMK1* expression by activating NRF3. To confirm this possibility, I examined NRF3 protein levels in the nucleus by immunoblot analysis using fractionated extracts of *HRD1* knockdown cells (FIG. 27). Unexpectedly, HRD1 inhibition did not fairly promote the nuclear entry of NRF3 but its cytoplasmic accumulation. This finding suggests that the induction of the *UHMK1*

expression upon *HRD1* siRNA treatment is not mainly due to the nuclear translocation of NRF3.

3.8 - HRD1 regulates proliferation of DLD-1 cells through the NRF3-independent pathway.

My results reveal that HRD1 inhibition induces *UHMK1* expression in DLD-1 cells through the NRF3-independent pathway. This finding indicates that HRD1 may regulates proliferation of cancer cells. To address this question, I examined the effects of *HRD1-NRF3* double knockdown on the proliferation of DLD-1 cells. At 36 and 72 hours after siRNA transfection, cell numbers of DLD-1 cells were counted. Interestingly, I found *HRD1-NRF3* double knockdown significantly increases proliferation of DLD-1 cells compared to that of NRF3 knockdown (FIG. 28). Cell cycle analysis using flow cytometer (FACS) demonstrated that *HRD1-NRF3* double knockdown compensates the NRF3 knockdown-mediated cell cycle arrest (G0/G1) of DLD-1 cells (FIG. 29). This result suggests that HRD1 regulates cell proliferation through the NRF3-independent pathway. Further examination is required to solve the detailed molecular mechanisms.

3.9 - VCP inhibition reduces NRF3 transcriptional activity.

It has been previously reported that the transcription factor Nrf1 is activated through VCP dependent re-localization from ER lumen to cytosol in cells with compromised proteasome activity (69). This finding provoked me to investigate the effects of VCP inhibition on the NRF3 transcriptional activity in colon cancer cells. The expression of *UHMK1* mRNA and protein in *VCP* or *VCP-NRF3* double knockdown DLD-1 cells was examined by real time PCR and immunoblot analyses. Both *VCP* and *VCP-NRF3* siRNA reduced *UHMK1*mRNA and protein expression levels in DLD-1 cells (FIG. 25B and 30). Next, I deciphered whether *VCP* knockdown promotes nuclear accumulation of NRF3 in colon cancer cells. For this end,

I conducted immunoblot analysis using fractionated extracts of *VCP* knockdown cells. Consequently, VCP inhibition did not cause nuclear accumulation of endogenous NRF3 protein (FIG. 31). Thus, I conclude that VCP is required for nuclear entry and thereby transcriptional activity of NRF3.

3.10 - Effects of *β-TRCP* knockdown on the expression of NRF3 target gene *UHMK1*.

The involvement of β-TRCP in the regulation of NRF3 influenced me to investigate the effects of β-TRCP on the transcriptional activity of NRF3. Unfortunately, I could not find whether *β-TRCP* knockdown enhances the *UHMK1* gene expression by stabilizing NRF3 in the nucleus (FIG. 32). In this experiment, MG132 treatment is required for promoting nuclear translocation of NRF3 and this treatment alone affected the *UHMK1* expression. Thus, I could not conclude this issue.

3.11 - Biological relationship between USP15 and CNC family proteins.

My laboratory identified that deubiquitinating enzyme USP15 stabilizes the transcription factor Nrf1 in the nucleus through its deubiquitination (70). In addition, proteome analysis conducted in my laboratory also revealed USP15 as one of the Nrf3-associated factors (data not shown). Hence this observation motivated me to examine the biological relationship between USP15 and other CNC family proteins p45, Nrf2 and Nrf3. V5-USP15 was transiently expressed in HEK293T cells, along with the CNC family proteins Nrf1, Nrf2, Nrf3 or p45, and the protein level of each was determined using immunoblot analysis. Under my experimental conditions, USP15 also stabilized p45, as well as Nrf1 (FIG. 33) (70). Unexpectedly, I did not observe a USP15-driven stabilization in Nrf3 and Nrf2 (FIG. 34), although USP15 has been reported to destabilize Nrf2 by activating the Keap1-mediated ubiquitination activity by its deubiquitination (62). The reason for this discrepancy between

my results and other is unknown. Further examination is required to determine the molecular basis of the USP15-mediated regulation of the CNC family proteins.

4. Discussion

4.1 - HRD1-VCP-mediated NRF3 regulation and its biological significance.

In this study, I have investigated the molecular regulatory mechanisms and biological function of NRF3 in cancer cells. I demonstrated that the ubiquitin ligase HRD1 and its cofactor VCP facilitates the molecular degradation of transcription factor NRF3 in the cytoplasm and that β-TRCP modulates that of NRF3 in the nucleus. These data are consistent with the results of my laboratory and others that the turnover of NRF3-related factor NRF1 is also under these regulatory systems (32, 69). From my research findings, it has been identified that HRD1 is a major E3 ligase which promotes the degradation of NRF3 through its ubiquitination in the ER lumen. After ubiquitination in the ER, NRF3 is translocated to the cytosolic side of the cell membrane by VCP and degraded by the proteasome.

An important issue regarding molecular regulation of NRF3 is to understand how this transcription factor liberates from the ER sequestration and translocates into the nucleus for inducing its target genes. My current study reveals that repression of proteasome activity promotes nuclear translocation of NRF3 (FIG. 13). This finding implies that escape from proteasomal degradation in the cytoplasm is one of the possible mechanism by which NRF3 is activated. It has been previously reported that, under stress condition the nature of activation of transcription factor NRF2 is the inhibition of KEAP1 mediated degradation of NRF2 (45, 71, 72). Accordingly, I investigated the involvement of HRD1 and its cofactor VCP on the activation of NRF3. Nevertheless, I discovered that inhibition of the HRD1-mediated cytoplasmic degradation of NRF3 does not markedly augment nuclear entry of NRF3 (FIG. 27). In addition, I found that the inhibition of VCP does not increase nuclear accumulation of endogenous NRF3 (FIG. 31), suggesting the involvement of VCP in NRF3 activation. Interestingly, the transcription factor NRF1 is also activated through VCP in cells

with compromised proteasome activity (69). Collectively, my research reveal that molecular mechanisms underlying the nuclear translocation of NRF3 is not due to regulation of the HRD1-mediated degradation.

Recently, regarding this NRF3 activation mechanism, it has been reported that ER associated degradation factors are important for aspartic protease DDI2 and DDI1 (*C. elegans* homologue) mediated nuclear translocation NRF1 and its homologue SKN-1, respectively (73, 74). DDI2 (DNA-damage inducible 1 homolog 2) is highly conserved throughout eukaryotes (75). Recently, my laboratory identified that the DDI2 is also required for the nuclear translocation of NRF3 (Atsushi Hatanaka, a personal communication). Identification of the NRF3-DDI2 axis further gives rise the next question as to how DDI2-mediated processing of NRF3 is regulated. This crucial question is related to the NRF3 activation mechanism. For this question, my speculation is that DDI2 is recruited to the VCP complex on the ER membrane and this step is a key mechanism for the NRF3 processing. Recent genome-wide screens have elegantly identified that UBXD8, a VCP-recruiting factor in ERAD involve molecular mechanisms of NRF1 and SKN1 activation (73, 74, 76). The ER membrane protein UBXD8 transfers ubiquitinated ERAD substrates to VCP, presumably indicating that it recognizes an ubiquitin chain conjugated to NRF3. Consistently, I found that HRD1 knockdown elicits cytoplasmic accumulation of NRF3 but not its DDI2-mediated processing (FIG. 27). This result may imply that the HRD1-mediated polyubiquitin chain of NRF3 is required for DDI2-mediated cleavage. Interestingly, UBXD8 associate with VCP (77, 78). Thus, it is assumed that DDI2 may recruit to the VCP complex for associating with NRF3. Alternatively, DDI2 also possesses the UBL domain and ubiquitin-interacting motif (UIM). These domains may be required for transfer of polyubiquitinated Nrf1 to VCP. A schematic model in FIG. 35, summarizes the biological significances of HRD1-VCP-mediated NRF3 regulation.

4.2 - β-TRCP mediates nuclear degradation of NRF3.

It is assumed that under the physiological condition the complete repression of NRF3 is mediated through its cytoplasmic sequestration and degradation mechanisms. However, under stress condition, NRF3 escape from cytoplasmic degradation and translocate to the nucleus upon the activation signal(s) that is identified yet. In the nucleus, the transcriptional activity of NRF3 is control by the nuclear degradation pathway. In this study I identified, β-TRCP promotes the nuclear degradation of NRF3. The β-TRCP-mediated degradation mechanism is also the common regulatory pathway for NRF3-related transcription factors NRF1 (32) and NRF2 (79). Given that a highly conserved DSGLS motif in NRF1 serves as a degron (32), it is possible that the DSGLS motif in NRF3 is also conserved and the target of β-TRCP for its degradation (8). So it is assumed that GSK-3β-mediated phosphorylation of DSGLS motif is essential for nuclear degradation of NRF3 as in the case of Nrf1 (32).

Previously my laboratory reported the involvement of β-TRCP on the transcriptional regulation of Nrf1 (32), but unexpectedly I could not conclude whether β-TRCP regulates NRF3 transcriptional activity or not. Because, while under my experimental conditions, β-TRCP promotes the nuclear degradation of NRF3 upon protesome inhibitor MG132 treatment but interestingly, MG132 treatment alone affected the mRNA expression of *NRF3* and its target gene *UHMK1* expression (FIG. 32). Further examination is required to determine the effects of β-TRCP on NRF3 transcriptional activity. Additionally, it has been reported that FBW7 (F-box WD repeat containing domain 7), a well-established tumor suppressor also involves in the nuclear degradation of NRF3 (80). Thus, the next question, which of β-TRCP or FBW7 dominantly determines the NRF3 stability in the nucleus remains to be elucidated.

4.3 - A viewpoint on similar molecular regulation of NRF1 and NRF3.

This study discovered that NRF3 and NRF1 are under the control of same regulatory systems including HRD-VCP and β-TRCP. It is very reasonable because NRF3 and NRF1 are believed to be derived from the common ancestral CNC gene (Drosophila) and they actually possess the NHB1 and NHB2 domains that are indispensable for their molecular regulation including the ER sequestration (10, 17). Thus, it further suggests that NRF3 and NRF1 share common biological function, whereas other CNC members such as NRF2 play distinct roles due to lacking NHB1 and NHB2 domains. Nevertheless, gene targeting experiments in mice suggest distinct physiological functions between Nrf3 and Nrf1. *Nrf3* knockout mice do not exhibit apparent abnormalities under normal conditions, while *Nrf1* knockout mice show embryonic lethality due to anemia (Table 1). Their functional differences might be due to a difference of their activation mechanisms. For example, my laboratory preliminarily found that NRF3 knockdown significantly induces NRF1 protein accumulation, which rescues loss of NRF3 function in human colon cancer HCT116 cells although NRF1 knockdown does not induce NRF3 protein (Dr. Tsuyoshi Waku. a personal communication). This compensatory mechanism by NRF1 might be a reason why Nrf3 knockout mice do not exhibit severe abnormalities. Accordingly, I consider that NRF3 and NRF1 are activated at least in part by distinct biological pathways. So, in future comprehensive analyses of NRF1 and NRF3 activation mechanisms, *i.e.* DDI2 processing mechanisms or other unidentified activation signals should provide us insights into their functional discrepancy. These observations also indicate that The CNC family proteins in higher eukaryotes might acquire diversity and complexity during the evolutionary progress from the common ancestral gene in *Drosophila.*

4.4 - Biological function of NRF3 in cancer cells.

As a member of CNC family transcription factor, NRF3 involves in the regulation of various cellular processes. However, the physiological functions of NRF3 are still elusive although there were some reports on involvement of NRF3 in inflammation (15) and smooth muscle

cell differentiation (23). Interestingly, high expression of NRF3 mRNA has been reported in several human cancers (20-22) and this indicates the potential physiological roles of NRF3 in cancer progressions. NRF3 exerts its function through the activation of target genes expression. Hence, identification of specific target genes of NRF3 in cancer cells is essential for understanding its physiological functions. Recently, my laboratory members further found that NRF3 activates proteasome genes (Nanami Kamada. a personal communication). In addition, some investigators also reported on potential target genes of NRF3 (15, 23, 81) but there are still no reports on NRF3 target genes which are directly related to cancer cells proliferation. In this study, I discovered that *UHMK1* is the target gene of NRF3 in colon cancer cells (DLD-1, HCT116) and NRF3 involves in the proliferation of colon cancer cells (DLD-1) by inducing the *UHMK1* gene expression. The UHMK1 kinase promotes cell proliferation by repressing the cyclin dependent kinase (CDK) inhibitor p27Kip1 through phosphorylation and then activating CDK in G1 phase (60, 61, 82). Unfortunately, I could not reveal by chromatin immunoprecipitation (ChIP) analysis that NRF3 directly mediates the *UHMK1* gene expression due to unknown reasons. Alternatively, the time course study of the *NRF3* and *UHMK1* gene expression upon NRF3 knockdown in DLD-1 cells strongly suggests that NRF3 is an upstream activator of the *UHMK1* gene expression (FIG. 22).

p27Kip1 is a tumor suppressor gene and an important regulator of the cell cycle (82). I found NRF3 knockdown increases cell cycle arrest of colon cancer cells (FIG. 24). This finding indicates that when *UHMK1* expression reduces through NRF3 inhibition, the activated p27Kip1 reduces cell proliferation by increasing cell cycle arrest. This finding may further provide us an answer to the question regarding NRF3 activation signal/stimuli. Given that the cell cycle regulator *UHMK1* gene is the NRF3 target gene, certain growth factors may activate NRF3 in cancer cells.

My finding on NRF3-mediated cancer cells proliferation is also consistent with a current research carrying in my laboratory regarding physiological roles of NRF3 in cancer (Dr. Tsuyoshi Waku. a personal communication). He found, NRF3 promotes cancer cells proliferation in the p53-dependent manner (data not shown). Collectively these findings indicate the physiological significance of the transcription factor NRF3 in cancer cells.

4.5 - The NRF3 regulatory system may be the potential target to design anticancer drug.

My study further proposes one attractive idea: development of a new anticancer therapeutic strategy by targeting the NRF3 regulatory systems. This research helps to understand the DD12-mediated activation of NRF3 (FIG. 35). The knockdown of DDI2 reduces the nuclear entry of NRF3 (Atsushi Hatanaka, a personal communication), presumably reducing NRF3-mediated cell growth. Accordingly, it is possible that DDI2 inhibitors work as anticancer drugs by repressing the NRF3 activity. Intriguingly, the retroviral protease-like (RVP) domain of yeast homolog Ddi1p structurally exhibits a similar fold to those of HIV protease domains (83). Thus, HIV therapeutic drugs that target the HIV protease might be repositioned as anticancer drugs that suppress the peptidase activity of DDI2.

5. Conclusion

This study reveals that two distinct proteasomal degradation systems including HRD1-VCP and β-TRCP regulate the protein stability of NRF3 in the cytoplasm and nucleus, respectively. Furthermore, from this study it has been revealed the nature of the activation mechanism of NRF3 is not the inhibition of its HRD1-VCP-mediated degradation. This study also identifies that NRF3 augments cell proliferation by inducing the cell cycle regulator gene *UHMK1*. A schematic model in Figure 36 summarizes the regulatory mechanism of NRF3-mediated cell proliferation, which is tightly coupled with multiple protein degradation and processing systems. Collectively, this study identifies multiple pathways regulating NRF3 with possible relevance to NRF3/*UHMK1*-dependent proliferation of cancer cells. Elucidation of these NRF3 regulatory pathways may provide possible molecular targets for the suppression of cancer cell proliferation.

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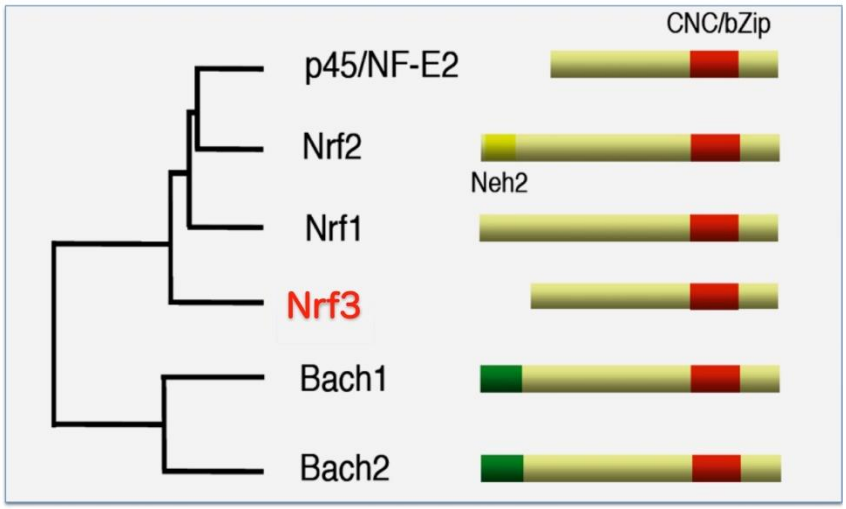
A.



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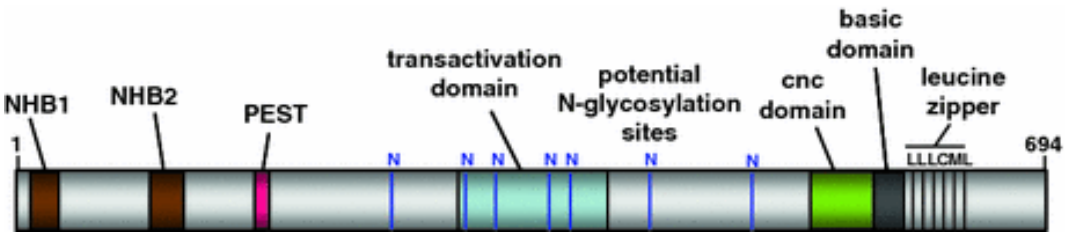

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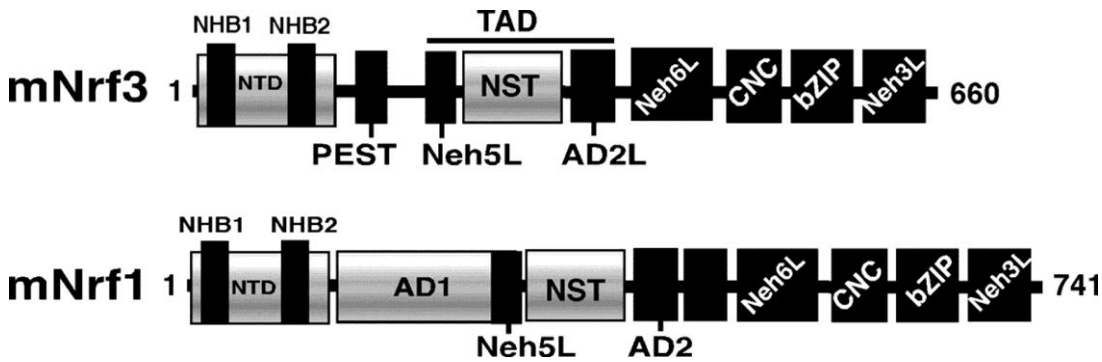

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FIG. 1. The CNC family proteins and structure of NRF3. (A) A phylogenetic tree of the vertebrate CNC family proteins. (B) Structure of human NRF3 consist of 694 amino acid protein. (C) The NHB1 and NHB2 domains of mouse Nrf1 and Nrf3 are conserved.

