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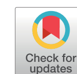
# Curcumin induces stabilization of Nrf2 protein through Keap1 cysteine modification



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ABSTRACT

The present study was aimed to investigate the effects of curcumin, a representative chemopreventive phytochemical with pronounced antioxidant and anti-inflammatory properties, on activation of Nrf2 and expression of its target protein heme oxygenase-1 (HO-1) in mouse skin *in vivo* and in cultured murine epidermal cells. Treatment of mouse epidermal JB6 cells with curcumin resulted in the induction of HO-1 expression, and this was abrogated in cells transiently transfected with *Nrf2* siRNA. While curcumin treatment increased protein expression of Nrf2, it did not alter the steady-state level of the *Nrf2* mRNA transcript. Treatment of cells with curcumin stabilized Nrf2 by inhibiting ubiquitination and subsequent 26S proteasomal degradation of this transcription factor. Tetrahydrocurcumin, a non-electrophilic analogue of curcumin that lacks the α,β-unsaturated carbonyl group, failed to induce HO-1 expression as well as nuclear translocation of Nrf2 and its binding to the antioxidant/electrophile response elements. Cells transfected with a mutant Keap1 protein in which cysteine 151 (Cys151) is replaced by serine exhibited marked reduction in curcumin-induced Nrf2 transactivation. Mass spectrometric analysis revealed that curcumin binds to Keap1 Cys151, supporting that this amino acid is a critical target for curcumin modification of Keap1, which facilitates the liberation of Nrf2. Thus, it is likely that the α,β-unsaturated carbonyl moiety of curcumin is essential for its binding to Keap1 and stabilization of Nrf2 by hampering ubiquitination and proteasomal degradation.

## 1. Introduction

In order to cope with a vast variety of noxious insults from the environment, our body is endowed with efficient defense systems that detoxify and eliminate those harmful chemicals, including carcinogens and their reactive metabolites and also neutralize/inactivate reactive oxygen species.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that plays a pivotal role as a master switch that turns on the transcription of genes encoding many carcinogen detoxifying enzymes as well as antioxidative and other cytoprotective proteins [1,2]. Under normal physiological conditions, Nrf2 is sequestered in the cytoplasm through interaction with Kelch-like ECH-associated protein 1 (Keap1). Keap1 serves as an adaptor protein between Nrf2 and Cullin3-Rbx1 E3 ligase complex, facilitating the ubiquitination and subsequent degradation of Nrf2 by 26S proteasomes [3–5]. Under stressed conditions, however, the ability of the Cullin3-Rbx1 E3 ligase to ubiquitinate Nrf2 is repressed, which allows Nrf2 to accumulate in the nucleus, leading to increased transcription of cytoprotective genes [2–5].

Skin is highly vulnerable to damage by environmental toxicants, such as UV, chemical carcinogens, and infectious agents. As Nrf2-mediated adaptive response is transient, it can be readily overwhelmed by extensive environmental stresses. In this context, maintenance of optimal level of defensive mechanisms is important in protecting skin

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against external insults.

Some natural products have been reported to induce activation of Nrf2 and its target gene expression [6,7]. Curcumin, a yellow colouring agent present in Indian spice turmeric (*Curcuma longa* L., Zingiberaceae), has strong antioxidant and anti-inflammatory activities which account for its chemopreventive effects [8–10]. Topical application of curcumin inhibited the chemically induced skin tumor formation in mice [11]. Our previous study has demonstrated that topically applied curcumin inhibited the expression of the cyclooxygenase-2 through inhibition of NF-κB activation in mouse skin [12].

In the present study, we investigated whether curcumin could induce activation of Nrf2 and cytoprotective gene expression in mouse skin *in vivo* and cultured murine epidermal cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Curcumin was purchased from LKT laboratory Inc. (St. Paul, MN, USA). Tetrahydrocurcumin was synthesized by conventional catalytic hydrogenation of curcumin in the presence of palladium on carbon and generously supplied by Prof. Jeewoo Lee (College of Pharmacy, Seoul National University). Dithiothreitol (DTT) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), gentamycin and antibiotic–antimycotic were purchased from Gibco BRL (Grand Island, NY, USA). Antibody against HO-1 was obtained from stressgen (Victoria, British Columbia, Canada). Antibody against ubiquitin was obtained from Calbiochem (Darmstadt, Germany). All other antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA)

### 2.2. Cells and animals

The mouse epidermal cell line (JB6) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Minimal Essential Medium (MEM) containing 5% FBS and 25 μg/ml gentamycin in an atmosphere of 95% air-5% $CO_2$ at 37 °C. HEK293T cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% FBS and antibiotic–antimycotic mixture at 37 °C in an atmosphere of 95% air-5% $CO_2$. Female ICR mice (6–7 weeks of age) were purchased from the Central Lab. Animal Inc. (Seoul, South Korea) and were housed in a climate-controlled quarter (24 ± 1 °C at 50% humidity) with a 12-h light/dark cycle and with free access to food and water. *Nrf2* knockout mice were kindly supplied by Dr. Jeffrey Johnson of the University of Wisconsin-Madison. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University and Keimyung University, South Korea.

### 2.3. Preparation and culturing mouse embryonic fibroblasts (MEFs)

MEFs were isolated from Nrf2 knockout ($Nrf2^{-/-}$) and wild type ($Nrf2^{+/+}$) mice. Male and female $Nrf2^{+/-}$ mice were paired, and the pregnancies were monitored. Embryos were obtained at day 13.5 after pairing under aseptic conditions. The heads of the embryos were used to confirm the *Nrf2* genotype by PCR, and the embryo bodies were minced into small pieces, and cultured in high glucose DMEM supplemented with 10% FBS and kept at 37 °C with 5% $CO_2$.

### 2.4. Western blot analysis

The treated cells were harvested, washed, and suspended in the cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Cell lysates were centrifuged at 13,000*g* for 15 min, and the aliquots of the supernatant containing protein were stored at −70 °C. Nuclear extract from JB6 cells was prepared as described previously [13]. For *in vivo* experiments, female ICR mice were treated topically on their shaven backs with curcumin (0.1 or 1 μmol) dissolved in 200 μl of acetone and were killed by cervical dislocation at the indicated time intervals. Proteins from the mouse epidermis were isolated as described previously [12], and subjected to SDS-PAGE and Western blot analysis.

### 2.5. Immunocytochemical analysis

For the immunocytochemical analysis of Nrf2, JB6 cells were plated on the chamber slide. After fixation with paraformaldehyde, cells were incubated with blocking agents (0.1% Tween-20 in PBS containing 5% bovine serum albumin), washed with PBS, and then incubated overnight in the presence of diluted (1:100) Nrf2 primary antibody. After washing with PBS, cells were then incubated with a FITC-conjugated secondary antibody for 1 h. Cells were also stained with propidium iodide (PI) and examined under a confocal microscope (Leika, Germany).

### 2.6. Electrophoretic mobility shift assay (EMSA) for assessment of Nrf2-ARE binding

A synthetic double-stranded oligonucleotide containing the Nrf2-binding domain (ARE) was labeled with [γ-$^{32}$P]ATP using a T4 polynucleotide kinase and separated from the free [γ-$^{32}$P]ATP by gel filtration using a NICK spin column (Amersham Biosciences, UK). The sequences of oligonucleotides in the double strands used in this study were 5′-TTT TCT GCT GAG TCA AGG TCC G-3′ and 3′-AAA AGA CGA CTC AGT TCC AGG C-5′. Prior to the addition of radiolabeled oligonucleotide (100,000 cpm), 10 μg of the nuclear extract was kept on ice for 15 min in gel shift binding buffer [4% glycerol, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mg/ml sonicated salmon sperm DNA]. The DNA protein complexes that formed were resolved on a 6% nondenaturing polyacrylamide gel at 145 V for 2 h and were subjected to autoradiography.

### 2.7. RNA isolation, cDNA synthesis, and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) for 50 min at 42 °C and again for 15 min at 72 °C. One μl of cDNA was amplified in sequential reactions using Maxime PCR PreMix Kit (iNtRON Biotechnology, Seoul, South Korea). For quantitation of *HO-1* mRNA, 20 cycles of 94 °C for 30 s, 53 °C for 35 s, and 72 °C for 30 s; for quantification of *Actin* mRNA, 20 cycle of 94 °C for 30 s, 59 °C for 35 s, and 72 °C for 30 s; for quantitation of *Nrf2* mRNA 20 cycle of 94 °C for 30 s, 59 °C for 35 s, and 72 °C for 30 s were conducted. These PCR cycles were followed by a final extension for 5 min at 72 °C. The primers used for each RT-PCR reactions are as follows: *HO-1*, 5′-TAC ACA TCC AAG CCG AGA AT-3′ and 5′-GTT CCT CTG TCA GCA TCA CC-3′; *Nrf2*, 5′-TCT CCT CGC TGG AAA AAG AA-3′ and 5′-AAT GTG CTG GCT GTG CTT TA-3′; *Actin*, 5′-AGA GCA TAG CCC TCG TAG AT-3′ and 5′-CCC AGA GCA AGA GAG GTA TC-3′ (forward and reverse, respectively). Amplification products were analyzed by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide, and then photographed under ultraviolet light.

### 2.8. Plasmids and transient transfection

Construction of plasmids pcDNA3-Myc3-Nrf2 (Addgene plasmid 21555) and pcDNA3-HA2-Rbx1/ROC1 (Addgene plasmid 19897) was described elsewhere [13,14]. The full-length human cDNA of *Keap1* was obtained from Thermo Fisher Scientific (Huntsville, AL, USA) and subcloned into pCMV-FLAG-MAT™-1 expression vector (Sigma-Aldrich, St. Louis, MO, USA) as *Hind*III/*Bam*HI fragment. The full-length human

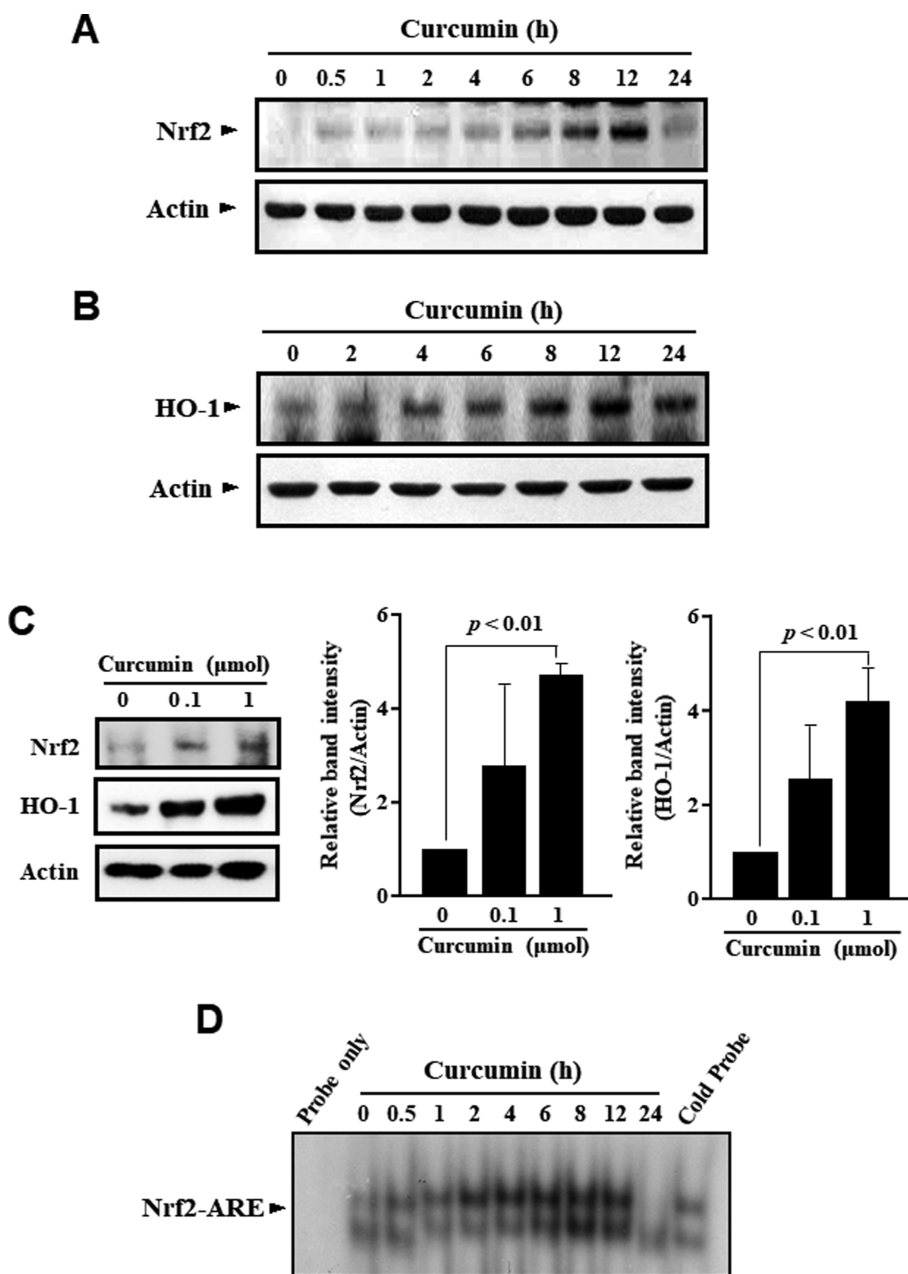



**Fig. 1.** Effects of curcumin on the activation of Nrf2 and expression of its target protein, HO-1 in mouse skin *in vivo*. Dorsal skin of female ICR mice was treated topically with 1 μmol of curcumin for indicated time periods. (A, B) After treatment, the protein levels of Nrf2 (A) and HO-1 (B) in the epidermal tissue lysates were measured by Western blot analysis as described in Materials and methods. (C) The expression of Nrf2 and HO-1 was measured under the same experimental conditions by Western blot analysis after 12-h topical curcumin treatment. The protein expression levels of Nrf2 and HO-1 were quantified using an image analysis software GelPro 3.1 and normalized to the vehicle (DMSO) control. A bar graph represents the mean of triplicates ± SD. (D) Mice were treated with 1 μmol curcumin and killed at the indicated times. The nuclear fraction was isolated as described in Materials and methods. The Nrf2-ARE binding activity was assessed by EMSA. Nuclear extracts were incubated with [$\gamma$-$^{32}$P]-labeled oligonucleotides harboring the ARE consensus sequence, and the Nrf2-ARE oligonucleotide complex was resolved by gel electrophoresis.

*Cullin3* was amplified by RT-PCR of the total RNA obtained from HEK293T cells with primers 5′-TAA GGT ACC AAT GTC GAA TCT GAG CAA-3′ (forward) and 5′-GCC TCG AGT TAT GCT ACA TAT GTG TAT AC-3′(reverse) and subcloned into His-tagged pcDNA6 expression vector (Invitrogen, Carlsbad, CA, USA) as KpnI/ApaI fragment. The reporter gene fusion constructs for wild type ARE were kindly provided by Prof. Jeffrey A. Johnson (University of Wisconsin-Madison). For plasmid transfection, cells were seeded and grown to 40–50% confluence growth media without antibiotics. Each plasmid was transfected into cells with lipofectamine LTX and PLUS reagent according to the instructions supplied by the manufacturer (Invitrogen, Carlsbad, CA, USA). For siRNA transfection, cells were transfected with *Nrf2* siRNA (Invitrogen, Carlsbad, CA, USA) by lipofectamine RNAi-MAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### *2.9. Luciferase reporter gene assay*

JB6 cells were grown up to 50% confluence in 12-well plates in complete media that do not contain antibiotics. Then, the cells were transfected with 100 ng of ARE-Luc vector (pGL2-NQO1) by using the Lipofectamine LTX according to the instructions supplied by the manufacturer (Invitrogen, Carlsbad, CA, USA). In all cases, the total amount of transfecting plasmid DNA was quantitated and adjusted using pcDNA3-β-galactosidase. After the following 24-h transfection, cells were treated for additional 6 h with 5 μM each of curcumin or tetrahydrocurcumin, and the lysis of transfected cells was carried out using the reporter lysis buffer. After mixing the cell extract with a luciferase substrate (Promega, Madison, WI, USA), the luciferase activity was measured by employing a luminometer (AntoLumat LB953, EG&G Berthold, Bad Widbad, Germany). The β-galactosidase assay was done according to the supplier's instructions (Promega β-galactosidase Enzyme Assay System) for normalizing the luciferase activity. For determining the effect of cysteine 151 (Cys151) mutation of Keap1 on