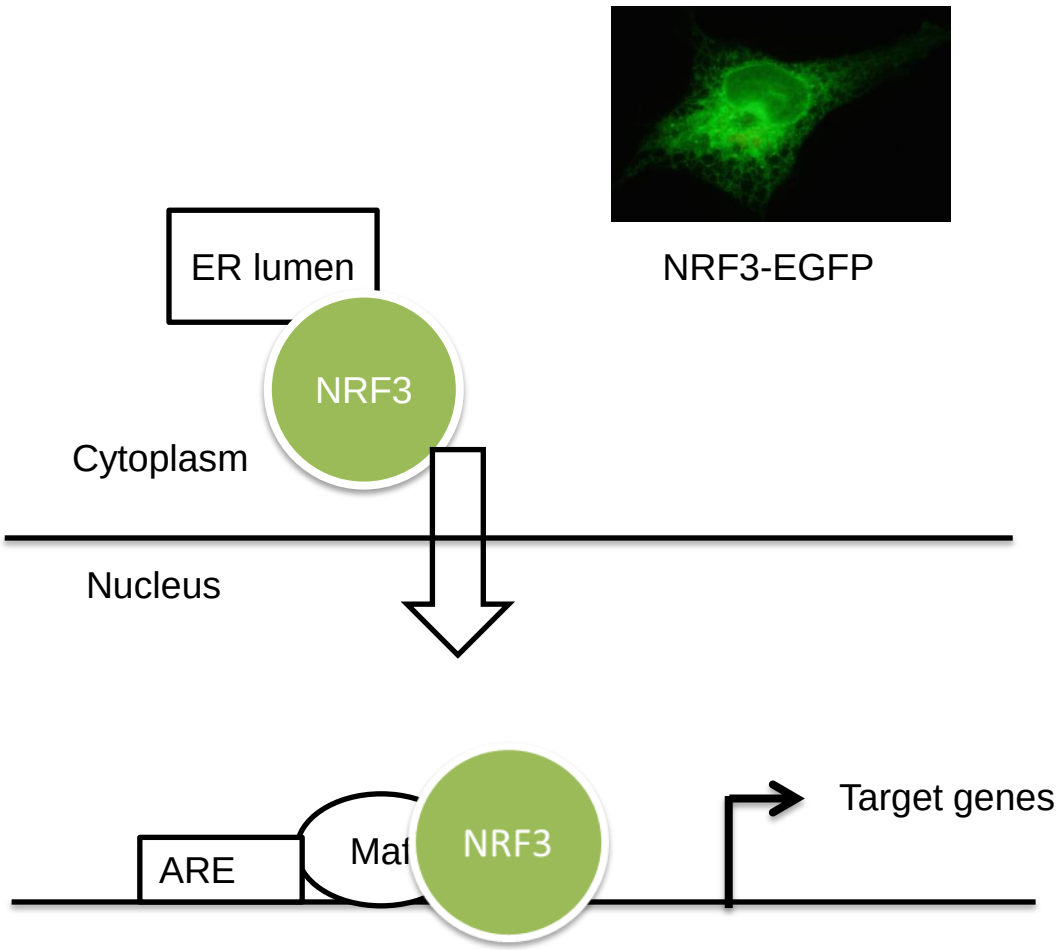

FIG. 2. A schematic model of NRF3 activity in the cells. Under physiological condition, NRF3 is localized in the ER (The image shows ER localization of NRF3-EGFP fusion protein in cells). Upon exposure to unknown signal(s), NRF3 translocates into the nucleus and activates gene expression through the ARE sequence along with a small Maf protein.

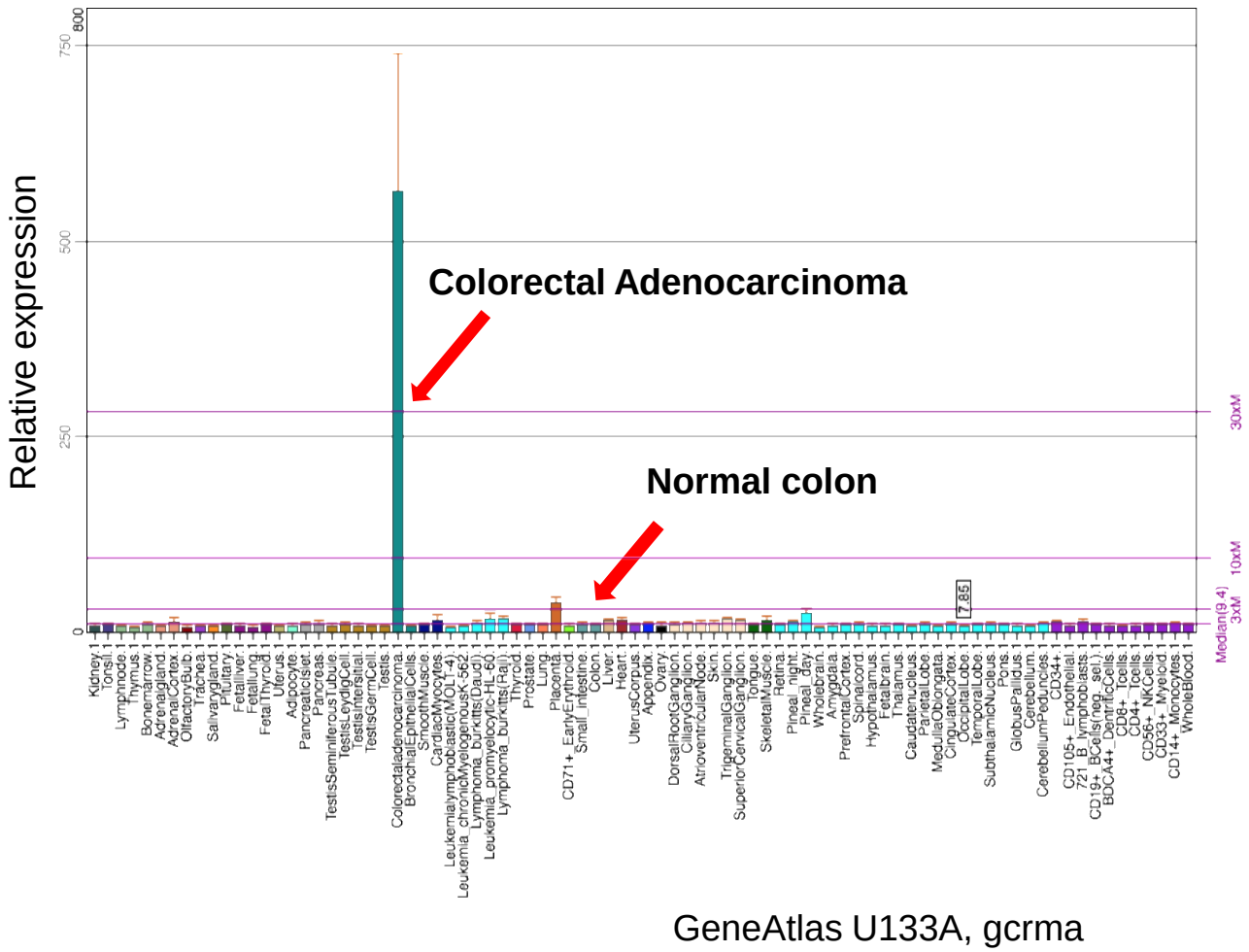

FIG. 3. High expression of NRF3 in colon cancer. This graph shows gene expression of NRF3 in a line of human tissues. Interestingly, NRF3 is highly expressed in colorectal adenocarcinoma compared to normal colon.

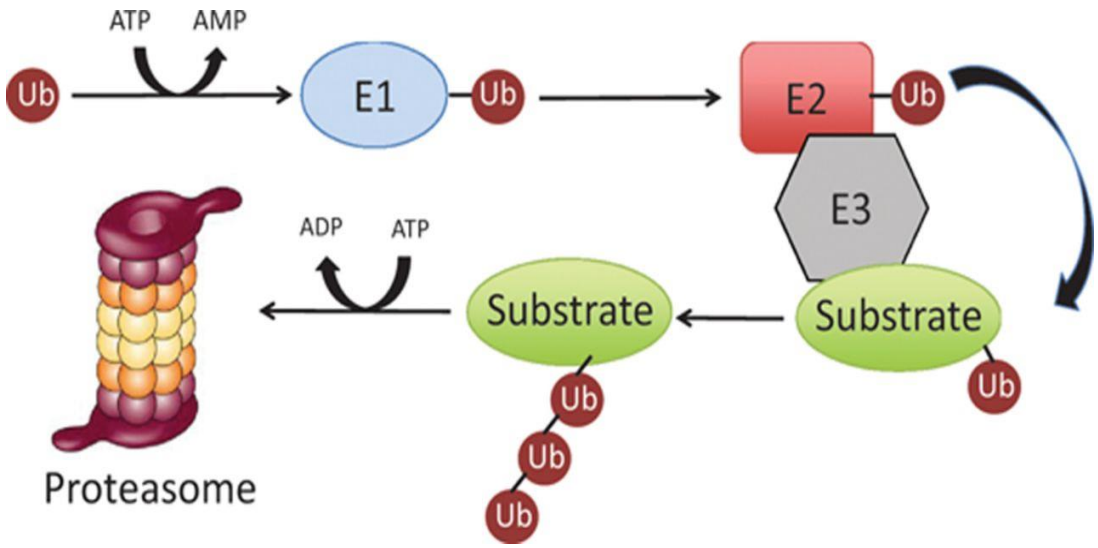

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FIG. 4. The Ubiquitin Proteasome System (UPS). The degradation of specific substrates occurs through sequential reactions, catalyzed by three enzymes (E1, E2 and E3).

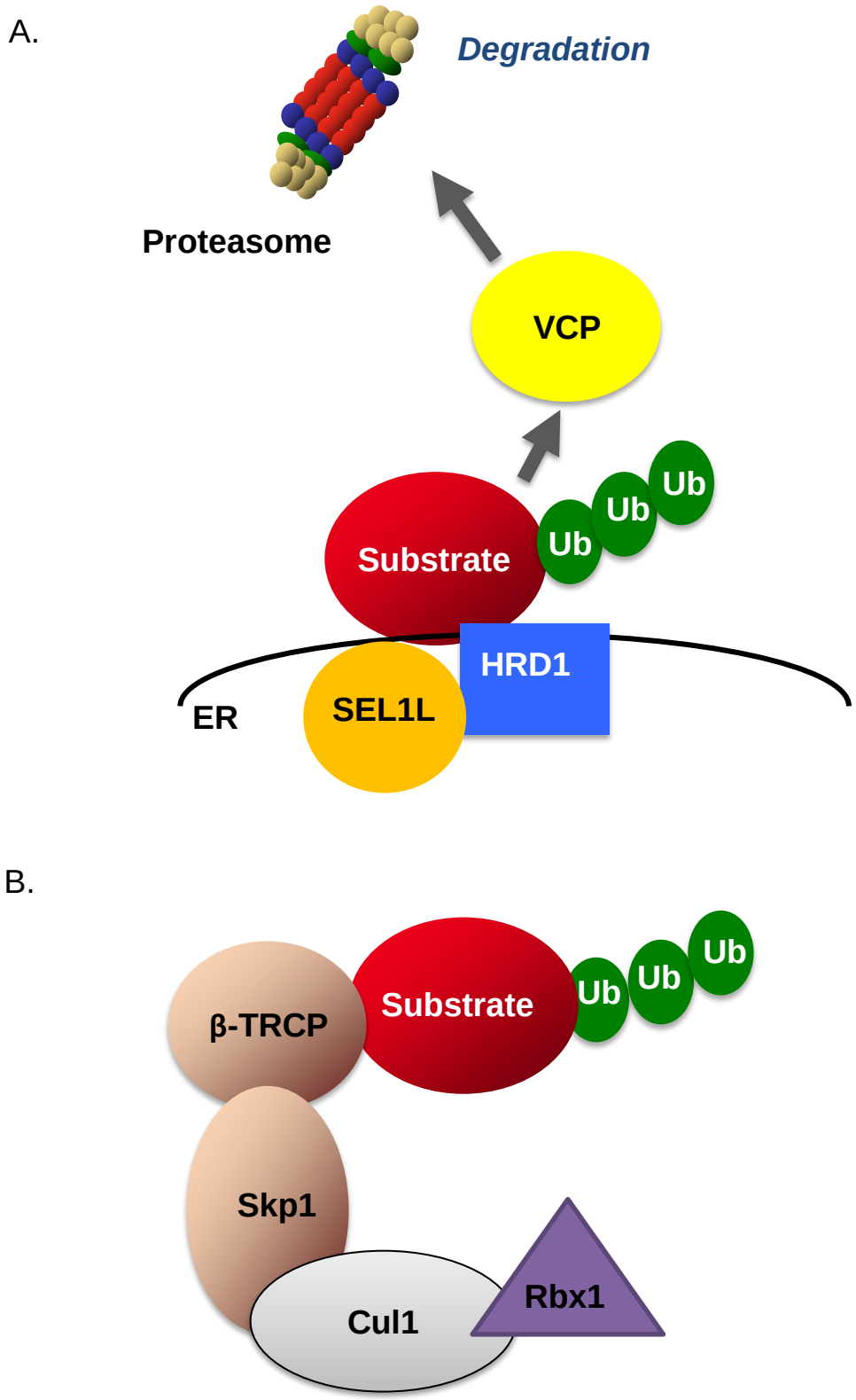


FIG. 5. Functions of E3 Ubiquitin ligases in UPS. (A) After being polyubiquitinated by the HRD1-SEL1L complex, the substrates are recognized by VCP and are transferred to the proteasome for degradation. (B) β-TRCP determines a substrate specificity and recruit target protein into Cul1 ub ligase complex along with SKP1.

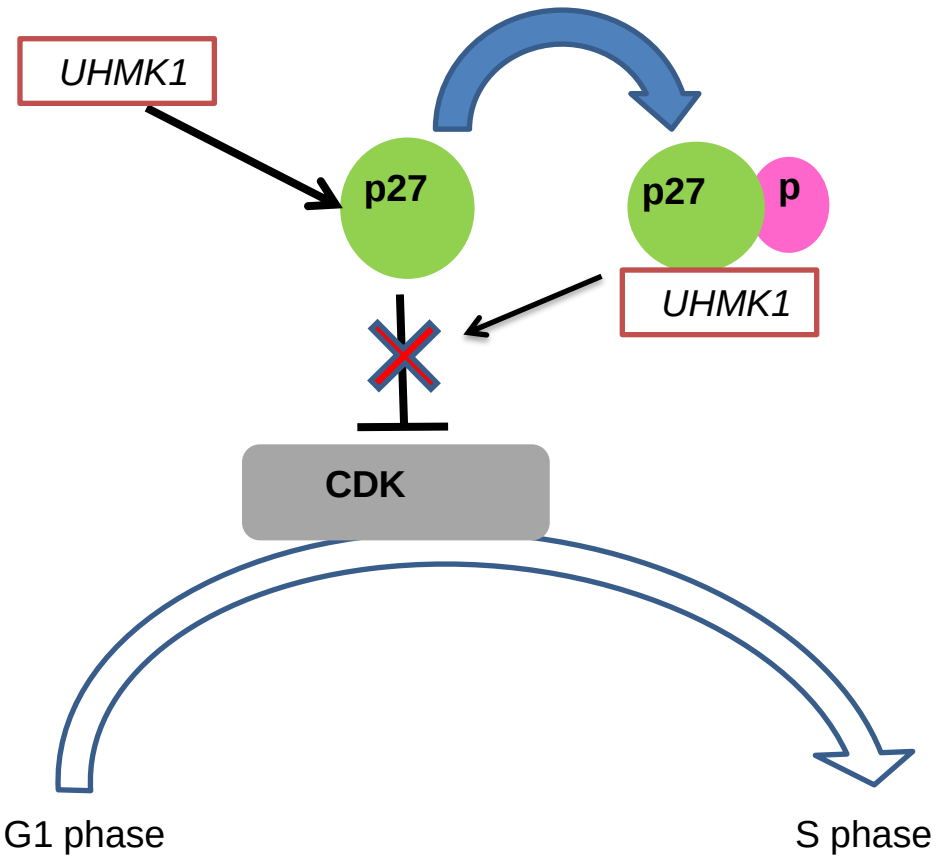


FIG. 6. *UHMK1* regulates cell cycle through G1/S check point. The cyclin dependent kinase (CDK) inhibitor p27 is one of the tumor suppressor genes and an important regulator of the cell cycle. *UHMK1* increases the activity of cyclin dependent kinase in G1 phase by phosphorylating its inhibitor p27 and promotes cell proliferation.

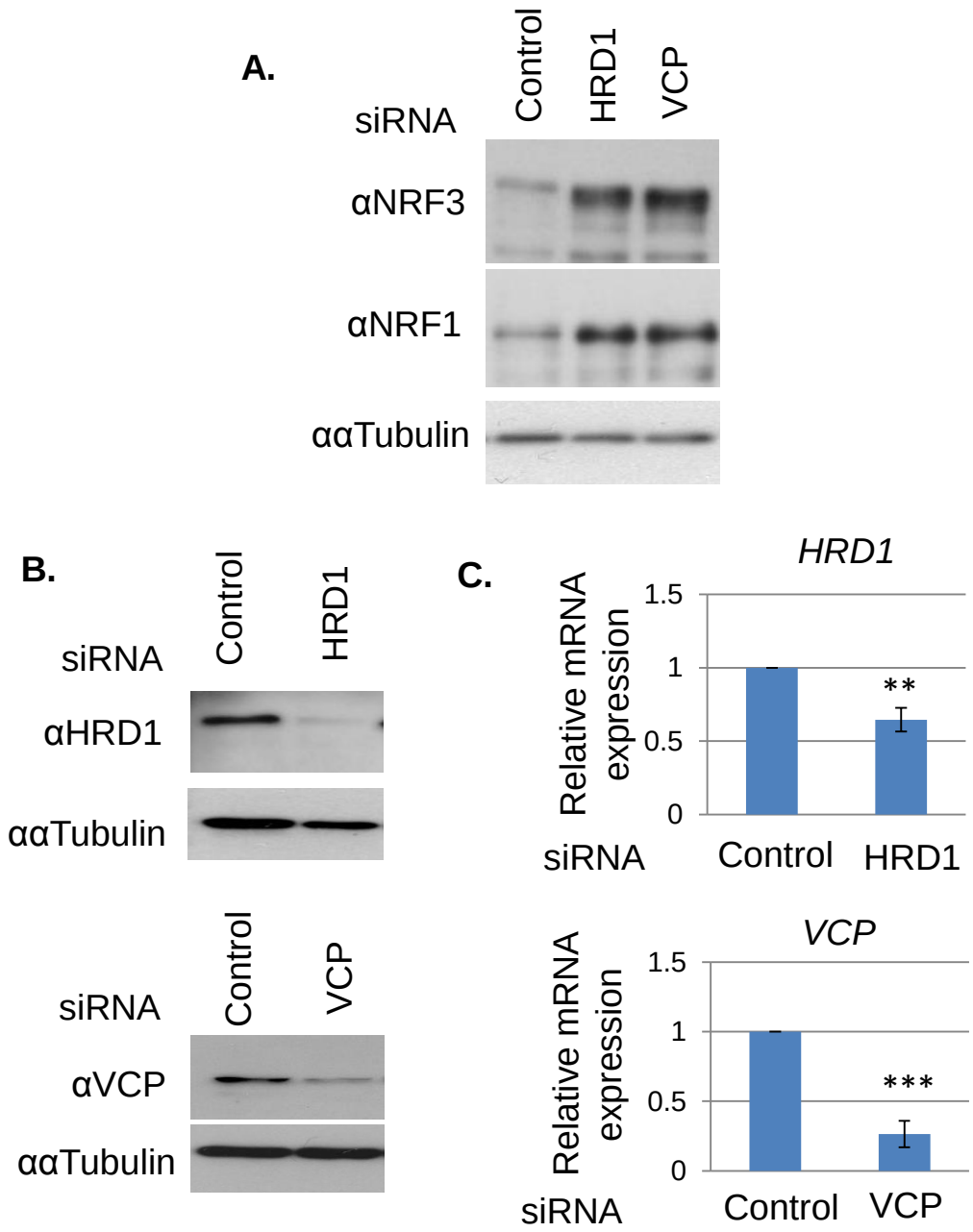


FIG. 7. HRD1 and VCP regulate the cytoplasmic degradation of NRF3. (A*)* *HRD1* or *VCP* siRNA stabilized endogenous NRF3 in DLD-1 cells. At 48 hr after the siRNA transfection, the whole-cell extracts were prepared and analyzed by immunoblotting with anti-NRF3 and anti-NRF1 antibodies. α-Tubulin was used as an internal control. (B, C) Knockdown efficiency of *HRD1* and *VCP* siRNA was determined by immunoblot analysis with the indicated antibodies on the figure and qRT-PCR analysis. The values of qRT-PCR analysis (C) were normalized to *18S rRNA* data. The error bars (C) represent data from three independent experiments (mean± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. ** $P < 0.01$ and *** $P < 0.001$ compared to the Control data.