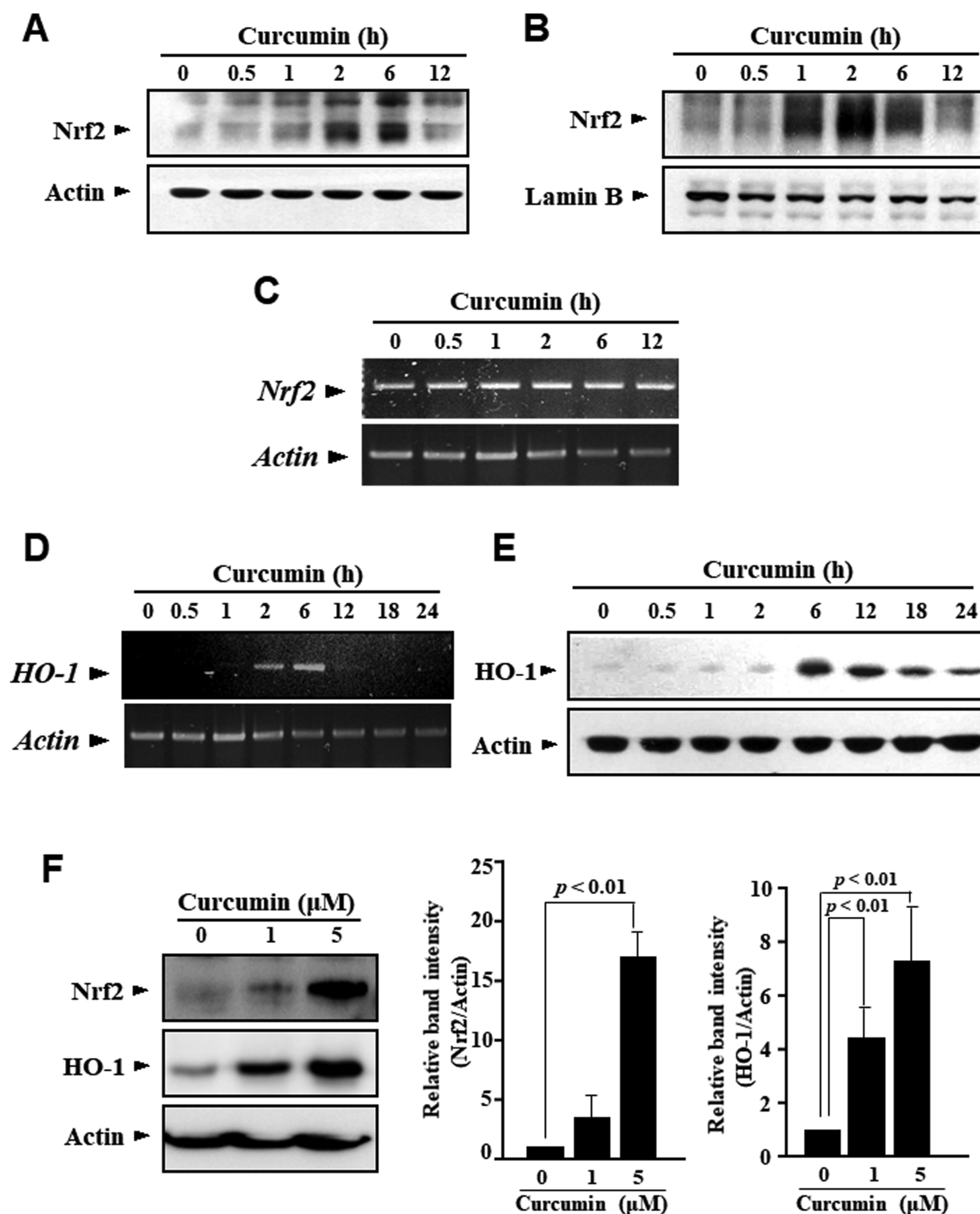



**Fig. 2.** Curcumin-induced expression of Nrf2 and HO-1 in JB6 cells. (A) JB6 cells were treated for the indicated time periods with 5 μM curcumin. The total protein was isolated and analyzed for the measurement of Nrf2 protein by Western blot analysis. (B) JB6 cells were treated with 5 μM curcumin and harvested at indicated time points. The nuclear fraction was isolated as described in Materials and methods, and Nrf2 levels were assessed by Western blot analysis. (C) JB6 cells were treated with 5 μM curcumin for the indicated time, and total mRNA were isolated and analyzed for the expression levels of *Nrf2* mRNA transcript by RT-PCR. (D) After treatment with curcumin (5 μM) for indicated time periods, RT-PCR analysis was performed to measure mRNA expression of *HO-1* as described in Materials and methods.(E) JB6 cells were treated for indicated time points with 5 μM curcumin. Total proteins were isolated and subjected to the Western blot analysis for the measurement of HO-1 protein expression. (F) The expression of Nrf2 and HO-1 was measured by Western blot analysis after 6-h curcumin treatment. Band intensities were quantified and normalized to the vehicle (DMSO) control. Data are means ± SD (n = 3).

Nrf2 transcriptional activity, HEK293T cells were transfected for 24 h with pGL2-NQO1 vector, expression plasmids for wild type Myc-Nrf2 and Flag-Keap1 or mutant Keap1. Transfected cells were treated with or without curcumin (15 μM) before the reporter assay.

### 2.10. Immunoprecipitation

Cells were treated with curcumin for indicated time and cells were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 μM NaF, 2 μM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Total protein (500 μg) was subjected to immunoprecipitation by shaking with Nrf2 primary antibody at 4°C for 2 h followed by the addition of protein G-agarose bead suspension (25% slurry, 40 μl) and additional shaking for 2 h at 4 °C. In another experiment, HEK 293 T cells were lysed in buffer described previously [15], and 400 μg of cellular proteins were subjected to

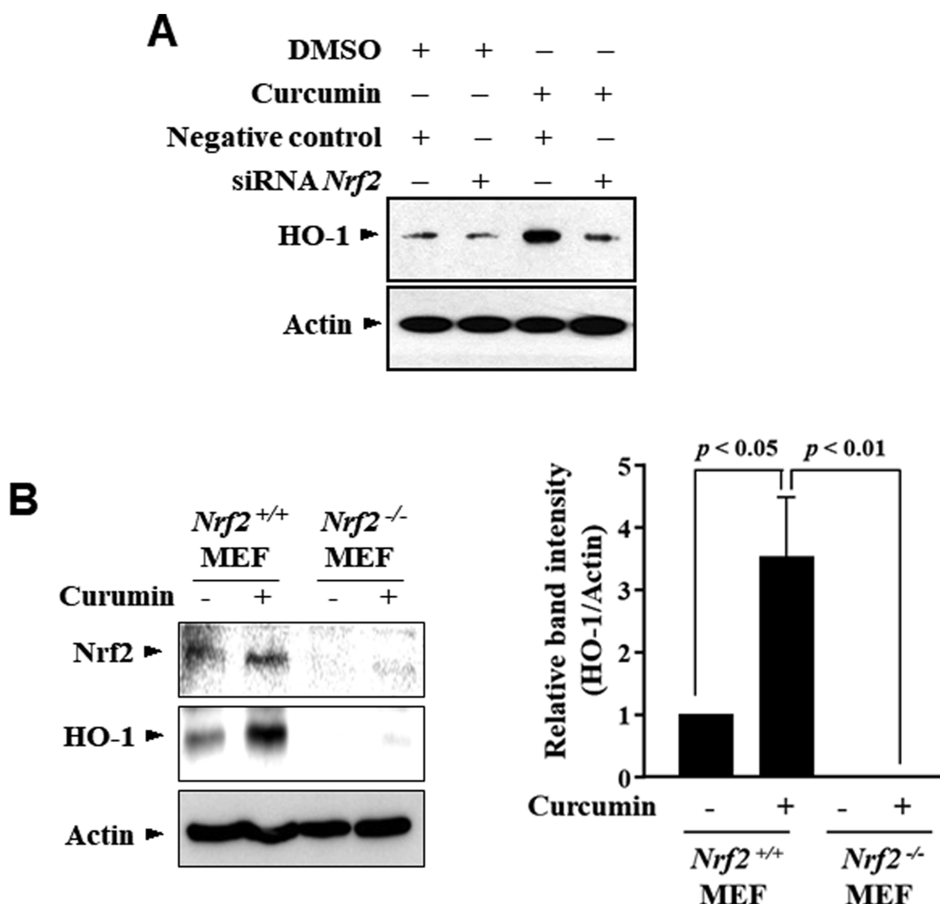



**Fig. 3.** Role of Nrf2 in curcumin-induced HO-1 expression in mouse epidermal cells. (A) JB6 cells were transfected transiently with siRNA-Nrf2 and treated with 5 μM curcumin for 6 h. Protein was isolated and analyzed for the measurement of HO-1 protein by Western blot analysis. (B) MEFs isolated from Nrf2 wild type or knock out mice were treated for 12 h with 20 μM of curcumin. After treatment, the cells were harvested, lysed and subjected to Western blot analysis to determine the levels of Nrf2 and HO-1. Data are means ± SD (n = 3).

immunoprecipitation by shaking with an indicated antibody at 4 °C for 2 h followed by shaking with protein G-agarose suspension (25% slurry, 40 μl) at same condition. After centrifugation at 3000 rpm for 30 s, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. After final wash, immunoprecipitate was resuspended in 40 μl of 1X SDS electrophoresis sample buffer and boiled for 3 min. Ten μl of supernatant from each sample was loaded on SDS-polyacrylamide gel.