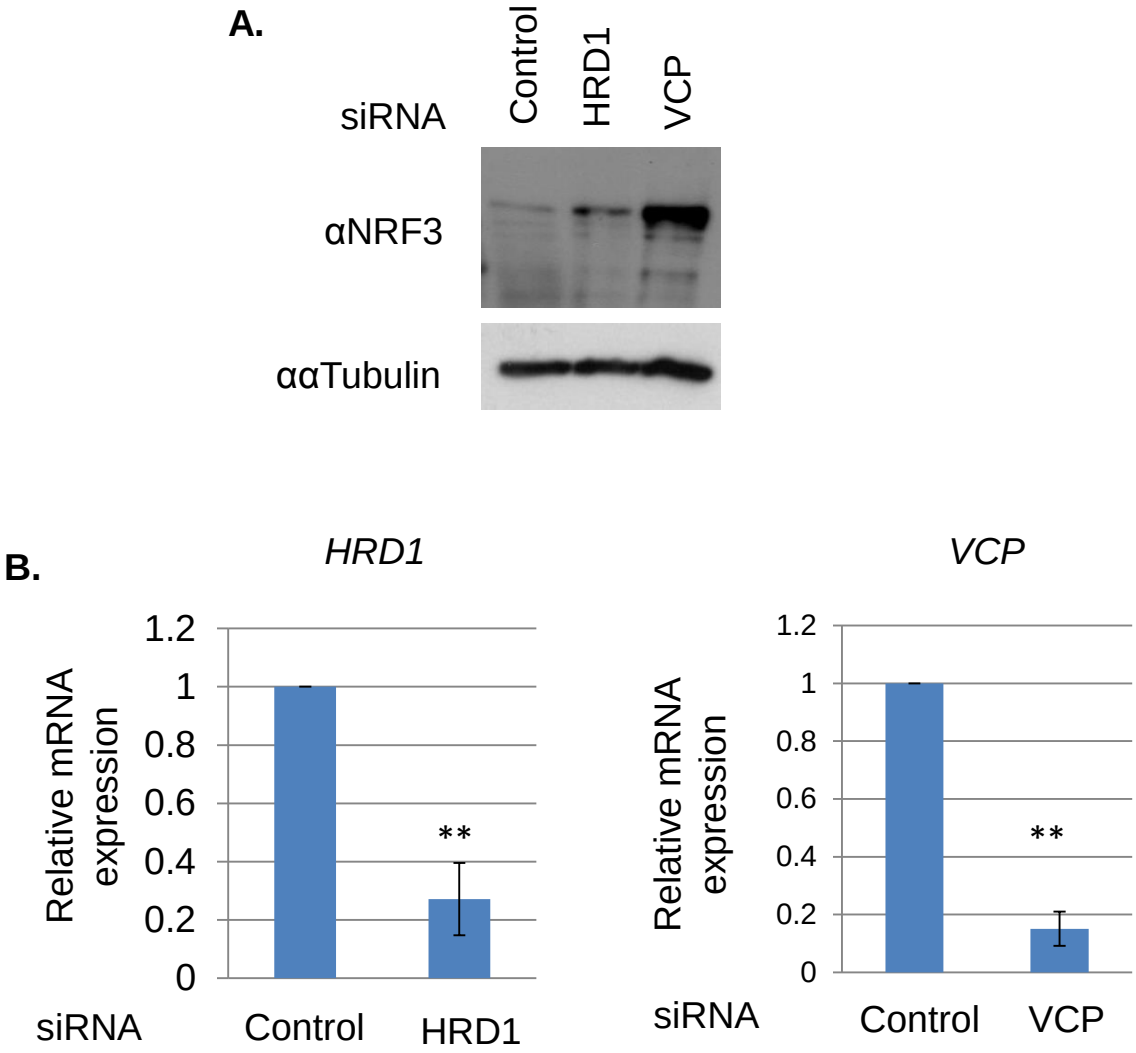


FIG. 8. HRD1 and VCP regulate the cytoplasmic degradation of NRF3 in HCT116 cells. (A) At 48 hr after siRNA transfection, the whole-cell extracts were prepared and analyzed by immunoblotting with anti-NRF3 antibody. α-Tubulin was an internal control. (B) Knockdown efficiency of *HRD1* and *VCP* siRNA was determined by real-time quantitative PCR analysis. The values were normalized to *β-actin* data. The error bars (B) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. ** $P< 0.01$ compared to the Control data.

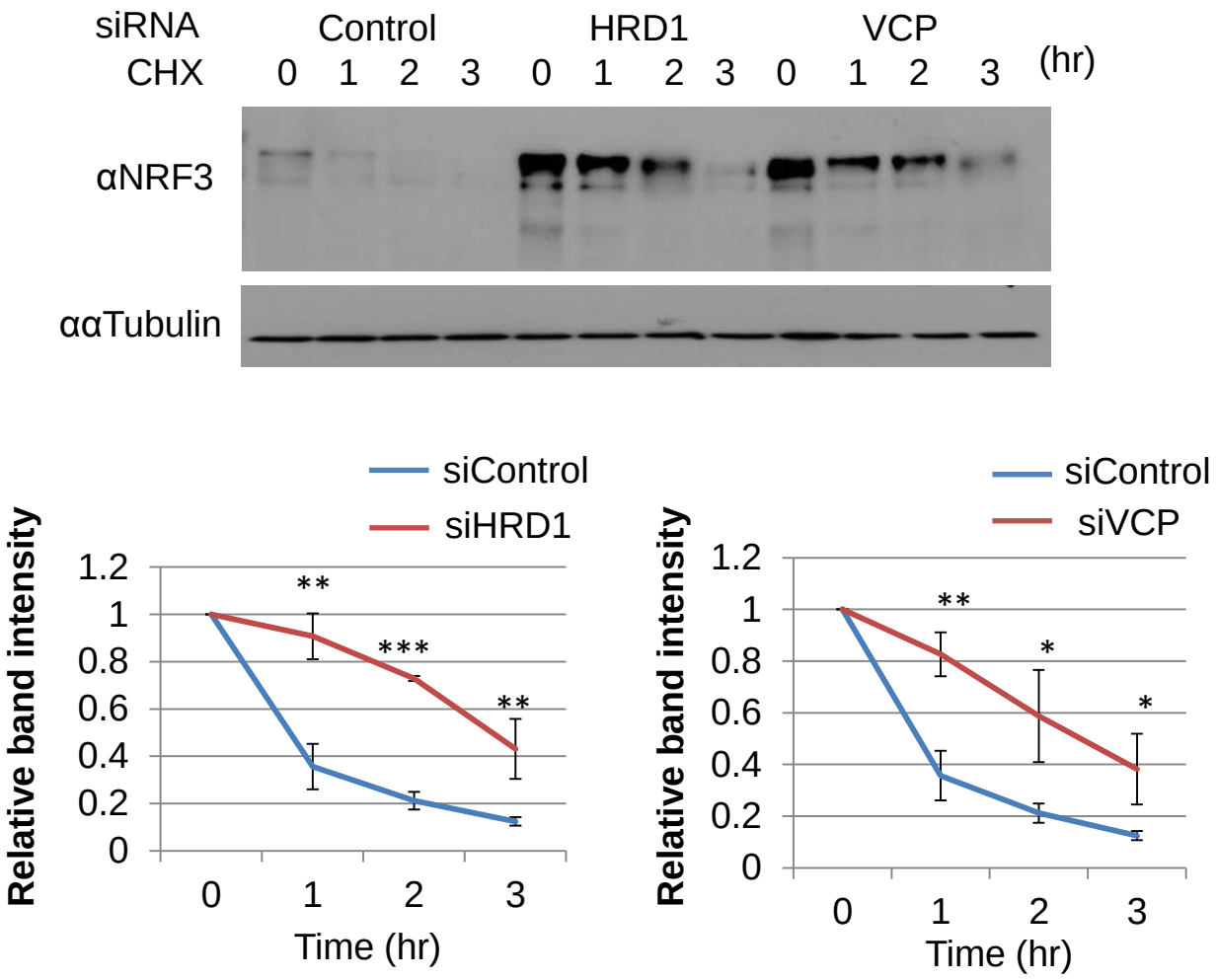


FIG. 9. Knockdown of VCP and HRD1 inhibits NRF3 degradation. (A) Cycloheximide chase experiment was performed. *Control*, *HRD1* and *VCP* siRNA were transfected into DLD-1 cells. At 48 hr after the transfection, the cells were treated with cycloheximide (CHX) (20 μg/ml) for indicated time points. Whole-cell extracts were prepared for immunoblot analysis with anti-NRF3 antibody. α-Tubulin was used as an internal control. The graphs depict the quantified band intensities of NRF3. The values were normalized with α-Tubulin. The error bars represent data from three independent experiments (mean± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the Control data.

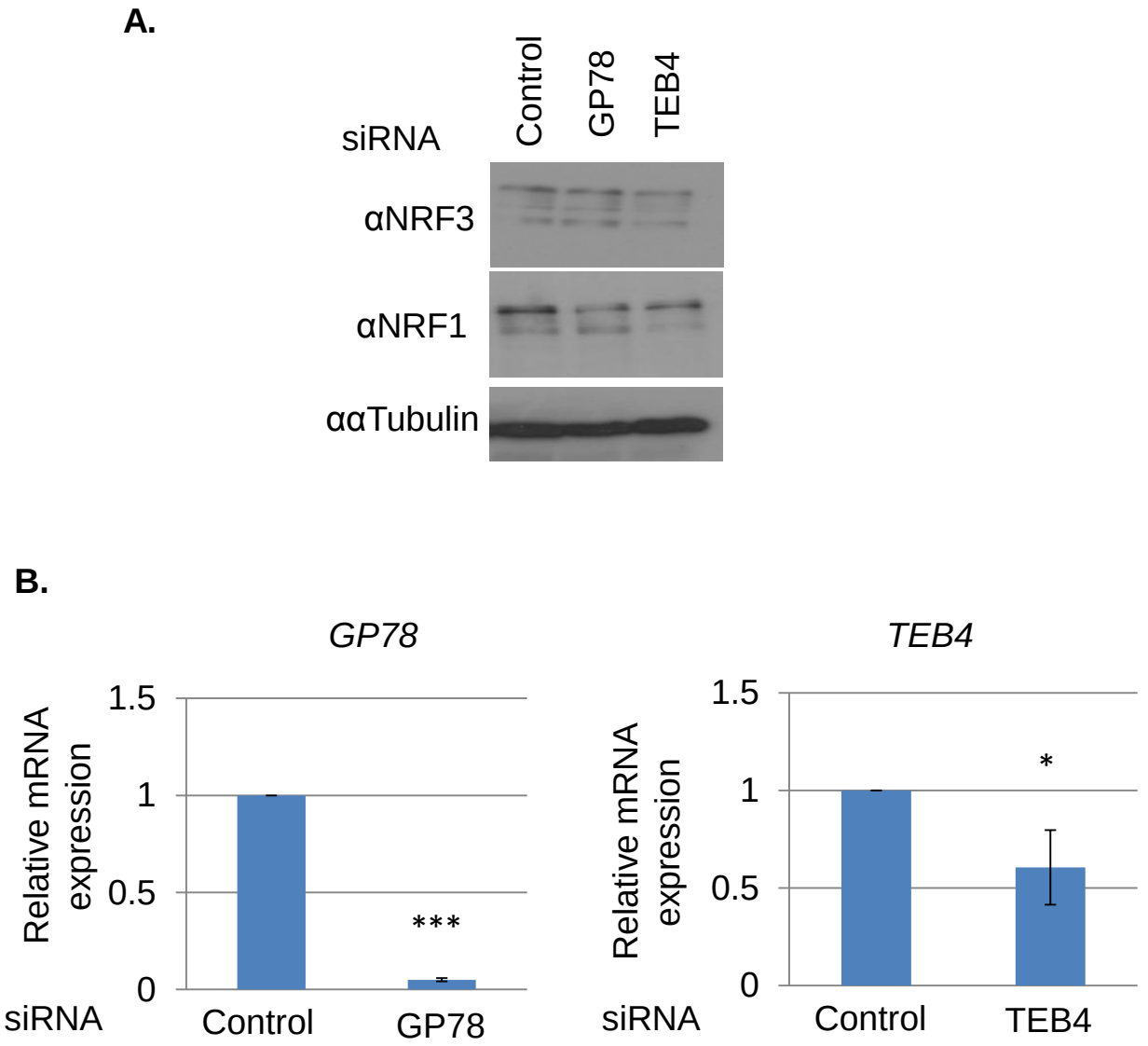


FIG. 10. GP78 or *TEB4* siRNA does not stabilize endogenous NRF3 in DLD-1 cells. (A) At 48 hr after siRNA transfection, the whole-cell extracts were prepared and analyzed by immunoblotting with anti-NRF3 and anti-Nrf1 antibodis. α-Tubulin was used as an internal control. (B) Knockdown efficiency of *GP78* and *TEB4* siRNA was determined by qRT-PCR analysis. The values were normalized to *18S rRNA* data. The error bars (B) represent data from three independent experiments (mean± standard deviation). Two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$ and *** $P < 0.001$ compared to the Control.

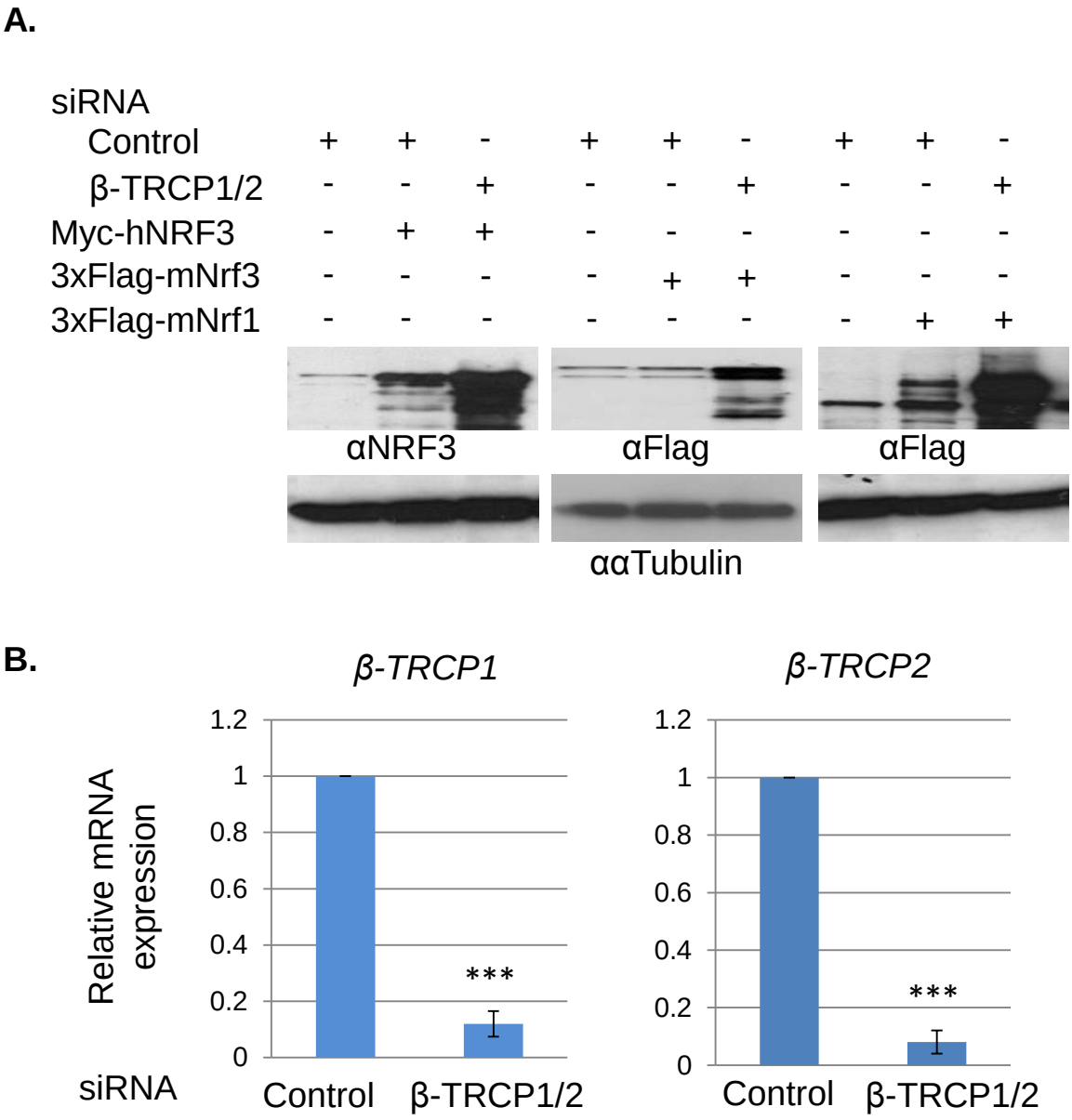


FIG. 11. β-TRCP regulates the degradation of overexpressed NRF3. (A) HeLa cells were transfected with Myc-hNRF3, 3×Flag-mNrf3 or 3×Flag-mNrf1 (as a positive control) expression vectors at 24 hr after two rounds of transfection with Control or *β-TRCP1/2* siRNA (simultaneously knockdown of both *β-TRCP1* and *β-TRCP2*). At 24 hr after the last transfection, whole-cell extracts from the cells were subjected to immunoblot analysis with anti-NRF3 and anti-Flag antibodies. α-Tubulin was used as an internal control. (B) The knockdown efficiency of *β-TRCP1/2* siRNA was determined by qRT-PCR analysis. The values were normalized to *18S rRNA* data. The Error bars represent data from three independent experiments (mean± standard deviation). The two-tailed student's t-test was used for the statistical analysis. *** $P < 0.001$ compared to the Control data.

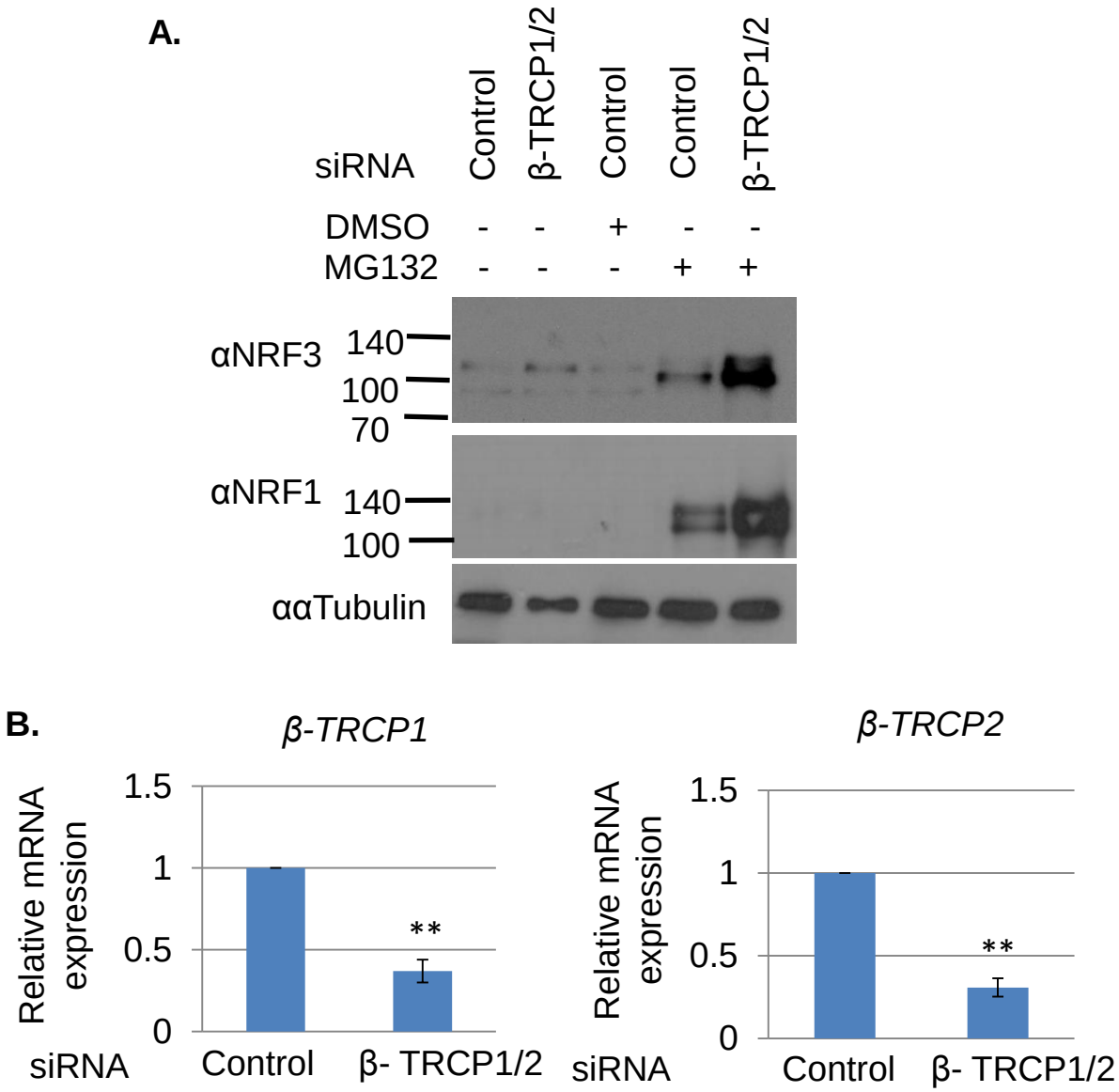


FIG. 12. β-TRCP modulates the nuclear degradation of endogenous NRF3. (A) Endogenous NRF3 is susceptible to the β-TRCP-mediated proteasomal degradation in the nucleus of DLD-1 cells. The cells were transfected with control or *β-TRCP1/2* siRNA. At 48 hr after transfection, the cells were subjected to two process; (1) their whole cell extracts were prepared for immunoblot analysis with anti-NRF3 and anti-NRF1 antibodies; (2) the cells were further treated with DMSO (dimethylsulfoxide) or MG132 (10 μM) for 6 hr, followed by similar immunoblot analysis. (B) The knockdown efficiency of siRNA for *β-TRCP1/2* was determined by qRT-PCR analysis. The values were normalized to *18S rRNA* data. The error bars (B) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. ** $P < 0.01$ compared to the Control data.

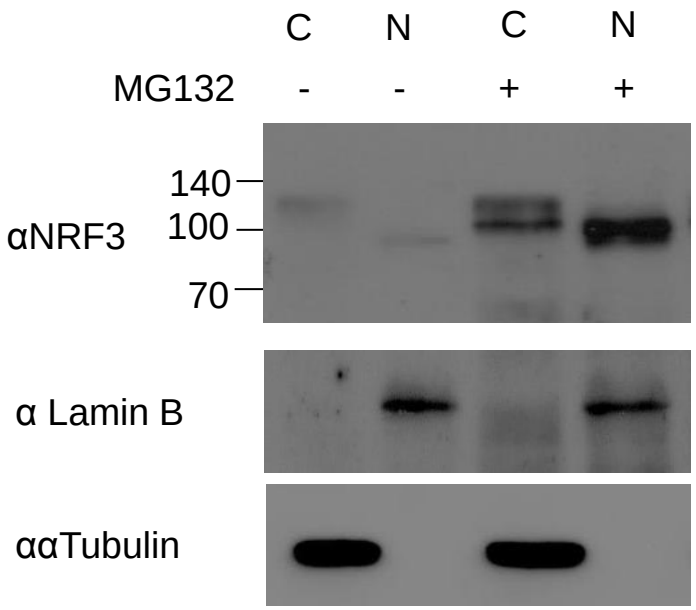


FIG. 13. Proteasome inhibitor MG132 treatment promotes the nuclear translocation of endogenous NRF3. DLD-1 cells were transfected with Control siRNA. At 48 hr after the transfection, the cells were treated with MG132 (10 μM) for 6 hr. After the MG132 treatment, cytoplasmic (C) and nuclear (N) fraction were extracted from the cells and subjected to immunoblot analysis with anti-NRF3 antibody. Lamin B and α-Tubulin were utilized as nuclear and cytoplasmic markers, respectively.

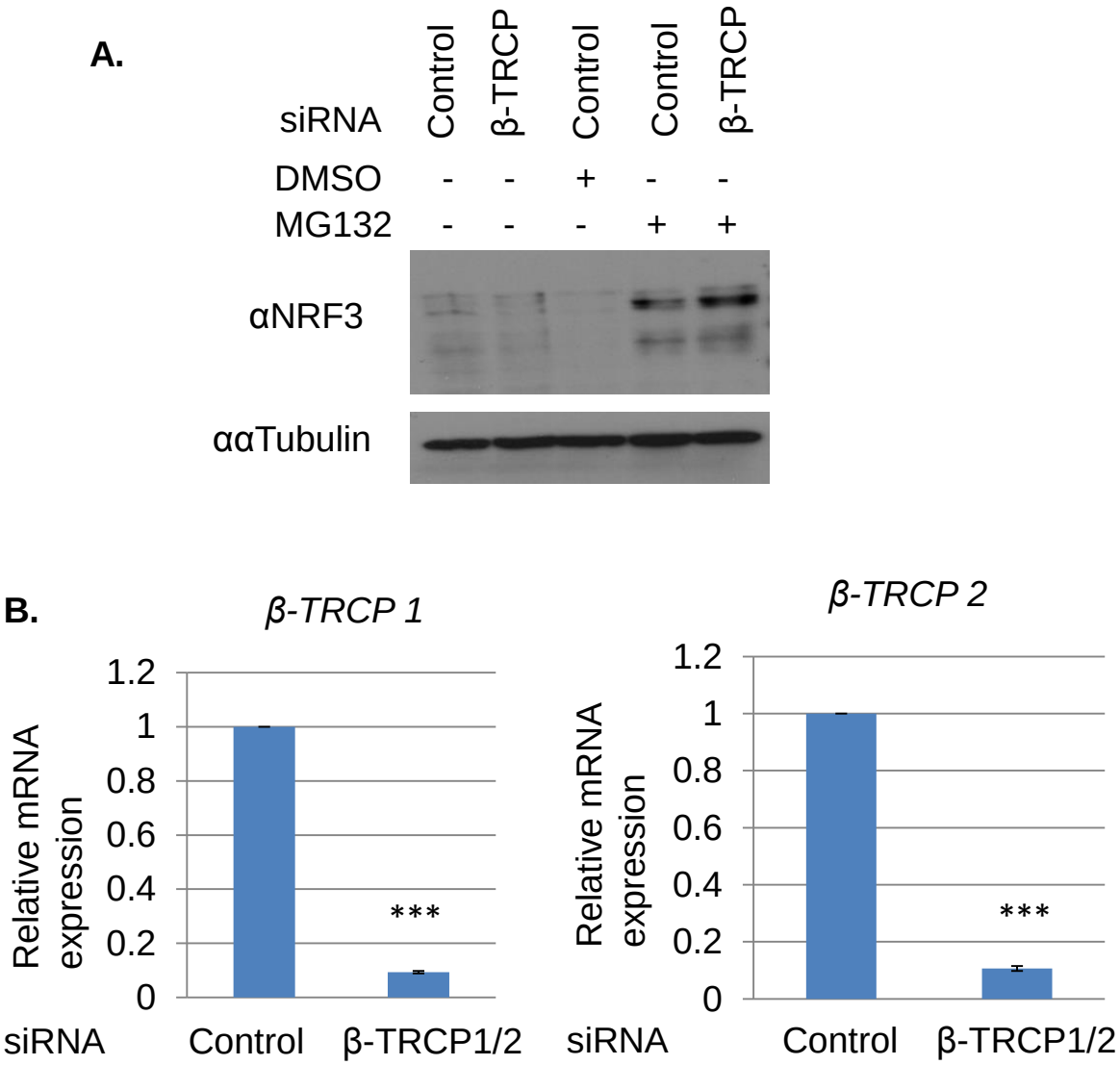


FIG. 14. β-TRCP promotes the degradation of endogenous NRF3 in HCT116 cells. (A) *β-TRCP 1/2* or *Control* siRNA was transfected into HCT116 cells. At 48 hr after the transfection, the cells were subjected to two process; (1) their whole cell extracts were prepared for immunoblot analysis with anti-NRF3 antibody; (2) the cells were further treated with DMSO or MG132 (10 μM) for 6 hr, followed by similar immunoblot analysis. (B) The knockdown efficiency of *β-TRCP1/2* siRNA was determined by real-time quantitative PCR analysis. The values were normalized to *β-actin* data. The error bars (B) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. *** $P < 0.001$ compared to the Control data.

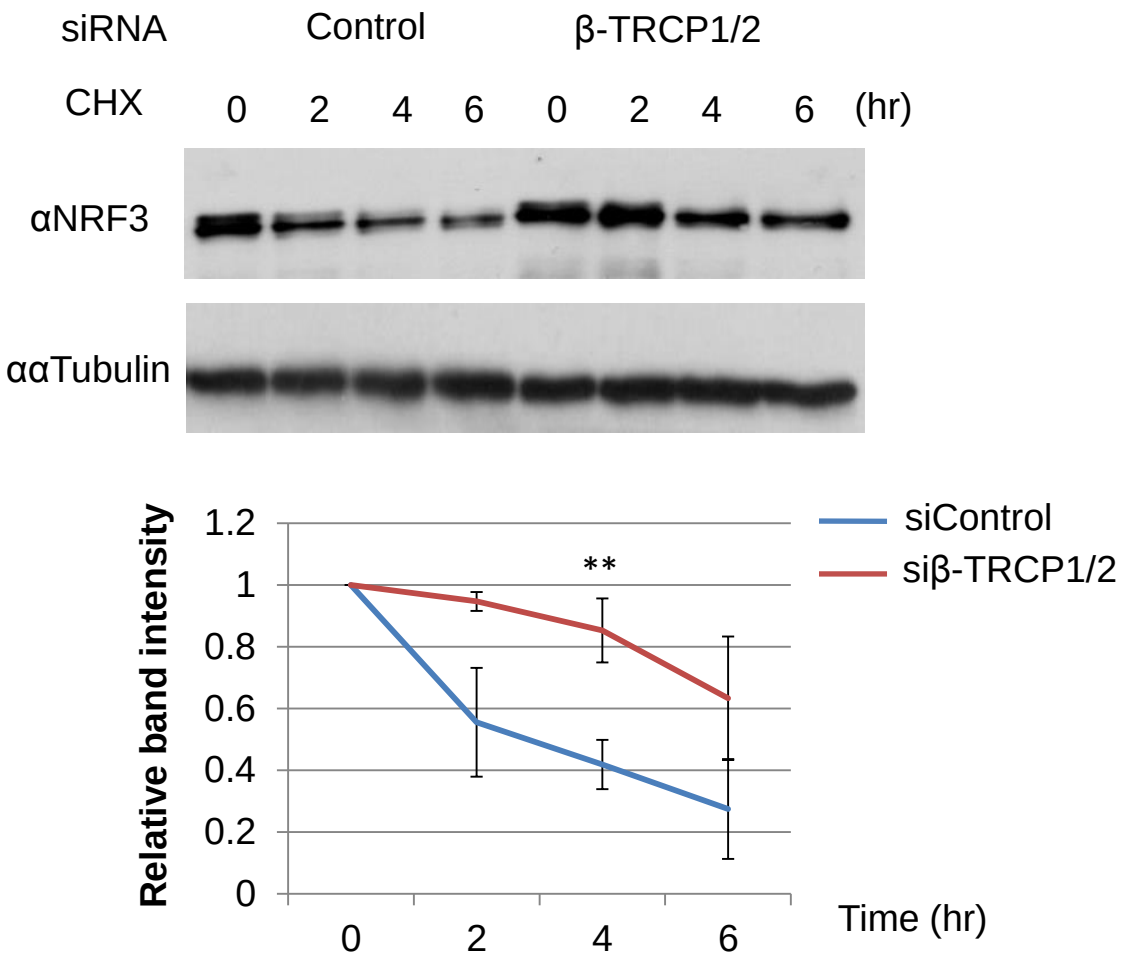


FIG. 15. β-TRCP promotes degradation endogenous NRF3 in DLD-1 cells. Cycloheximide chase experiment was performed. At 48 hr after the Control or *β-TRCP1/2* siRNA transfection, the cells were treated with MG132 (10 μM) for 6 hr prior treatment with cycloheximide (CHX) (20 μg/ml). Whole-cell extracts were prepared for immunoblot analysis with anti-NRF3 antibody. α-Tubulin was used as an internal control. The graph depicts the quantified band intensities of NRF3. The values were normalized with α-Tubulin. The error bars represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. ** $P < 0.01$ compared to the Control data.

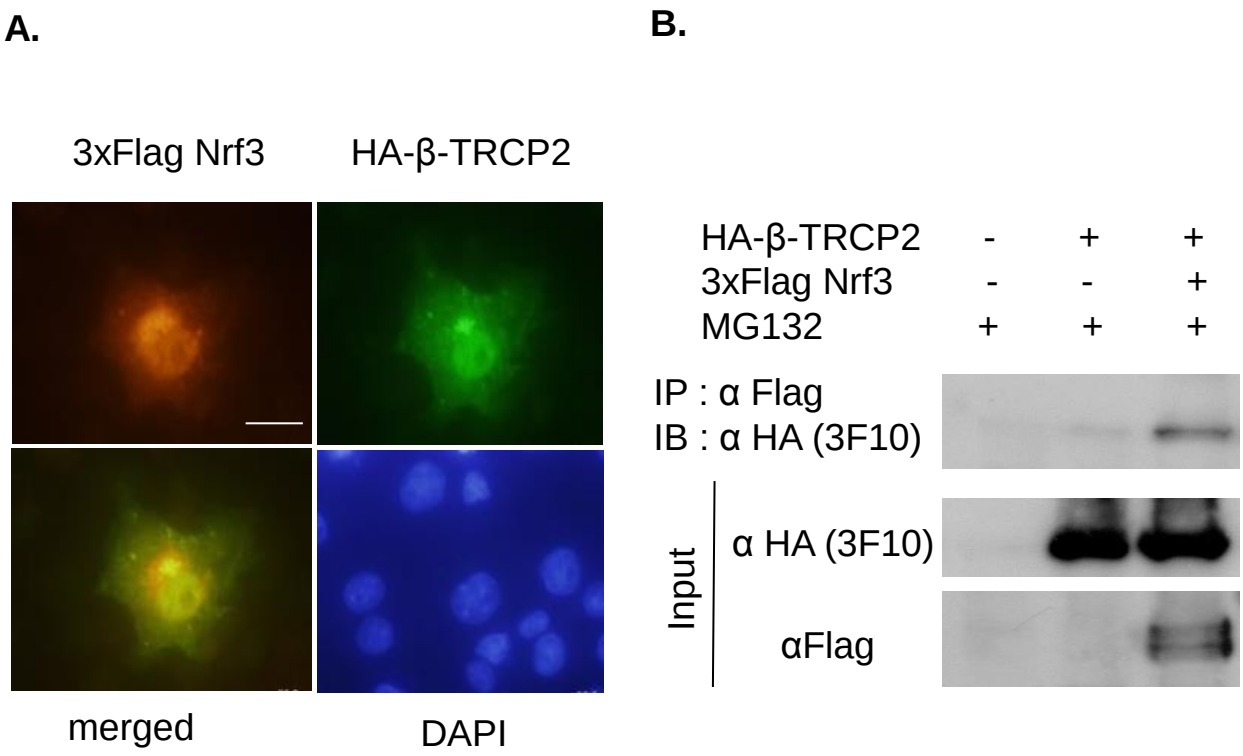


FIG. 16. Colocalization and physical interaction of Nrf3 with β-TRCP2. (A) Colocalization of Nrf3 with β-TRCP2 in COS7 cells. The cells were transfected with the 3xFlag-tagged Nrf3 or HA–β-TRCP2 plasmids. At 24 hr after transfection, the cells were treated with MG132 (10 μM) for 6 hr. After MG132 treatment, the cells were immunostained with the indicated antibodies. Bar, 20 μm. (B) Physical interaction of Nrf3 with β-TRCP2. Whole-cell extracts of COS7 cells expressing 3xFlag-Nrf3 and HA-taggedβ-TrCP2 were subjected to immunoprecipitation (IP) with anti-Flag antibody, followed by immunoblot (IB) analysis with the indicated antibodies.