### *2.11. Covalent docking simulation of interaction between curcumin and Keap1*

Covalent docking simulation was performed to predict binding pose of curcumin with selected reactive cysteine residues of mouse Keap1 protein using CovDock module implemented in Schrödinger program (Schrödinger, L.L.C., New York, NY, USA). In our previous study [16], a homology model of mouse Keap1 structure based on human Keap1 BTB domain (PDB ID: 4CXI) and human KLKL11 BTB and BACK domains (PDB ID: 3I3N) was used for covalent docking simulation. The same approach was adopted to predict the interaction between curcumin and Keap1 selected cysteine residues. Preparation of the protein structure was processed through “Protein Preparation Wizard” in Schrodinger program. The minimization was terminated when the root mean square deviation of the heavy atoms in the energy minimized structure relative to the starting coordinates exceeded 0.3 Angstrom using OPLS3e force field. The grid size for covalent docking simulation was set to 15 Å of the centroid of cysteine residue in position of 151, 273 and 288 amino acids. Curcumin was sketched and was minimized with MMFFs force field through “LigPrep” in Schrodinger program. Flexible ligand sampling with SP docking mode was considered in the docking procedure. The predefined “Michael Addition” reaction was used to form covalent bonds. All other parameters were set to defaults for the CovDock docking procedure.

### *2.12. Detection of modified Keap1*

Recombinant human Keap1 (0.06 μM, Abnova, Taipei, Taiwan) was incubated with 0.6 μM of curcumin in 100 μl of recombinant protein storage buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) for 2 h at room temperature. The reaction was quenched by adding 60 μM of DTT, and the mixture was incubated for another 15 min. Proteins were separated by NuPAGE® 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). For the identification of curcumin-modified Keap1, gels were treated with endoproteinase Chymotrypsin (Roche, Mannheim, Germany) at 25 °C overnight. Following digestion, proteolytic peptides were extracted and dried with SpeedVac. Resuspended samples in 0.1% formic acid were purified and concentrated using C18 ZipTips (Millipore, Burlington, MA, USA) before MS analysis. The proteolytic peptides were loaded onto a fused silica microcapillary column (12 cm × 75 μm) packed with $C_{18}$ reversed-phase resin (5 μm, 200 Å). Peptide separation was conducted in a series of step gradients composed of initial isobaric flow for 5 min with 3% solvent B (0.1% formic acid in acetonitrile), then linear gradient from 3% to 35% for 30 min, followed by linear gradient to 40% for 5 min. At the end of each running, 90% of solvent B was eluted for 10 min with a rate of 250 nl/min. The % gradient of solvent B was against solvent A (0.1% formic acid in $H_2O$). The column was directly connected to LTQ linear ion-trap mass spectrometer (Finnigan, CA, USA) equipped with a nano-electrospray ion source. Each full MS scan was followed by five MS/MS scan from the most intense to the fifth intense peaks of full MS scan. The acquired LC-ESI-MS/MS fragment spectra were searched in the BioWorksBrowserTM (version Rev. 3.3.1 SP1, Thermo Fisher Scientific Inc., CA, USA) with the SEQUEST search engines against the data in FASTA format generated from Keap1, transcript variant 1, mRNA (NCBI

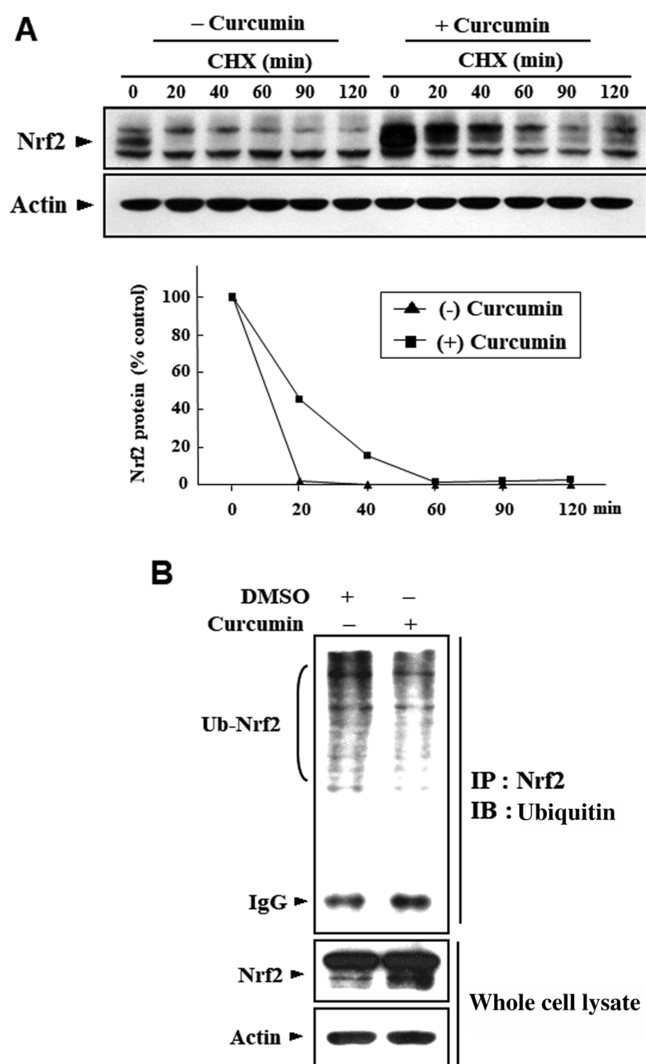



**Fig. 4.** Curcumin-induced stabilization of Nrf2 protein. (A) JB6 cells were treated with 5 μM of curcumin for 6 h and then challenged with 0.1 mg/ml CHX for the indicated time periods. Nrf2 was detected by Western blot analysis. (B) After treatment of JB6 cells with 5 μM of curcumin for 6 h, 500 μg of each protein sample was immunoprecipitated with anti-Nrf2 antibody and visualized by Western blot analysis with anti-ubiquitin antibody.

accession number NM_203500) in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The conditions for the search were as follows; Chymotrypsin as enzyme specificity, a permissible level for two missed cleavages, peptide tolerance; ± 2 amu, a mass error of ± 1 amu on fragment ions and variable modification of cysteine (+57 Da), oxidation of methionine (+16 Da) and curcumin modification of cysteine (+372.14 Da) residues.

## 3. Results

### 3.1. Topically applied curcumin induces expression of Nrf2 and HO-1 in mouse skin

Topical application of 1 μmol curcumin onto the back of mouse induced the expression of Nrf2 (Fig. 1A) and its target cytoprotective protein, HO-1 (Fig. 1B) in a time-dependent manner. In another experiment, topically applied curcumin (0.1 and 1 μmol) for 12 h upregulated expression of Nrf2 and HO-1 in a dose-dependent manner (Fig. 1C). Nrf2, once translocated into the nucleus, binds to ARE present in the promoter regions of target genes. Topically applied curcumin induced transient increase in the binding of Nrf2 to the oligonucleotide harbouring the ARE consensus sequence (Fig. 1D).

### 3.2. Curcumin induces transcription and translation of HO-1 through Nrf2 activation in mouse epidermal cells

Like that observed in mouse skin, the treatment of mouse epidermal JB6 cells with curcumin (5 μM) induced the expression of Nrf2 protein in a time-dependent manner (Fig. 2A), predominantly in the nucleus (Fig. 2B). However, the steady-state levels of *Nrf2* mRNA remained unchanged (Fig. 2C). In another experiment, curcumin treatment increased the expression of HO-1 at both transcriptional (Fig. 2D) and translational (Fig. 2E) levels in JB6 cells in a time-dependent manner. The curcumin-induced expression of Nrf2 and HO-1 was concentration-dependent as well (Fig. 2F). To determine whether curcumin-induced HO-1 expression is mediated by Nrf2, JB6 cells were transiently transfected with *Nrf2* siRNA or control siRNA, and the expression of both Nrf2 and HO-1 was compared by Western blot analysis. Results shown in Fig. 3A demonstrates that siRNA knockdown of *Nrf2* gene abrogated the expression of HO-1. Similarly, embryo fibroblasts from *Nrf2* deficient mice were not responsive to curcumin-induced enhancement of HO-1 expression (Fig. 3B). This finding indicates that Nrf2 is essential for the curcumin-induced up-regulation of HO-1 expression in mouse epidermal cells.