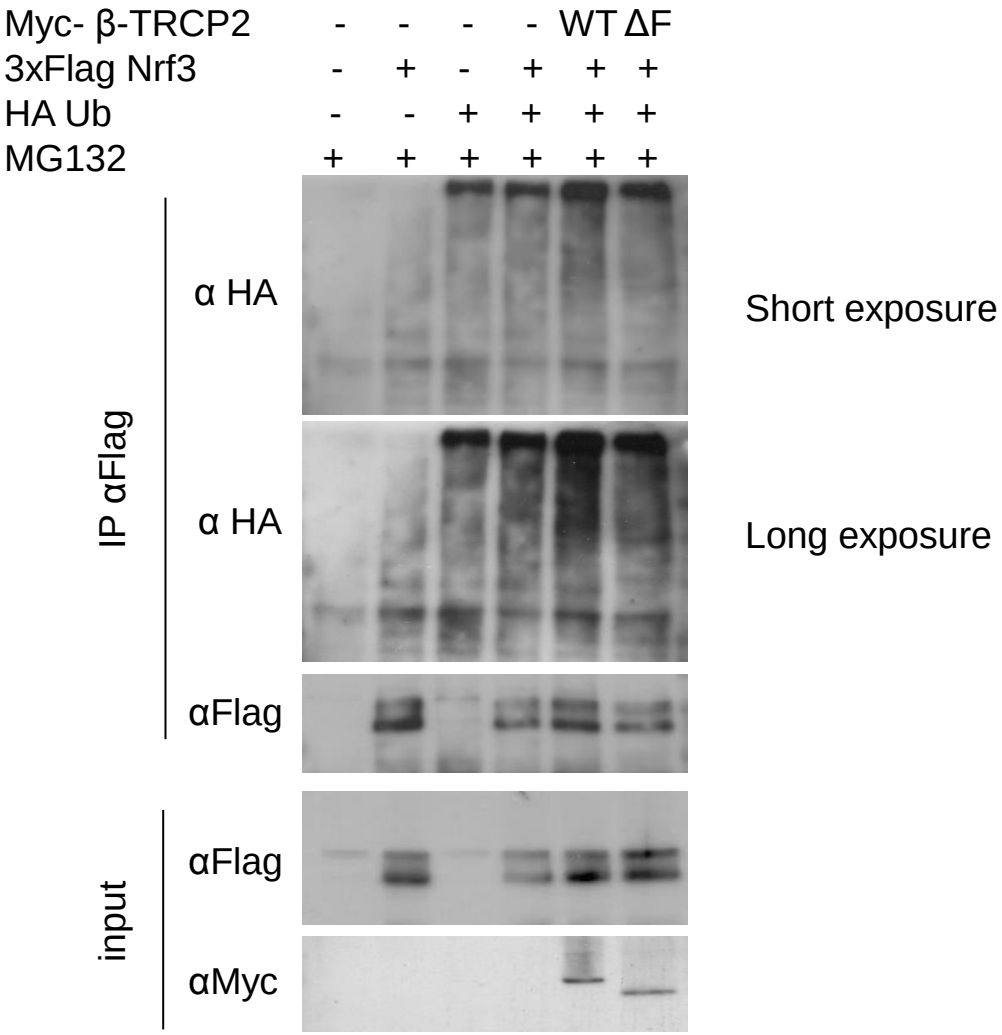


FIG. 17. β-TRCP mediates the polyubiquitination of Nrf3 in cultured cells. HCT116 cells were transfected with expression plasmids encoding 3xFlag-Nrf3, HA-tagged ubiquitin along with plasmids expressing wild-type Myc-tagged β-TRCP2 or ΔF-box β-TRCP2 mutant. At 24 hr after transfection, the cells were treated with MG132 (10 μM) for 6 hr. The cells were then subjected to immunoprecipitation by anti-Flag antibody. After immunoprecipitation, ubiquitination of overexpressed Nrf3 was detected by immunoblot analysis with anti-HA antibody.

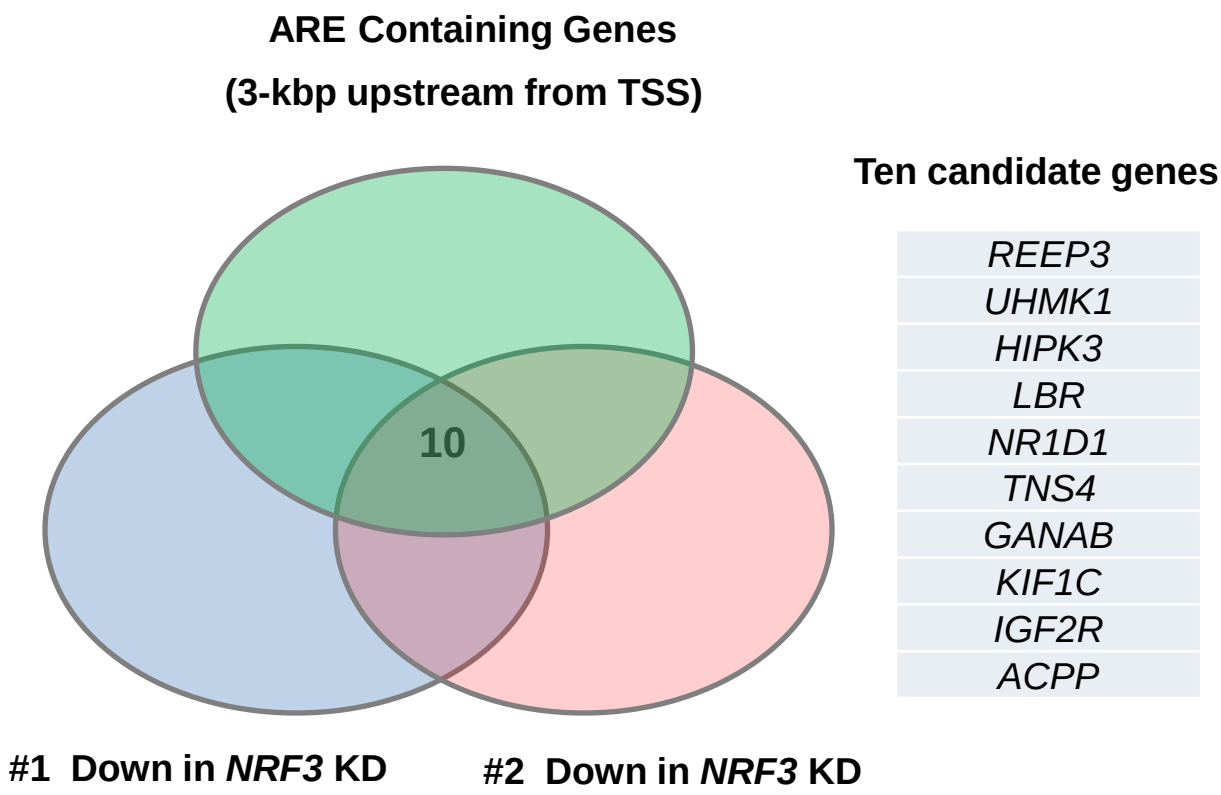


FIG. 18. Identification of *UHMK1* gene as a target of NRF3. Venn diagram combining of two independent microarray data of NRF3 siRNA-transfected DLD-1 cells (#1 and #2) and a list of genes possessing the species-conserved ARE within the 3-kbp upstream form the transcriptional start site (TSS). Ten candidate genes from this analysis are shown.

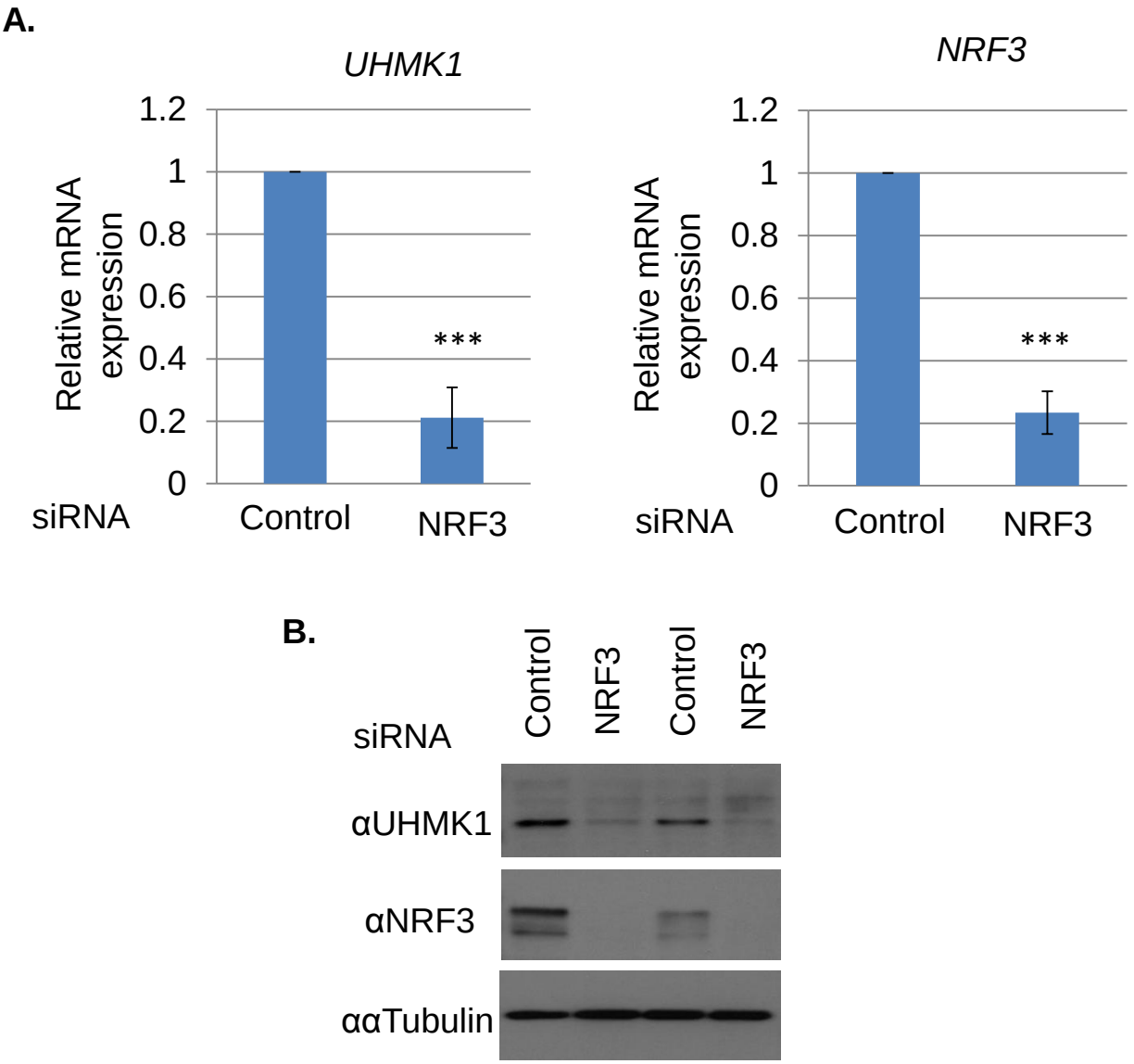


FIG. 19. NRF3 regulates the expression of *UHMK1* in DLD-1 cells. *NRF3* knockdown significantly reduces mRNA and protein levels of UHMK1 in DLD-1 cells. At 48 hr after the transfection of control or *NRF3* siRNA, the mRNA expression of *UHMK1* and *NRF3* was determined by real-time quantitative PCR analysis. The values were normalized to *18S rRNA* data (A). Immunoblotting of the whole cell extracts with anti-NRF3 and anti-UHMK1 antibodies was performed (B). α-Tubulin was used as an internal control. The figure (B) showed immunoblotting analysis of two independent experimental samples. The error bars (A) represent data from three independent experiments (mean± standard deviation).The two-tailed Student's t-test was used for the statistical analysis. *** $P < 0.001$ compared to the Control.

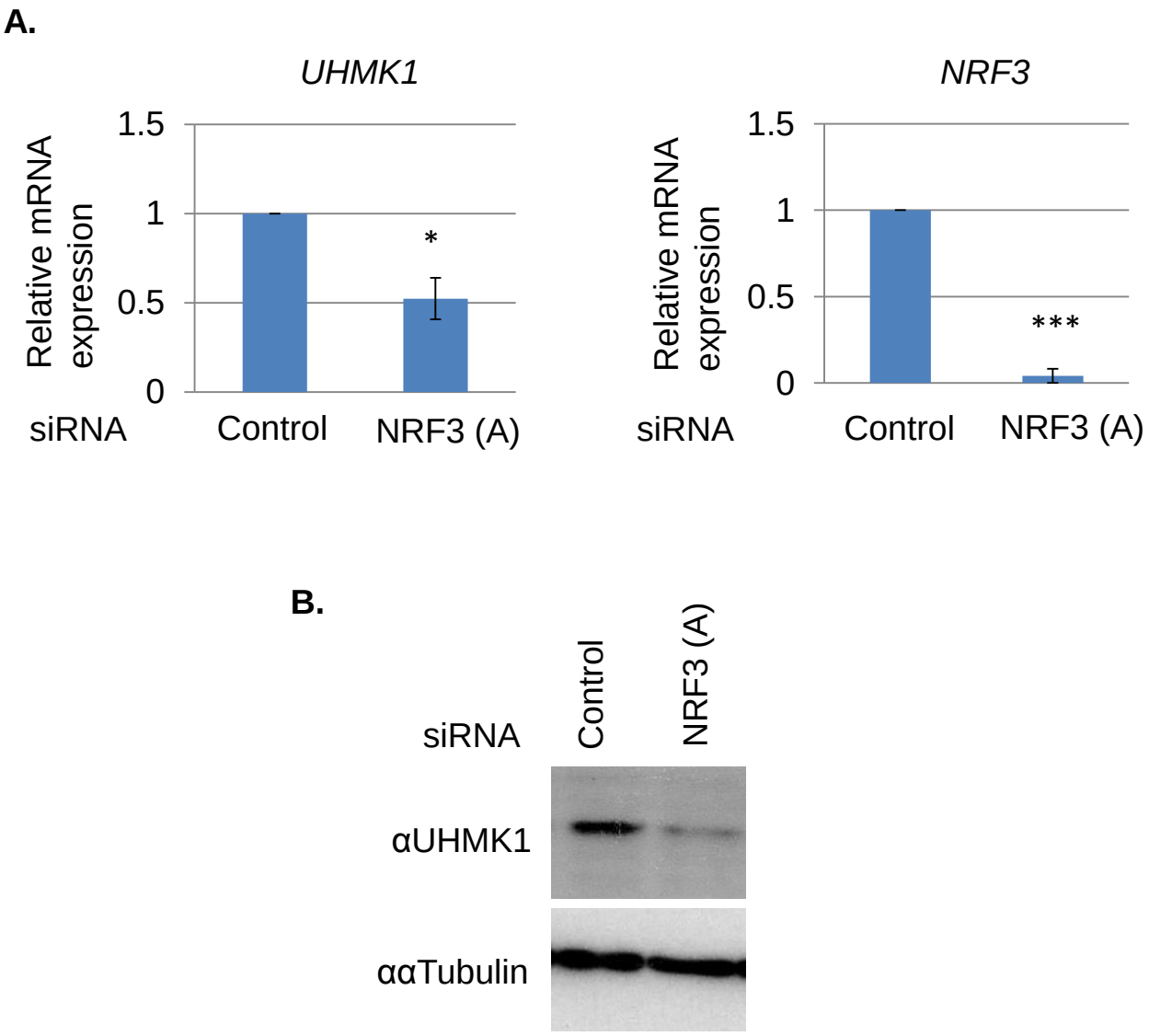


FIG. 20. Additional *NRF3* siRNA also reduces the *UHMK1* expression in DLD-1 cells. (A and B) Effects of additional *NRF3* siRNA (NRF3 (A)) on the mRNA and protein expression were determined by qRT-PCR and immunoblot analysis. The detailed experiment procedure was described in the legend of Figure 19A and B. The error bars (A) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. *$P < 0.05$ and *** $P < 0.001$ compared to the Control data.

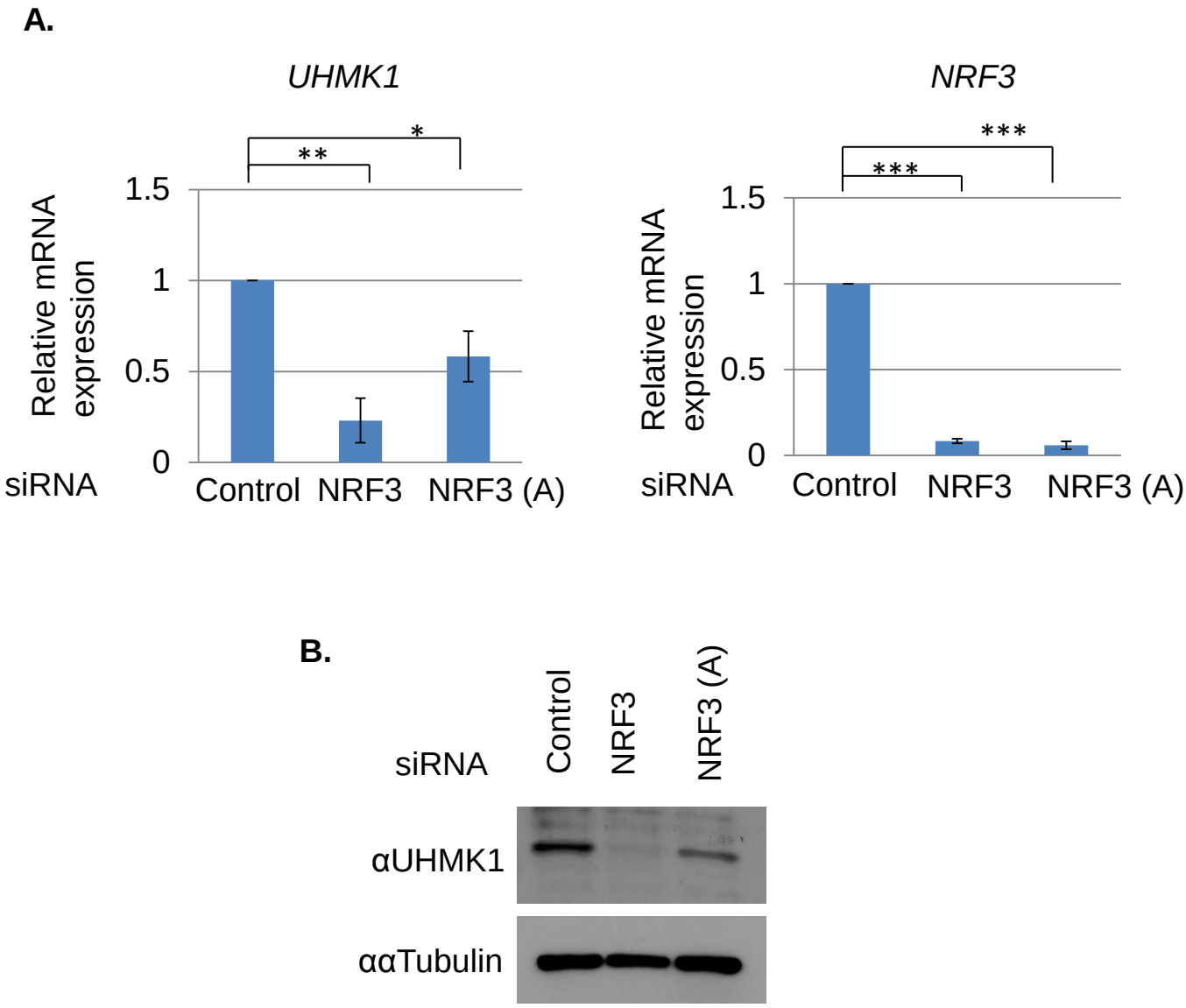


FIG. 21. NRF3 also regulates the expression of *UHMK1* in HCT116 cell. (A and B) NRF3-mediated *UHMK1* expression was observed by NRF3 siRNA in HCT116 cells. mRNA and protein expression of *UHMK1* were determined by qRT-PCR and immunoblot analyses. The detailed experiment procedure was described in the legend of Figure 19A and B. The error bars (A) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the Control data.

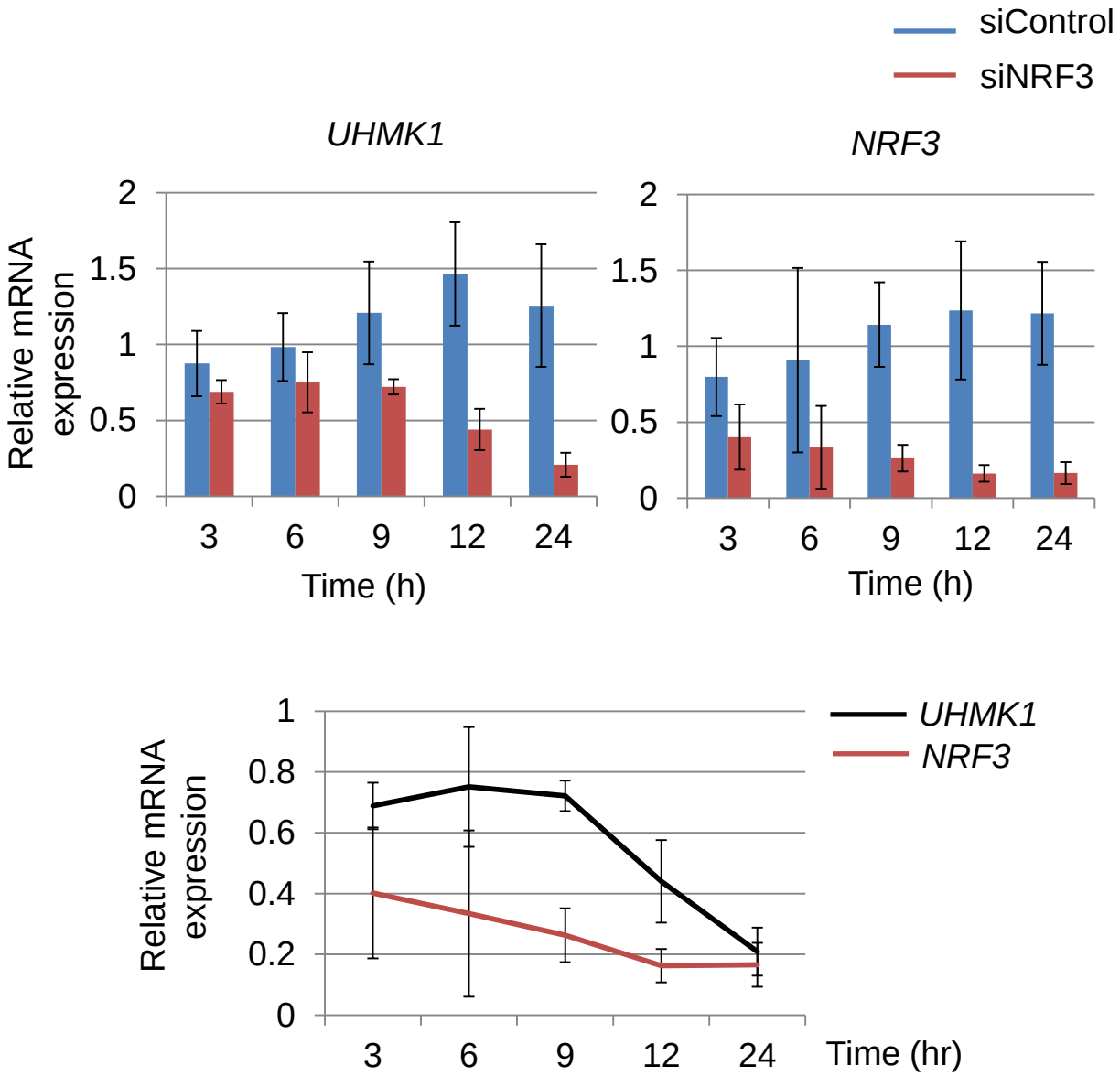


FIG. 22. A time course study of *UHMK1* mRNA expression after the *NRF3* knockdown. DLD-1 cells were transfected with control or *NRF3* siRNA, followed by mRNA extraction of the cells at indicated times and qRT-PCR analysis. The values were normalized to *18S rRNA* data. The line bar represent the lavel of *UHMK1* and *NRF3* mRNA expression after *NRF3* siRNA treatment at indicated time points. The error bars represent data from four independent experiments (mean± standard deviation).

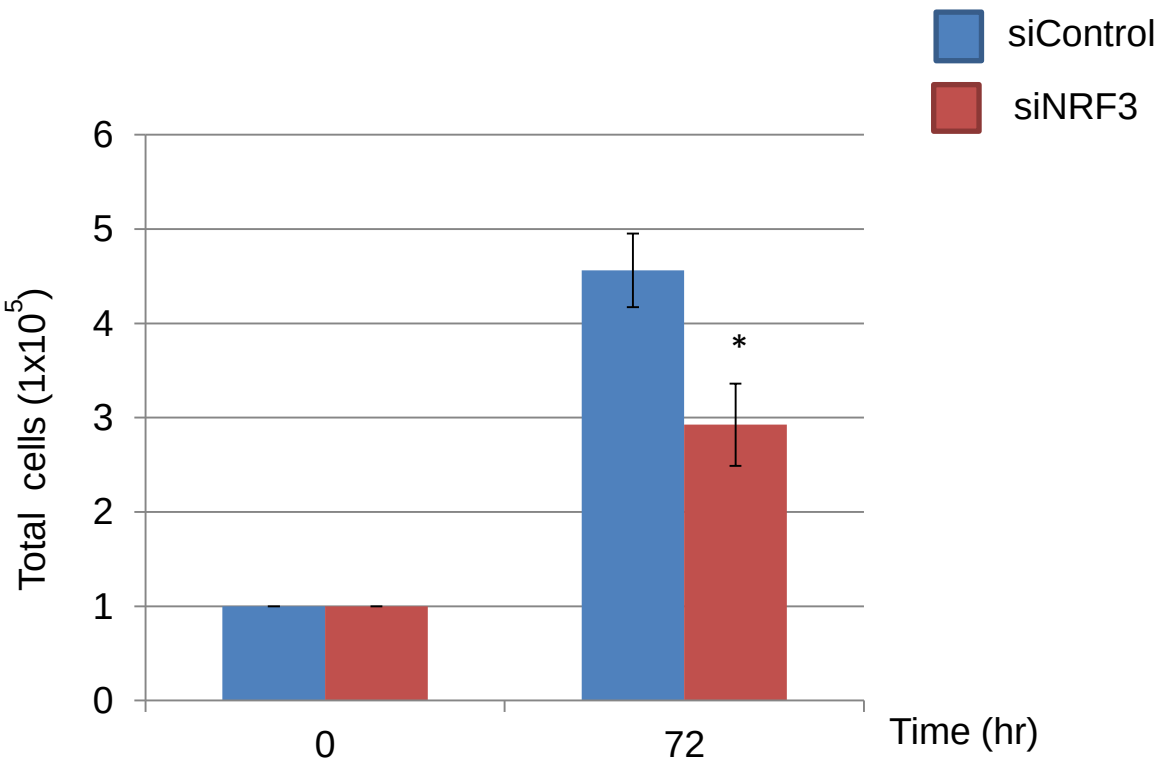
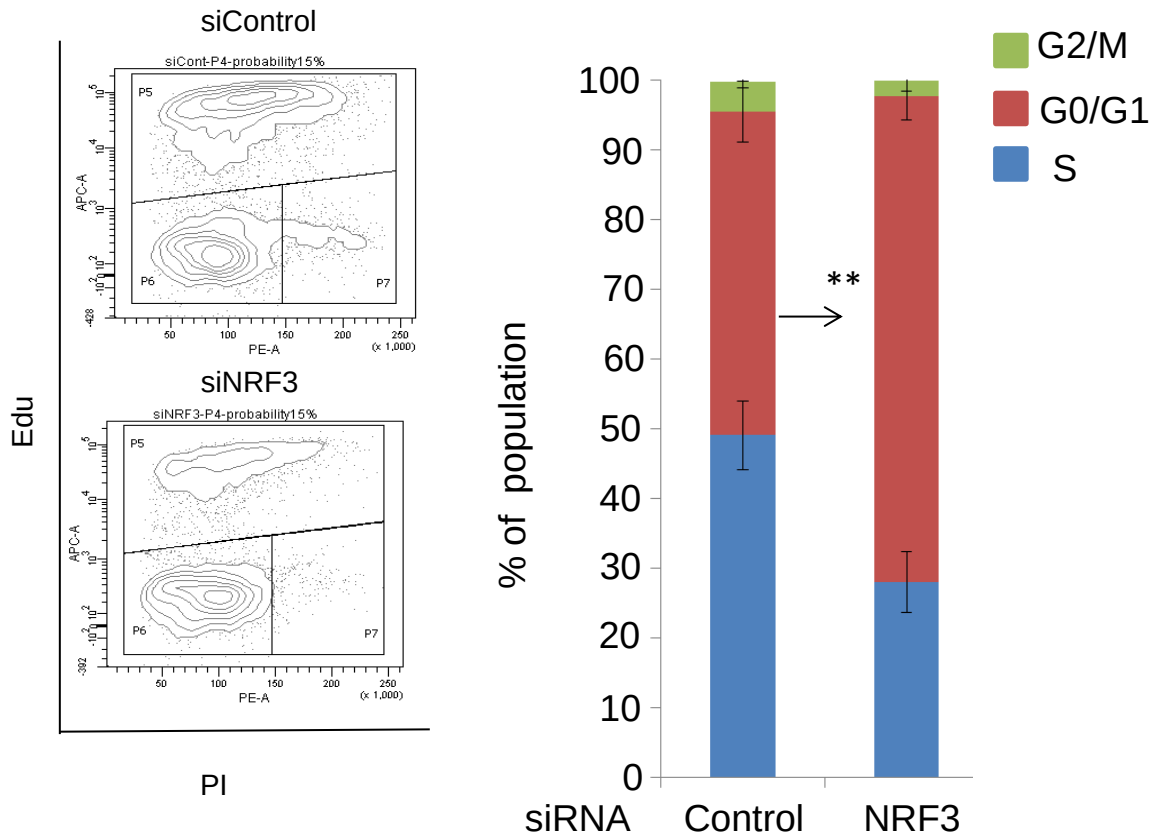


FIG. 23. NRF3 promotes the proliferation of colon cancer cells. *NRF3* knockdown significantly reduces the proliferation of DLD-1 cells. The cells were transfected with *Control* or *NRF3* siRNA. At 72 hr after the transfection, cell numbers were counted by a hemocytometer. The initial cell numbers at the time of transfection were $1x10^5$. The error bars represent data from three independent experiments (mean± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$ compared to the Control data.

A.



B.

siRNA	S	G0/G1	G2/M
siControl	49 (± 4.9)	46.4 (± 4.3)	4.4 (±0.9)
siNRF3	28.1 (± 4.4)	69.6 (±3.5)	2.3 (±1.5)

FIG. 24. *NRF3* knockdown significantly arrests DLD-1 cells to the G0/G1 phase. (A) At 48 hr after transfection with *Control* or *NRF3* siRNA, the cells were subjected to FACS analysis to determine the fraction of their populations in different cell cycle stages (G0/G1, S and G2/M). The representative data from three independent experiments is shown (A, left). The percentages of cell population in each phase are shown as mean ± standard deviation (B). The error bars (A) represent data from three independent experiments (mean± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. ** $P < 0.01$ compared to the Control data.

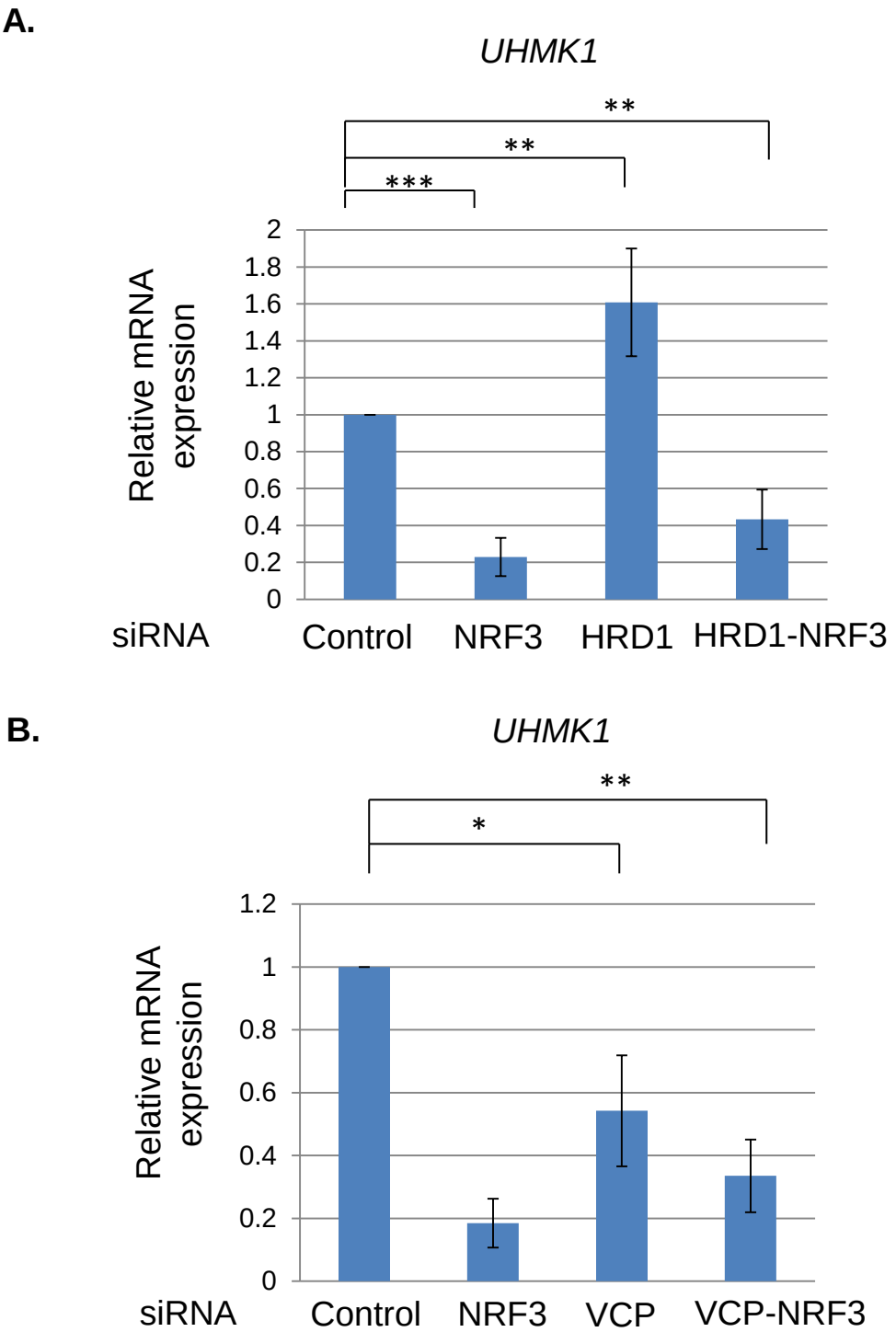


FIG. 25. *HRD1* or *VCP* knockdown effects on *UHMK1* mRNA expression. Expression levels of *UHMK1* mRNA in DLD-1 cells transfected with *HRD1* or *VCP* siRNA (A and B, respectively) were examined by qRT-PCR analysis. The error bars represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. NS, no significant difference; ** $P < 0.01$ and *** $P < 0.001$ compared to the Control data (A), * $P < 0.05$ and ** $P < 0.01$ compared to the Control data (B).

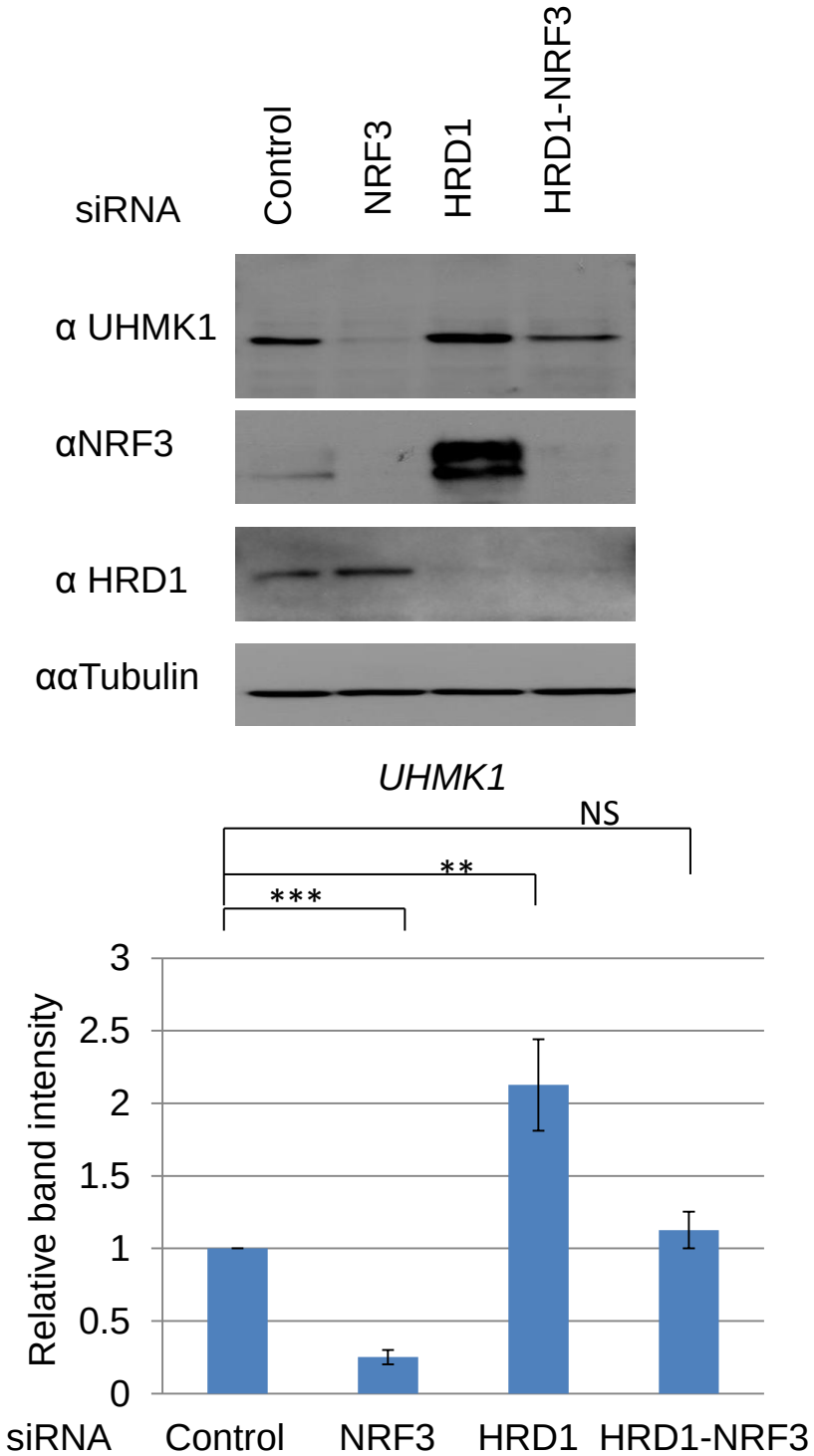


FIG. 26. *HRD1* knockdown affects the UHMK1 protein expression. DLD-1 cells were transfected with *Control*, *NRF3*, *HRD1* or *HRD-NRF3* siRNA for 48 hours. After transfection, whole-cell extracts were prepared and analyzed by immunoblotting with anti-NRF3, anti-UHMK1 or anti HRD1 antibodies. α-Tubulin was used as an internal control. The graph (B) represents the quantified band intensities of UHMK1. The values were normalized with α-tubulin. The error bars represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. NS, no significant difference; ** $P < 0.01$ and *** $P < 0.001$ compared to the Control data.