### 3.3. Curcumin stabilizes Nrf2 by blocking ubiquitin-dependent 26S proteasomal degradation

As illustrated in Fig. 2A and B, curcumin induced protein expression of Nrf2 without altering the steady-state level of its mRNA transcript (Fig. 2C). This prompted us to suggest protein stabilization as a plausible mechanism underlying Nrf2 activation by curcumin. To test this supposition, we monitored the degradation of basal and curcumin-induced Nrf2 protein in JB6 cells after blockade of *de novo* protein synthesis by CHX. Residual Nrf2 protein existing in the untreated cells underwent rapid degradation after the addition of CHX. Nrf2 in curcumin-treated JB6 cells, however, was more stable than that of untreated cells (Fig. 4A). Like the majority of other regulatory proteins, Nrf2, is mainly degraded by the 26S proteasomes. Nrf2 is covalently modified by ubiquitin molecules, a highly conserved ~8 kDa polypeptide, at the ε-amino group of Nrf2 lysine residues before degraded by the 26S proteasomes. As shown in Fig. 4B, ubiquitination of Nrf2 was decreased in cells treated with curcumin.

### 3.4. The α,β-unsaturated carbonyl group of curcumin is essential for its activation of Nrf2 and subsequent HO-1 expression

It is well known that Keap1 plays an essential role in Cullin3-Rbx1 E3 ligase as a sensor of oxidative and electrophilic stresses. Keap1 contains several highly conserved cysteine residues that are molecular targets of chemopreventive agents for the activation of Nrf2. As curcumin has an electrophilic α,β-unsaturated carbonyl moiety (Fig. 5A), it may covalently modify one or more of these cysteine residues present in Keap1. As an initial approach to explore this possibility, JB6 cells were co-incubated with curcumin and DTT, a thiol reducing agent that can modify cysteine residues. DTT treatment attenuated curcumin-induced expression of Nrf2 and HO-1 in JB6 cells (data not shown). In addition, tetrahydrocurcumin, which lacks the α,β-unsaturated carbonyl moiety (Fig. 5A), failed to induce the nuclear accumulation of Nrf2 as assessed by Western blot (Fig. 5B) and immunocytochemical (Fig. 5C) analyses. Likewise, tetrahydrocurcumin was unable to promote ARE-driven transcriptional activity of Nrf2 (Fig. 5D) and its target protein expression (Fig. 5E).

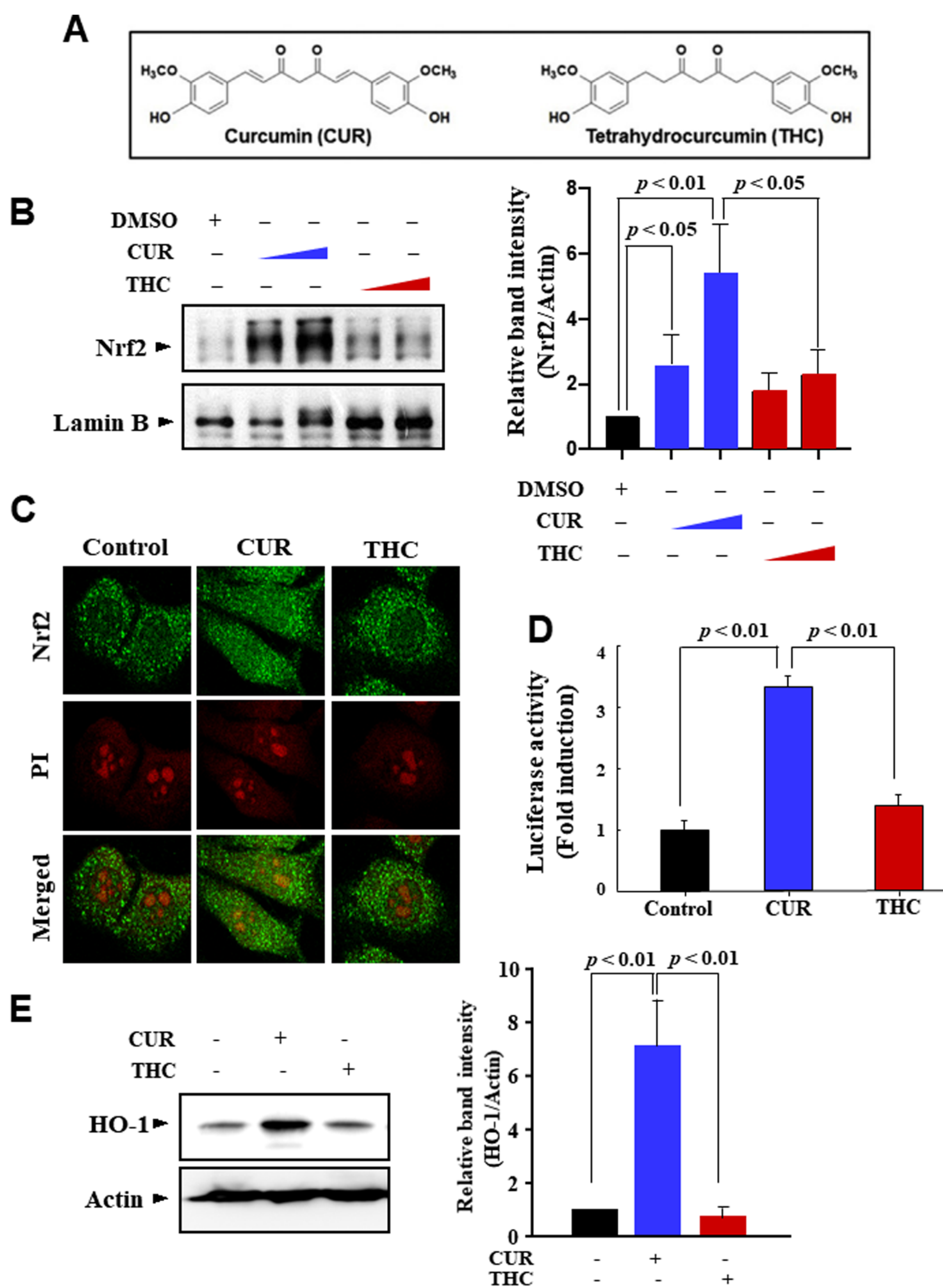



**Fig. 5.** Comparative effects of curcumin and tetrahydrocurcumin on nuclear translocation and transcriptional activity of Nrf2 in JB6 cells. (A) Chemical structures of curcumin and tetrahydrocurcumin. Curcumin has an α,β-unsaturated carbon which is considered to target nucleophiles whereas tetrahydrocurcumin has no such electrophilic moiety. (B) JB6 cells were treated with 2.5 or 5 μM of curcumin or its non-electrophilic analog tetrahydrocurcumin. Nuclear protein was isolated and analyzed for the measurement of Nrf2 expression by Western blot analysis, and band intensities were quantified. Data are means ± SD (n = 3). (C) JB6 cells (1 × $10^4$ per well) were seeded onto 4-chamber coverglasses immersed in 500 μl of 5% FBS-MEM. After 24 h incubation, cells were treated with curcumin or tetrahydrocurcumin at 5 μM concentration each for 6 h, and immunocytochemical analysis of Nrf2 was conducted. (D) JB6 cells were transfected transiently with 100 ng of pGL2-NQO1, and 24 h later, the cells were treated with 5 μM each of curcumin or tetrahydrocurcumin for additional 6 h. Cell lysates were then assayed for the luciferase activity. (E) JB6 cells were treated with 2.5 or 5 μM of curcumin or its non-electrophilic analog tetrahydrocurcumin. Total proteins were isolated and subjected to the Western blot analysis for the measurement of HO-1 protein expression and band intensities were quantified. Data are means ± SD (n = 3).