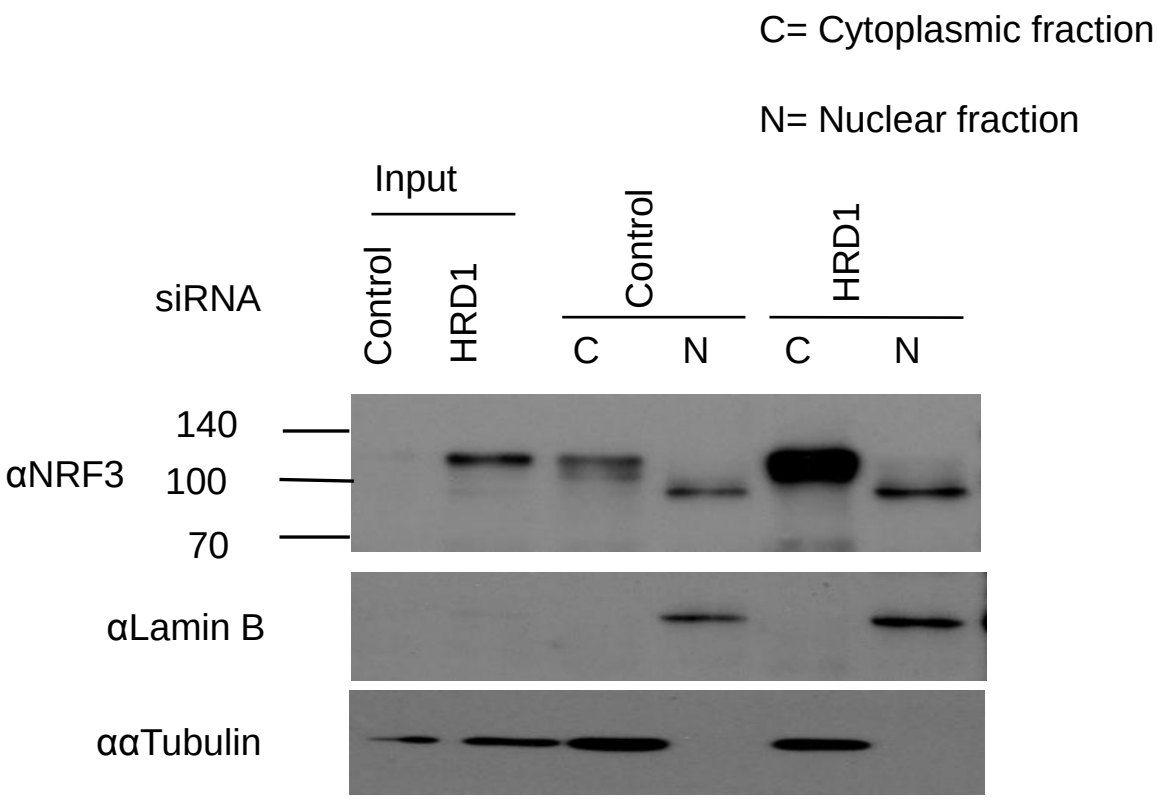


FIG. 27. *HRD1* knockdown does not promote the nuclear translocation of NRF3. DLD-1 cells were transfected with *Control* and *HRD1* siRNA for 48 hr. After transfection, cytoplasmic and nuclear fractions were extracted from the cells and subjected to immunoblot analysis with anti-NRF3 antibody. Lamin B and α-Tubulin were utilized as nuclear and cytoplasmic markers, respectively.

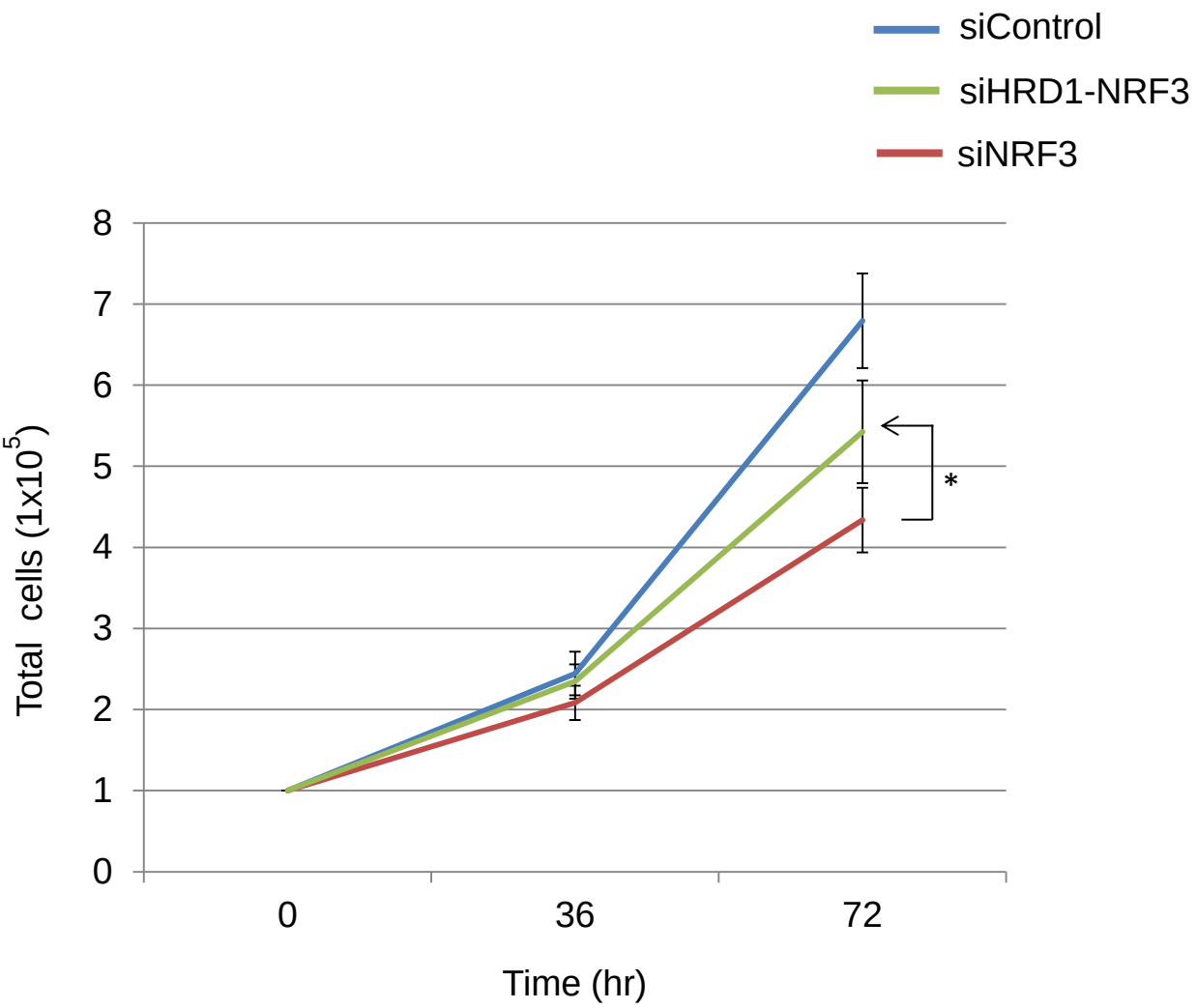
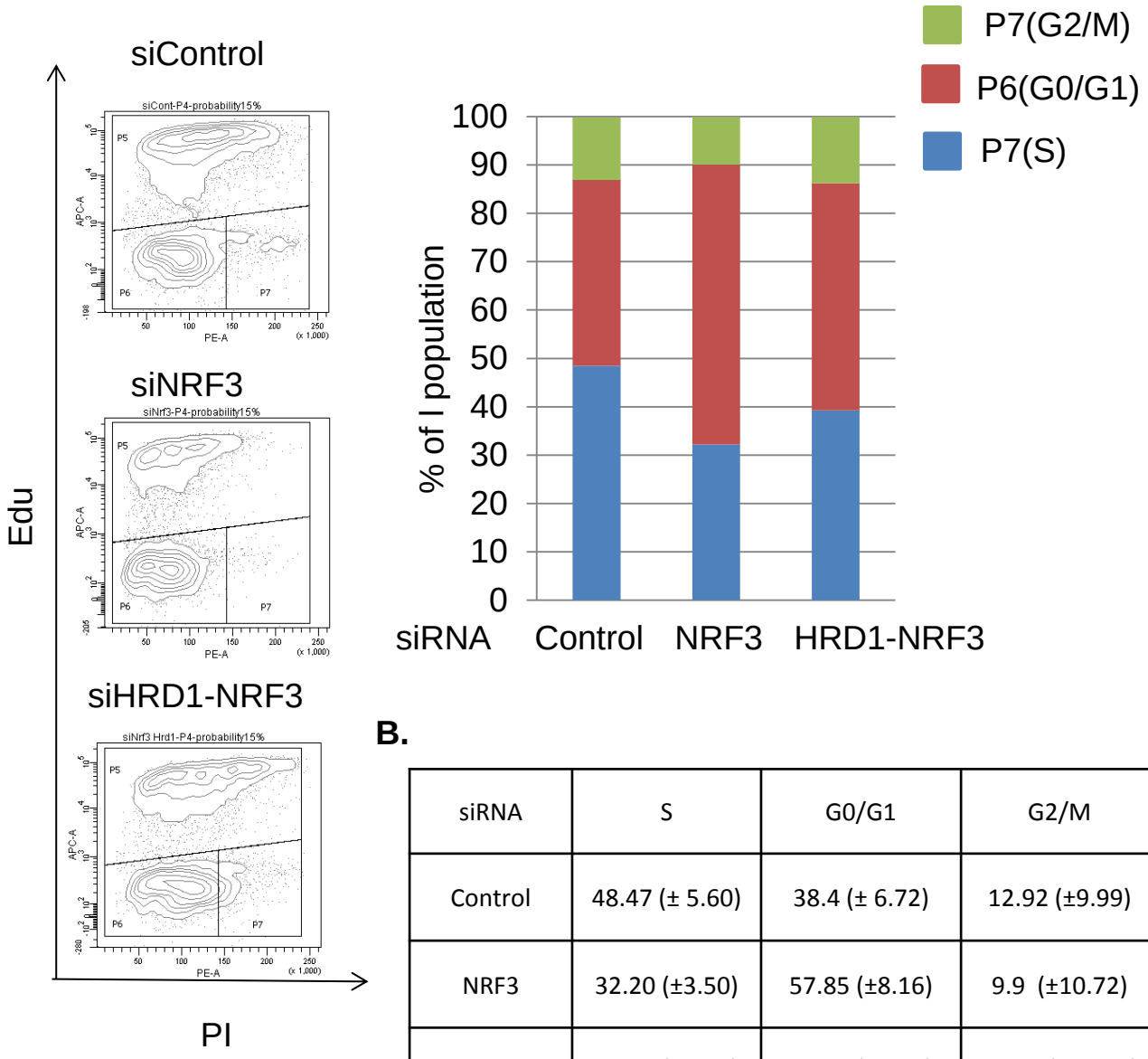


FIG. 28. HRD1 regulates proliferation of DLD-1 cells through the NRF3-independent pathway. DLD-1 cells were transfected with *Control*, *NRF3* and *HRD1-NRF3* siRNA. At 36 and 72 hr after transfection, cell numbers were counted by a hemocytometer. The initial cell numbers at the time of transfection were $1x10^5$. The error bars represent data from four independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$ compared to the Control data.

A.



B.

siRNA	S	G0/G1	G2/M
Control	48.47 (± 5.60)	38.4 (± 6.72)	12.92 (±9.99)
NRF3	32.20 (±3.50)	57.85 (±8.16)	9.9 (±10.72)
HRD1-NRF3	39.27 (±3.02)	46.97 (±7.65)	13.67 (±10.70)

FIG. 29. HRD1-NRF3 double knockdown compensates the NRF3 knockdown-mediated cell cycle arrest. DLD-1 cells were transected with *Control*, *NRF3* and *HRD1-NRF3* siRNA. At 48 hr after transfection, the cells were subjected to FACS analysis to determine the fraction of their populations in different cell cycle stages (G0/G1, S and G2/M). The representative data from four independent experiments is shown (A, left). The percentages of cell population in each phase are shown as mean± standard deviation (B).

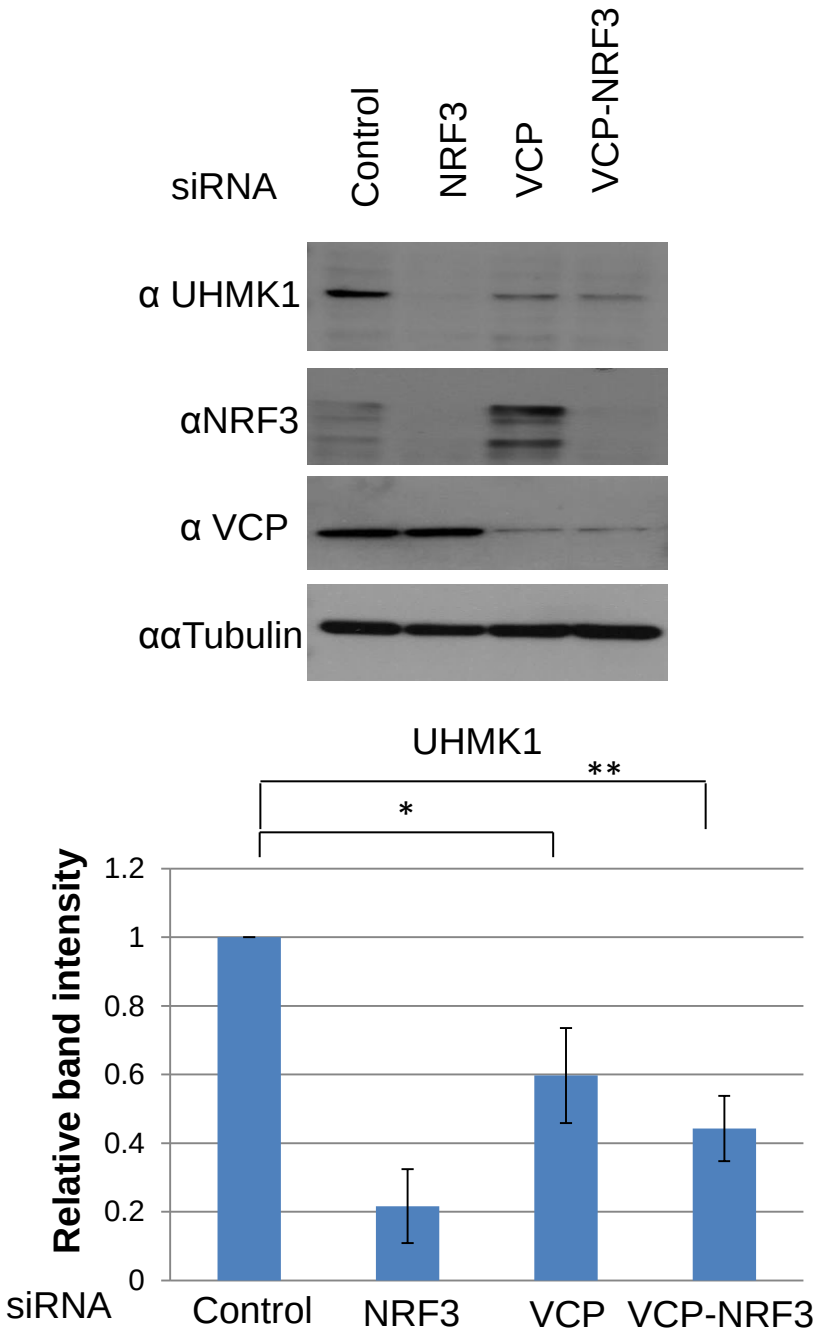


FIG. 30. VCP inhibition reduces transcriptional activity of NRF3. *VCP* knockdown effects on UHMK1 protein expression. DLD-1 cells transfected with *Control*, *NRF3*, *VCP* and *VCP-NRF3* siRNA for 48 hr. After transfection, whole-cell extracts were prepared and subjected to immunoblot analysis with anti-NRF3, anti-UHMK1 or anti-VCP antibodies. α-Tubulin was used as an internal control. The graph represents the quantified band intensities of UHMK1. The values were normalized with α-Tubulin. The error bars (B) represent data from three independent experiments (mean ± standard deviation). The two-tailed Student's t-test was used for the statistical analysis. * $P < 0.05$ and ** $P < 0.01$ compared to the Control data.