### *3.5. Modification of the cysteine 151 residue in Keap1 by curcumin is important for its stimulation of Nrf2 transcriptional activity*

Among the cysteine residues present in murine Keap1, Cys151, Cys273 and Cys288 are of particular importance to regulate Nrf2 activity. Prediction of covalent docking pose of curcumin was performed with these reactive cysteine residues in a mouse Keap1 homology model [16]. According to this docking model, Cys151 is located at the tip of the flexible loop in the BTB domain, while Cys273 and Cys288 reside in the intervening region of the IVR domain (Fig. 6A). Molecular docking simulation represents that the α,β-unsaturated carbonyl group of curcumin covalently binds to the reactive sulfur atom of Keap1 cysteine residues by Michael addition (Fig. 6B–D). Two carbonyl groups and the phenolic hydroxyl group of curcumin adducted to Cys151 residue form hydrogen bonds with the ε-amino group of Lys131 and the carboxyl group of Pro89 residues, respectively and the other phenyl moiety interacts with the His154 residue through π-π stacking interaction (Fig. 6B). Curcumin shows unfavorable binding pose to the Cys273 residue (Fig. 6C) while it has a stable interaction with Cys288 via two hydrogen bonds between its two phenolic hydroxyl groups and Ser243 and Glu289 residues (Fig. 6D). Cdock affinity of curcumin to Cys151, Cys273 and Cys288 was −3.31, −2.37 and −3.41 kcal/mol, respectively.

We further conducted LC-ESI-MS/MS analysis to identify the binding site(s) of curcumin. Human recombinant Keap1 incubated with curcumin was digested with chymotrypsin, and analyzed by tandem mass spectrometry. An increment of molecular mass by curcumin modification was observed in the peptide fragments ($y_5^+$ and $[b_{10}-H_2O]^+$) containing Cys151 (Fig. 7A).

Accordingly, the effects of curcumin on Nrf2 transcriptional activity were examined in the cells transfected with wild type or cysteine mutant Keap1 together with Nrf2. As shown in Fig. 7B, curcumin treatment enhanced Nrf2 activity in the cells transfected with Nrf2 and wild type Keap1, but not in those cells harbouring Cys151 mutant Keap1. These findings suggest that curcumin interacts with Cys151 residue in Keap1, thereby hampering Keap1-mediated Nrf2 suppression.

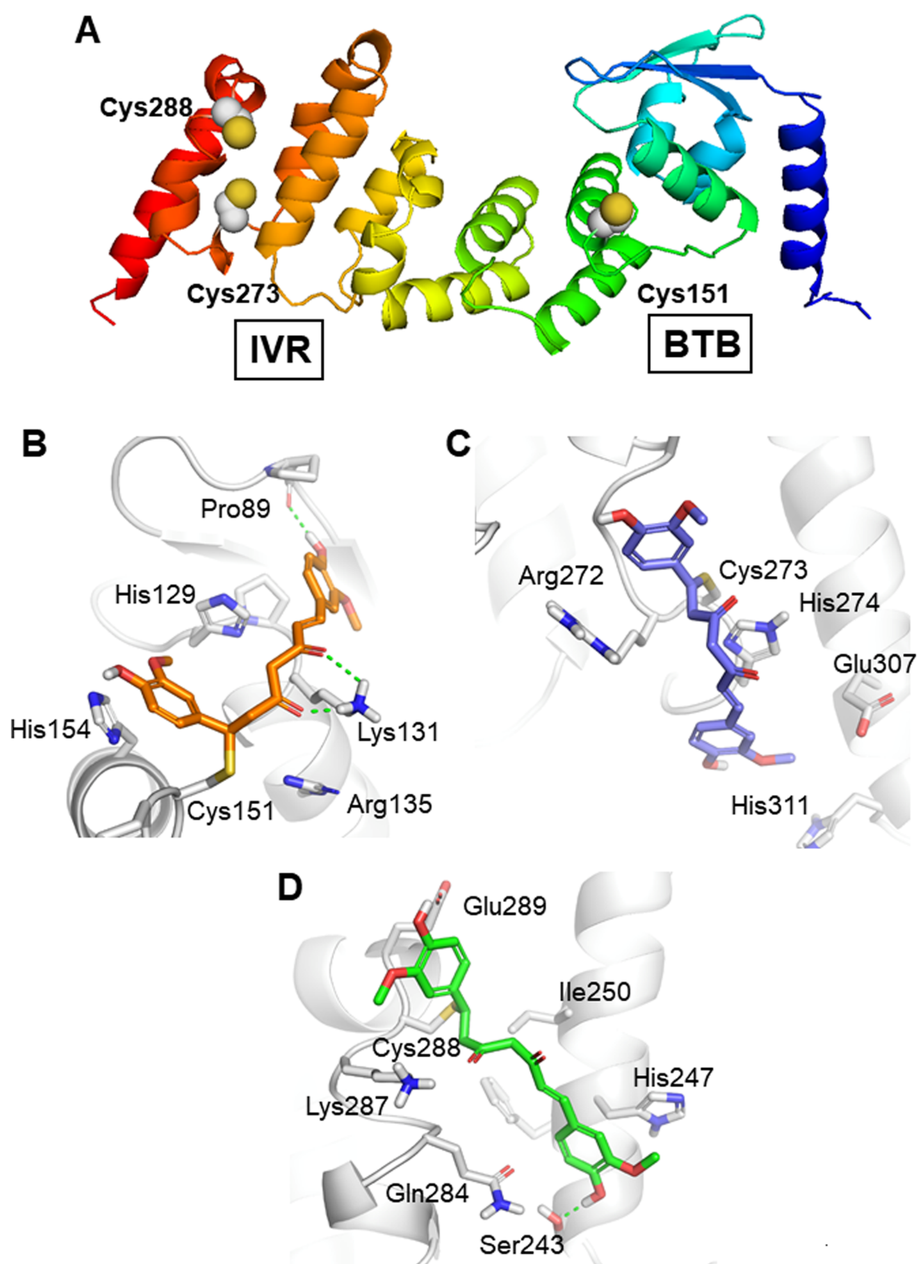


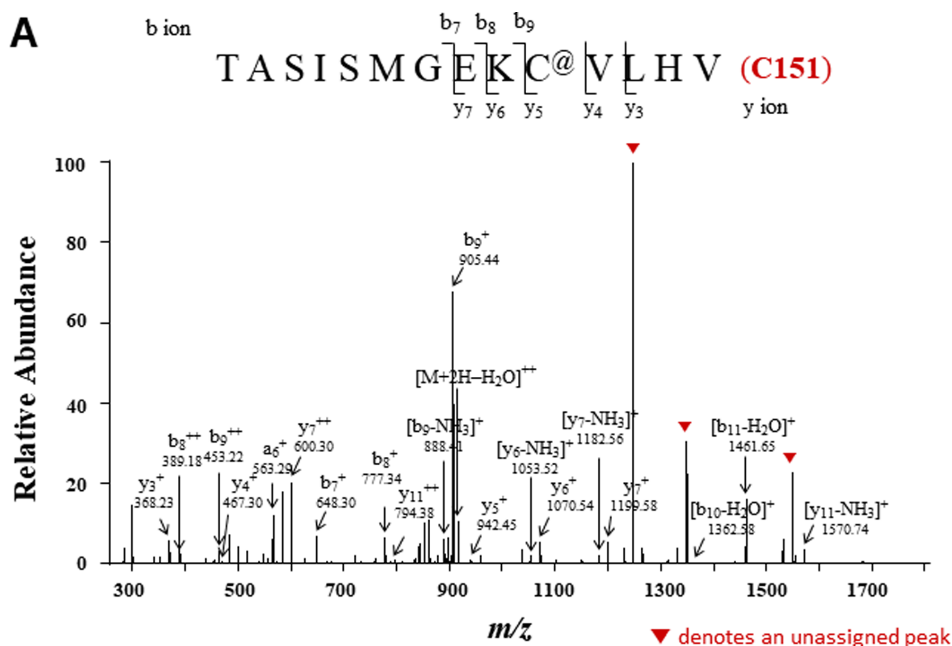

**Fig. 6.** Covalent docking pose of curcumin in BTB and IVR domains of mouse Keap1 protein. (A) A model of Keap1 BTB and IVR structures with three cysteine residues in position of 151, 273 and 288. The covalent binding pose of the α,β-unsaturated carbonyl group of curcumin to Cys151 (B), Cys273 (C) and Cys288 (D) residues is shown. Gray carbon-capped sticks are the amino acids of Keap1. Green dashed lines are hydrogen bonds.

### *3.6. Curcumin increases interaction between Nrf2 and Keap1 without altering the association between the components of Cullin3-Rbx1 E3 ligase complex*

Under normal physiological conditions, Keap1 sequesters Nrf2 in the cytoplasm targeting it for ubiquitination and proteasomal degradation. To determine whether increased accumulation of Nrf2 by curcumin results from decreased interaction between Nrf2 and Keap1, the levels of Keap1-associated Nrf2 were determined after curcumin treatment. When HEK293T cells were transfected with expression vectors for Nrf2 and Keap1 followed by immunoprecipitation with Keap1 antibody, an increase in Keap1-associated Nrf2 was observed (Fig. 8A). It is known that disruption of binding between Keap1 and Cullin3 results in decreased activity of Cullin3-Rbx1 E3 ubiquitin ligase. This prompted us to examine the effects of curcumin on Cullin3-Rbx1 E3 ligase complex assembly. Curcumin treatment, however, didn't alter the interaction between Cullin3-Keap1 (Fig. 8B) and Cullin3-Rbx1 (Fig. 8C). These results suggest that curcumin increases Nrf2 stability by repressing the ability of Keap1 to efficiently target Nrf2 for ubiquitin-mediated degradation rather than release Nrf2 from Keap1.

## 4. Discussion

Nrf2, as a master switch in turning on transcription of many carcinogen detoxifying and antioxidant genes, is considered a prime target for chemoprevention by a wide spectrum of edible phytochemicals [6,7]. Under basal conditions, Keap1 binding to Cullin3 enables the complex to degrade Nrf2 through ubiquitination and subsequent 26S proteasomal degradation [2–5]. Some electrophiles bind to Keap1 and disrupt proteasome-mediated degradation of Nrf2. For instance, the adduction of Keap1 cysteine(s) by such electrophilic substances as sulforaphane and *tert*-butyl hydroquinone changes the conformation of



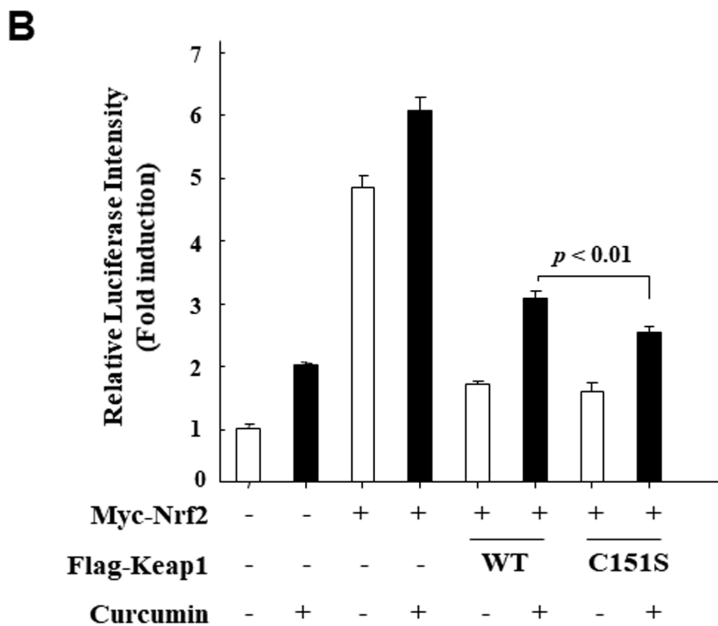




**Fig. 7.** Cysteine 151 of Keap1 as a putative binding site of curcumin for its activation of Nrf2. (A) Human recombinant Keap1 (5.64 μg) was incubated for 1 h at room temperature in final volume of 100 μl containing 600 nM curcumin, followed by adding 60 μM DTT for 15 min. Proteolytic Keap1 peptides were analyzed by mass spectrometry. (B) HEK293T cells were transfected with plasmids containing an ARE-driven luciferase reporter gene, expression plasmids for wild type Myc-Nrf2 and Flag-Keap1 or mutant Keap1. Transfected cells were treated with vehicle or curcumin (15 μM) for 6 h before the reporter assay as described in Materials and methods. Data are means ± SD (n = 3).

ligase and promotes the escape of Nrf2 from proteasomal degradation [17–19]. Curcumin bears two electrophilic α,β-unsaturated carbonyl moieties. Therefore, modification of Keap1 cysteine(s) by this electrophilic phytochemical through Michael addition reaction is speculated to provoke conformational changes in Keap1, impairing its ability to suppress Nrf2 activation.

Several different mechanisms appear to be involved in Nrf2 activation by Keap1 cysteine modifications. It is noticeable that Keap1 is not merely a repressor that sequesters Nrf2 in the cytoplasm, but rather acts as an active mediator that targets Nrf2 for ubiquitination and proteasomal degradation. Keap1 functions as a substrate adaptor protein for the Cullin3-Rbx1 E3 ubiquitin ligase complex (3–5). Keap1 protein contains several cysteine(s), which are highly conserved across species. Among these, Cys151, Cys273, and Cys288 are considered as sensors for electrophiles and oxidants that are capable of inducing the transcriptional activity of Nrf2 [17]. In particular, mutation of Cys151 to serine has been shown to abolish electrophile- and oxidant-mediated Nrf2 ubiquitination [20]. In line with this observation, our results indicate that Cys151 is required for transcriptional activity of Nrf2 in response to curcumin treatment. The other two cysteine residues, Cys273 and Cys288 are required for Keap1 to repress Nrf2-dependent transcriptional activation [17]. However, curcumin-induced Nrf2 transcriptional activity was barely impaired in cells harbouring mutant Keap1-C273S and Keap1-C288S (data not shown). Based on these findings, we speculate that Cys151 may be a putative binding site of curcumin. Mass spectral analysis revealed that Keap1 Cys151 was modified by curcumin, corroborating the above speculation.

According to the current paradigm, electrophilic adduction of Keap1 Cys151 may dissociate Keap1 from Cullin3 and decrease the activity of Cullin3-Rbx1 E3 ubiquitin ligase. An early independent study suggested that modification of Keap1 cysteine(s) by ARE inducers directly disrupts the interaction between Keap1 and Nrf2 [21]. However, subsequent experiments demonstrated that the disruption of the Keap1-Nrf2 complex did not occur upon modification of Keap1 cysteine [22,23]. While