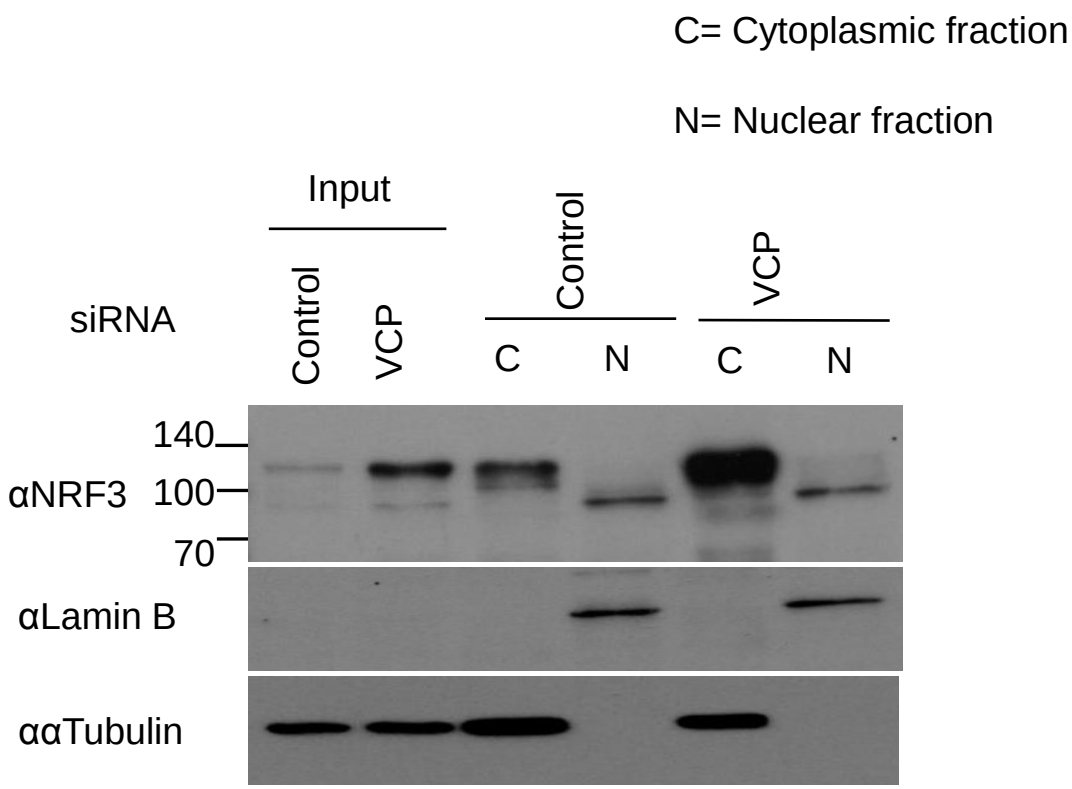


FIG. 31. *VCP* knockdown does not promote nuclear accumulation of NRF3. DLD-1 cells were transfected with Control and VCP siRNA for 48 hr. After transfection, cytoplasmic and nuclear fractions were extracted from the cells and subjected to immunoblot analysis with anti-NRF3 antibody. Lamin B and α-Tubulin were utilized as nuclear and cytoplasmic markers, respectively.

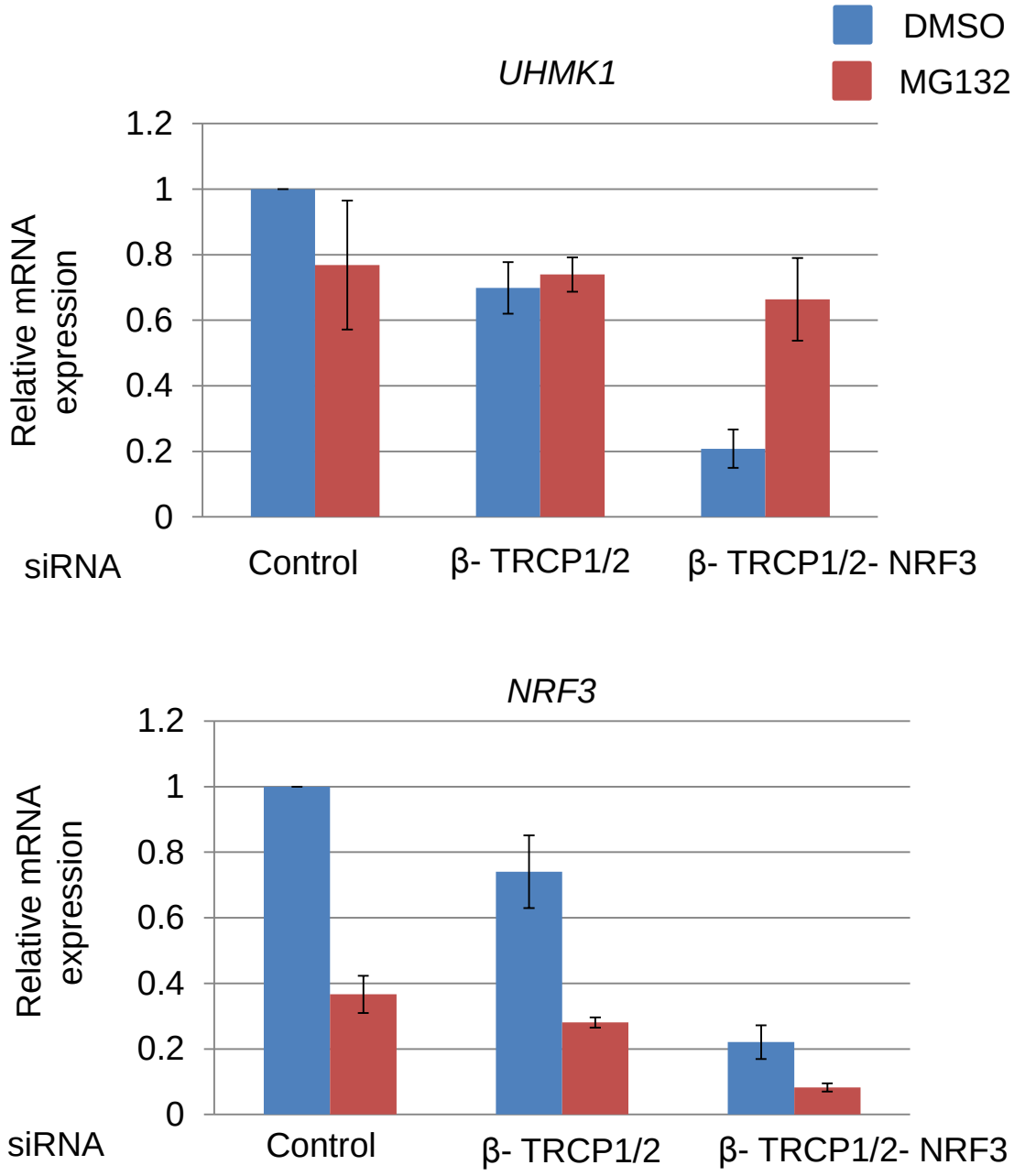


FIG. 32. Effects of β-TRCP knockdown on NRF3 target gene *UHMK1*. DLD-1 cells transfected with indicated siRNA. At 48 hr after siRNA transfection, the cells were treated with DMSO (dimethylsulfoxide) or MG132 (1 uM) for 16 hr . The mRNA expression levels of *UHMK1* and *NRF3* were determined by real time quantitative PCR analysis. The values were normalized to *18S rRNA* data. The error bars represent data from two independent experiments (mean ± standard deviation).

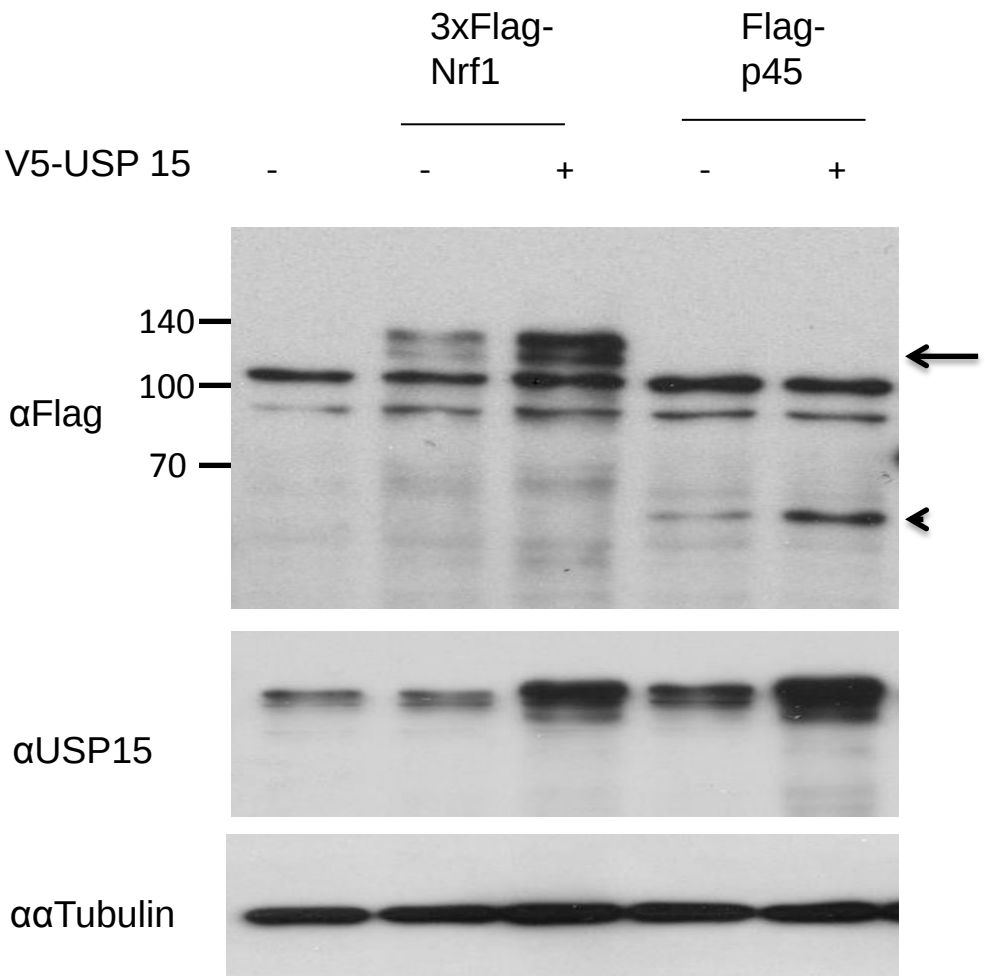


FIG. 33. USP15 stabilizes p45 of the CNC family proteins. The expression vectors of 3×Flag-Nrf1, Flag-p45 along with V5-USP15 were transfected into HEK293T cells. The whole cell extracts were subjected to immunoblot analysis with the indicated antibodies. The arrow and arrowhead stand for 3xFlag-Nrf1 and Flag-p45 expression, respectively.

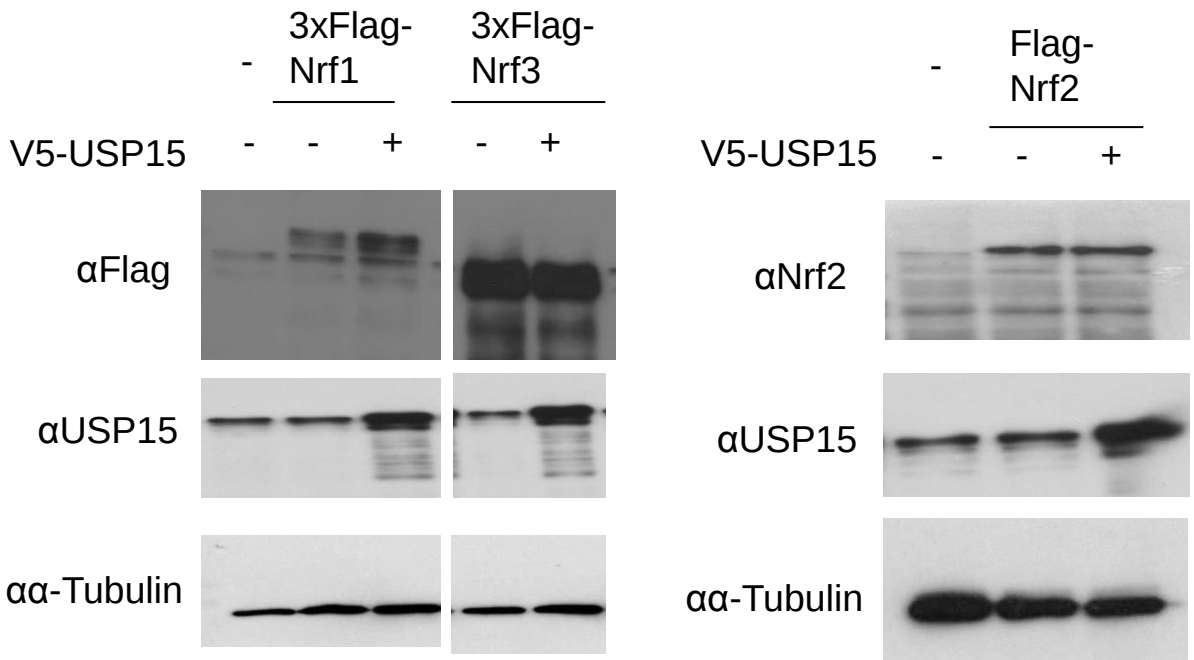


FIG. 34. USP15 effects on the CNC family proteins Nrf2 and Nrf3. The expression vectors of 3×Flag-Nrf1, Flag-Nrf2 or 3xFlag-Nrf3 along with V5-USP15 were transfected into HEK293T cells. The whole cell extracts were subjected to immunoblot analysis with the indicated antibodies.

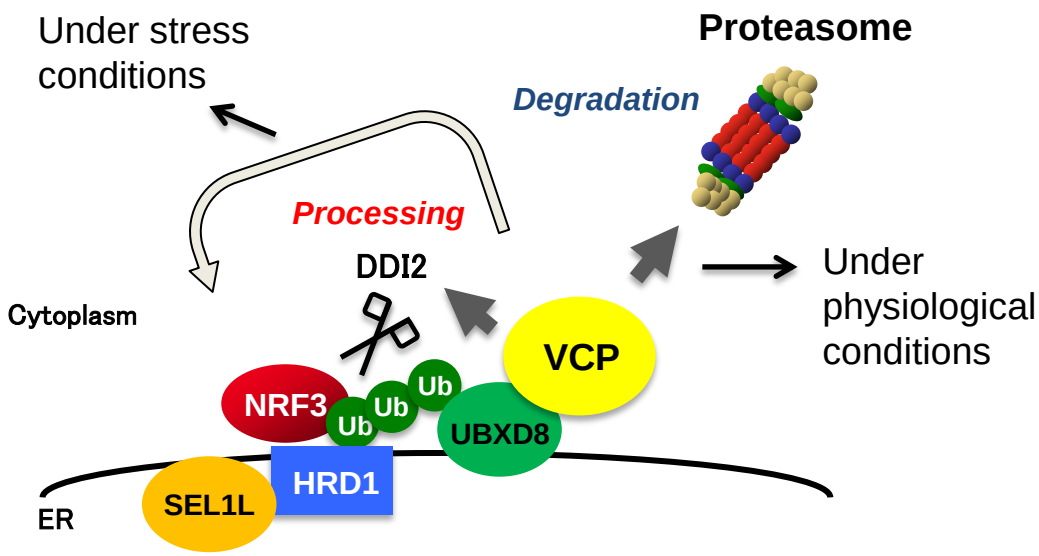


FIG. 35. A schematic model of biological significances of HRD1-VCP-mediated NRF3 regulation. The transcription factor NRF3 undergoes cytoplasmic degradation via HRD1 and VCP under physiological conditions. After being polyubiquitinated by the HRD1-SEL1L complex, NRF3 is recognized by VCP and transferred to the proteasome for degradation. Simultaneously, under stress conditions (in cancer cells), the polyubiquitinated NRF3 is processed by DDI2 for its nuclear translocation (activation).

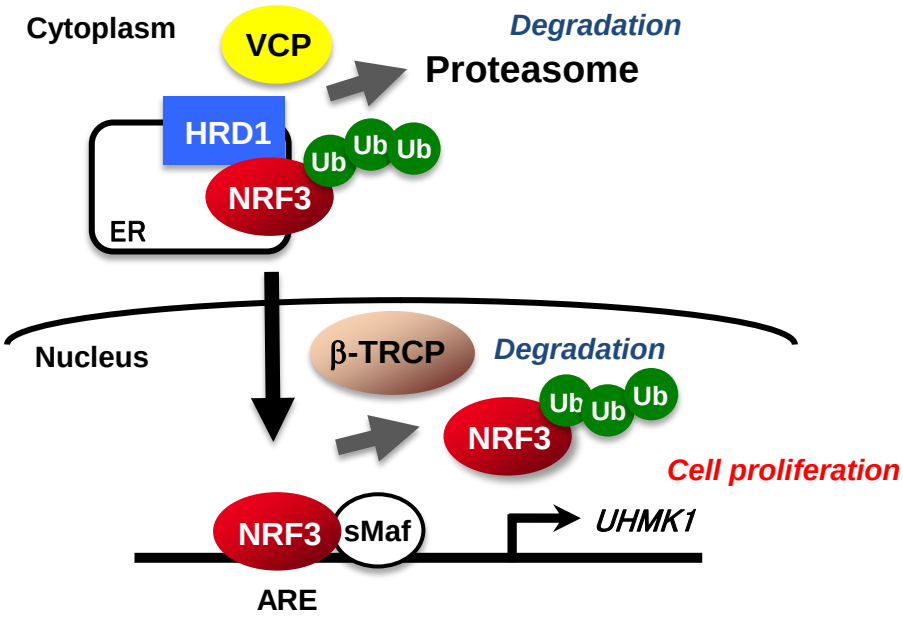


FIG. 36. A schematic model of multiple regulation of the biological function of the transcription factor NRF3. Under normal conditions, Nrf3 is degraded by the ERAD ubiquitin ligase HRD1 and VCP in the cytoplasm. In the nucleus, NRF3 activates the gene expression of the *UHMK1* gene for cell proliferation. Alternatively, the β-TRCP-based E3 ubiquitin ligase suppresses the NRF3 function by mediating its nuclear degradation.

Table 1　A comparison of the mouse CNC protein knockout phenotypes.

Genotype	Phenotypes of Knockout mice	References
nrf3−/−	No obvious phenotype	(12, 18)
nrf1−/−	Mid- to late embryonic lethal; defective erythropoiesis; oxidative stress	(36)
nrf2−/−	Age-related lupus-like syndrome (♀) and neurodegeneration (♀ and ♂); sensitivity to oxidative and ER stress	(1)
p45−/−	Viable; mild anemia; thrombocytopenia (lack of platelets); death from hemorrhage	(27)

Sykiotis *et al.* (2010) Sci.Signal, with minor modification.

Table 2　Sequences of primers for real time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
NRF3	CTGACTGGGAAGGCAGAAAAG	TCAGGCTGTGATGAAAGCAA
UHMK1	AGAGAAACCATGGGCAGAAG	CAAGCCATGAAACAGCATCT
HRD1	TGCAACCACATTTTCCATACCA	GCGATGCACGAAGGACATC
VCP	TACCAACCGGCCTGACAT	TGGCAACACGGGACTTCT
ß-TRCP1	TGCCGAAGTGAAACAAGC	CCTGTGAGAATTCGCTTG
ß-TRCP2	TCAGTGGCCTACGAGATA	ACACGCTCATCATACTGCA
GP78	GGTGCAGCGTAAGGACGAA	GCATCATCTTCAGAACTTTTGTTCA
TEB4	TTGTCCTTCCAAGTCCGCCAG	GACTGTGGAGGTGGTGGAGATG
18S rRNA	CGCCGCTAGAGGTGAAATTC	CGAACCTCCGACTTTCGTTCT
ß-Actin	CCAACCGCGAGAAGAT	CCAGAGGCGTACAGGG

Acknowledgment

Thanks to merciful GOD for all the innumerable favors you have offered me.

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