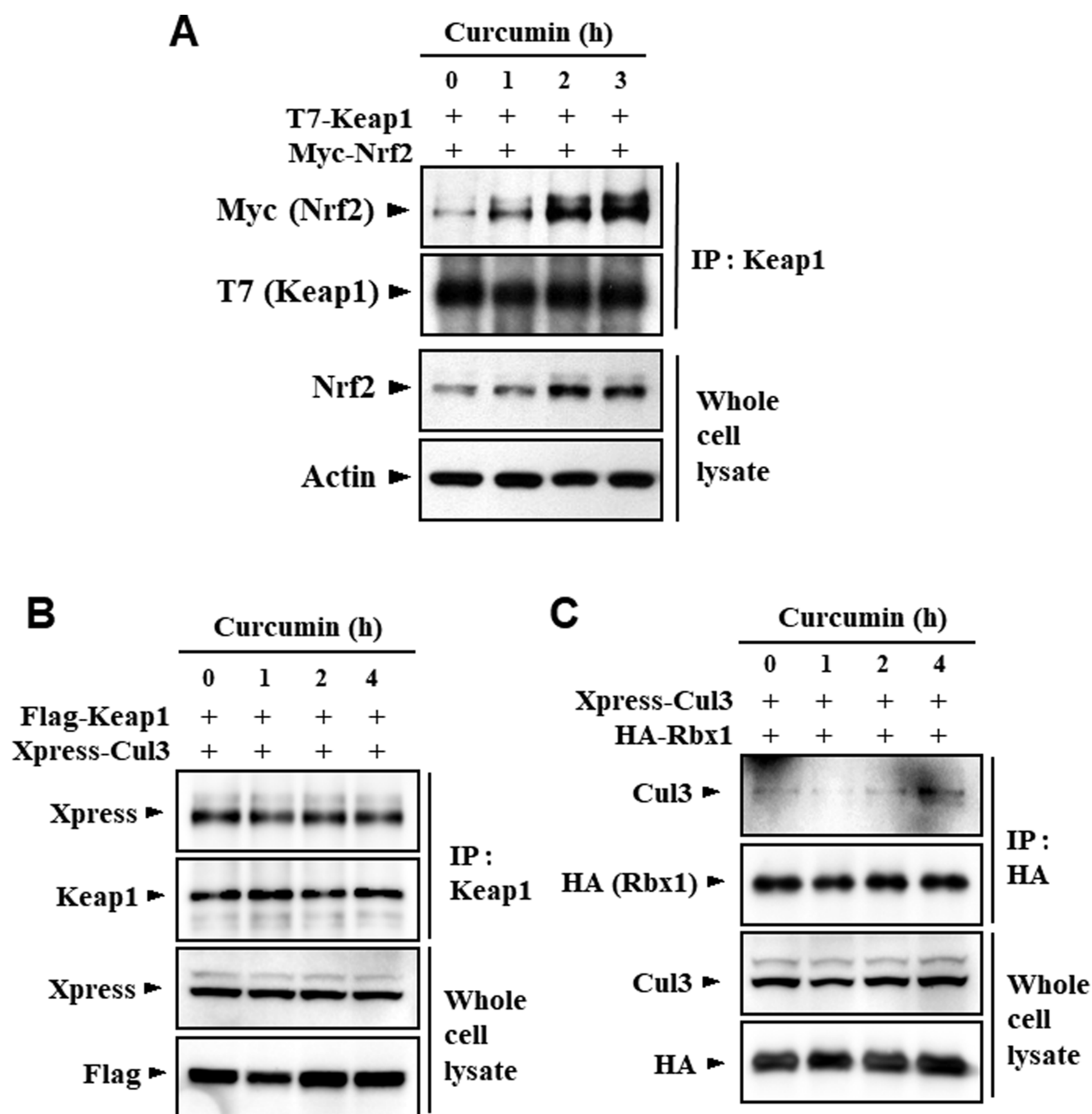



**Fig. 8.** Effects of curcumin on interaction between Nrf2 and Keap1, Keap1 and Cullin3 and Cullian3 and Rbx1. (A, B and C) HEK293T cells were transfected with plasmids of 500 ng Myc-Nrf2 (A), 1,000 ng Flag-Keap1 (B), 1,000 ng Xpress-Cullin3 (B,C), 1,000 ng HA-Rbx1 (C) and 1,000 ng T7-Keap1 (A) for 24 h as indicated, followed by treatment with curcumin (15 μM) for the indicated time. Cell lysates were subjected to immunoprecipitated and immunoblotting using antibodies against Keap1, Cullin3, HA, Myc and T7.

modification of Keap1 cysteine does not alter the affinity of Keap1 for Nrf2, recent data indicate that the Keap1-Cullin3 interaction is altered by Keap1 cysteine modification [23,24]. These results suggest that the reaction of ARE inducers with Keap1 cysteine leads to reduced association between Keap1 and Cullin3, thereby repressing Nrf2 ubiquitination. This would, in turn, lead to Nrf2 accumulation.

Although the Cullin3-Keap1 interaction among the components of Cullin3-Rbx1 E3 ligase complex is an important aspect of its ability to ubiquitinate Nrf2, this cannot fully account for the mechanisms regulating the activation of Nrf2 signaling. Cope and Deshaies proposed an interesting model that Cullin-dependent ubiquitin ligase is a very dynamic structure regulated by cycles of assembly and disassembly for efficient degradation of target proteins [25]. The main point of this model is that the Cullin-Rbx1 core complex cycles between active and inactive states. In addition, several studies suggest that cyclical assembly and disassembly of Cullin dependent E3 ligase complex is partially mediated by the antagonistic action of Nedd8 modification of the Cullin protein and association of Cullin protein with Cullin-associated NEDD8-dissociated protein 1 (CAND1) [26–28]. In the active state, the Cullin protein is modified by Nedd8 conjugation, and the conjugated Nedd8 polypeptide may also promote the ubiquitination of targets and prevent binding of the inhibitor CAND1 [29,30]. Thus it is possible that modification of Keap1 Cys151 by curcumin causes a conformational change of Cullin3 to enhance deneddylation states of Cullin3 or interaction between CAND1 and Cullin3, leading to the reduced activity of Cullin3-Rbx1 E3 ubiquitin ligase. However, further experiments are required to elucidate precise molecular mechanisms responsible for the regulation of Cullin3 activity by curcumin.

While covalent modification of Keap1 cysteine thiol appears to be a principal mechanism by which curcumin stabilizes Nrf2, other regulatory mechanisms are also involved in Nrf2 activation. Some studies demonstrated that phosphorylation of Nrf2 also appears to play an important role in activating Nrf2 by ARE inducers. The activity of protein kinase C (PKC) was stimulated by various ARE inducers, and the sole site of phosphorylation by PKC was identified as Ser40 [31–33]. The result from co-IP experiments demonstrated that phosphorylation of Nrf2 by PKC *in vitro* promotes its dissociation from Keap1, and this effect was largely hampered by S40A mutation [32]. In addition, p38 mitogen-activated protein kinase (MAPK) isoforms have been implicated in Nrf2 signaling, and alteration of the Keap1-Nrf2 affinity has

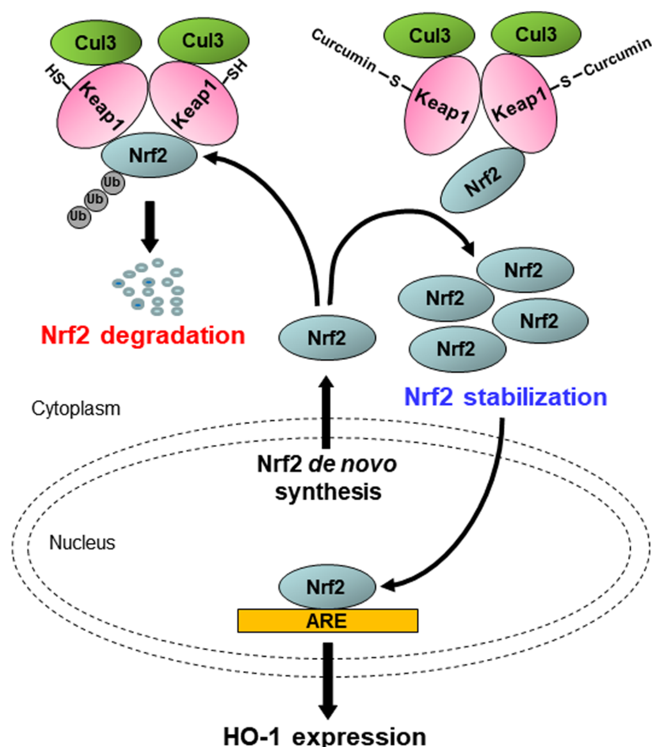



**Fig. 9.** A proposed scheme for the activation of Nrf2 by curcumin. In its basal state, Nrf2 is sequestered in the cytoplasm as an inactive complex with Keap1, an adaptor protein that brings a Cullin3-Rbx1 E3 ligase complex into close proximity to Nrf2, facilitating the ubiquitination and proteasomal degradation of Nrf2. Under stressed conditions, however, the ability of the Cullin3-Rbx1 E3 ligase to ubiquitinate Nrf2 is inhibited. As a result, Nrf2 is stabilized and accumulated. Keap1 protein contains highly conserved cysteines, some of which are sensors for electrophiles and oxidants. Curcumin has two electrophilic α,β-unsaturated carbonyl moieties capable of covalently binding to a cysteine residue (e.g., Cys151) of Keap1. Covalent modification of Keap1 by curcumin causes conformational change of the Keap1-Cullin3-Rbx1 E3 ligase complex, leading to a decrease in the ubiquitin ligase activity. This will result in stabilization of Nrf2.

been proposed as the mechanistic explanation [34]. Specific inhibitors of p38 MAPK, PKC and phosphatidylinositol 3-kinase have been reported to attenuate curcumin-induced Nrf2 activation [35–37].

In conclusion, our study revealed that curcumin inhibits the ability of the Cullin3-Rbx1 E3 ubiquitin ligase complex to target Nrf2 for ubiquitination by modifying Keap1 Cys151 residue. This modification may change the conformation of the complex and saturates the binding capacity of Keap1 to Nrf2, facilitating nuclear translocation of *de novo* synthesized Nrf2 (Fig. 9).

## CRediT authorship contribution statement

Jun-Wan Shin and Young-Joon Surh conceived and designed the experiments; Jun-Wan Shin, Kyung-Soo Chun, Seong Hoon Kim, Su-Jung Kim, and Do-Hee Kim performed the experiments; Su-Jung Kim and Nam-Chul Cho conducted computer-based docking analysis; Jun-Wan Shin, Kyung-Soo Chun, and Do-Hee Kim analyzed the data; Hye-Kyung Na helped to get special reagents and participate in discussion on the results; Jun-Wan Shin and Young-Joon Surh wrote the paper. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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