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Curcumin partly prevents ISG15 activation via ubiquitin-activating enzyme E1-like protein and decreases ISGylation

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ABSTRACT

The expression of the ubiquitin-like molecule interferon-stimulated gene 15 kDa (ISG15) and post-translational protein modification by ISG15 (ISGylation) are strongly activated by interferons or pathogen infection, suggesting that ISG15 and ISGylation play an important role in innate immune responses. More than 400 proteins have been found to be ISGylated. ISG15 is removed from substrates by interferon-induced ubiquitin-specific peptidase 18 or severe acute respiratory syndrome coronavirus 2-derived papain-like protease. Therefore, maintaining strong ISGylation may help prevent the spread of coronavirus disease 2019 (COVID-19). However, it is unknown whether nutrients or chemicals affect ISGylation level. Curcumin is the major constituent of turmeric and functions as an immunomodulator. Here, we investigated the effect of curcumin on ISGylation. MCF10A and A549 cells were treated with interferon α and curcumin after which the expression levels of various proteins were determined. The effect of curcumin on ubiquitylation was also determined. Curcumin treatment was found to reduce ISGylation in a dose-dependent manner. The findings suggested that curcumin partly prevents disulfide bond-mediated ISG15 dimerization directly or indirectly, thereby increasing monomer ISG15 levels. Reduced ISGylation may also occur via the prevention of ISG15 activation by ubiquitin-activating enzyme E1-like protein. In conclusion, curcumin treatment was found to reduce ISGylation, suggesting that it may contribute to severe COVID-19. This is the first study to report a relationship between ISGylation and a food component.

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1. Introduction

Interferons (IFNs) are a group of cytokines that play a role in the defense against bacterial and viral infections. IFNs are induced by diverse stimuli, such as lipopolysaccharides, lipoproteins, lipopeptides, double-stranded RNA, single-stranded RNA, or unmethylated CpG motifs mainly via Toll-like receptors [1,2]. IFN-

stimulated gene, 15 kDa (ISG15) is the first reported ubiquitin-like molecule induced by IFNs [3–6]. It forms covalent conjugates with at least 434 proteins [7], which is similar to protein ubiquitylation [8]. ISG15 is activated by the E1 enzyme, ubiquitin-activating enzyme E1-like protein (UBE1L), in an ATP-dependent manner [9–15]. Activated ISG15 is transferred to the E2 enzyme, ubiquitin-conjugating enzyme E2 L6 (UBE2L6), followed by ISG15 modification (ISGylation) of substrates, which is recognized by ISG15 ligases [9–15]. ISG15 expression and ISGylation are strongly induced by type I IFNs [3,6,16]. Since mainly viral proteins are translated in the infected cells and newly synthesized proteins can be modified by ISG15 [17], ISGylation may interfere with pathogen protein function. For example, it has been shown that ISGylation of the human papillomavirus (HPV) L1 capsid protein has a dominant inhibitory effect on the infectivity of HPV16 pseudoviruses [3,17]. Furthermore, cellular proteins involved in antiviral defense or

Abbreviations: COVID-19, coronavirus disease 2019; FBS, fetal bovine serum; HPV, human papillomavirus; IFN, interferon; IL-1, interleukin-1; IRAK, interleukin-1 receptor-associated kinase; ISG15, interferon-stimulated gene 15 kDa; NO, nitrogen monoxide; PLpro, papain-like protease; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TBST, Tris-buffered saline with Tween-20; UBE1L, ubiquitin-activating enzyme E1-like protein; UBE2L6, ubiquitin-conjugating enzyme E2 L6; USP18, ubiquitin-specific peptidase 18; 2-ME, 2-mercaptoethanol.

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export of viral particles have been shown to be ISGylated [4]. IFNs are key cytokines involved in innate immune responses [18,19]. Ubiquitin-specific peptidase 18 (USP18) is an IFN-induced protein, which acts as an ISG15 isopeptidase that removes ISG15 from substrates [20–23]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as a novel human betacoronavirus that causes coronavirus disease 2019 (COVID-19) [24]. Similar to USP18, SARS-CoV-2-derived papain-like protease (PLpro) can remove ISG15 from substrates [25–29]. Therefore, it was hypothesized that ISGylation may delay SARS-CoV-2 propagation, although the substrate protein has not been identified. ISGylation may modulate certain immune responses during infections and various stresses.

Turmeric is the rhizome of the plant *Curcuma longa* and a gold-colored spice commonly used in the Indian subcontinent [30]. Curcumin is a bioactive compound present in turmeric. It exhibits multiple activities, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities [30]. Some of its polypharmacological effects may be attributed to covalent adduction to cellular proteins. Curcumin forms covalent bonds with the thiol of glutathione, N-acetylcysteine, and 2-mercaptoethanol (2-ME) [31]. Moreover, it has been shown to block the thiols of interleukin-1 (IL-1) receptor-associated kinase (IRAK) in a murine T-cell line, resulting in the inhibition of early events in inflammatory signaling cascades, such as IRAK recruitment to the IL-1 receptor and phosphorylation of IRAK and IL-1 receptor-associated proteins [32].

ISGylation represents a well-known protein modification induced by IFNs; however, the environment suitable for effective ISGylation has not been fully elucidated, except for nitrosylation of ISG15 [33]. Nitrosylation of ISG15 prevents ISG15 homodimerization, which increases the levels of monomer ISG15 that participates in ISGylation [33]. Here, we report that curcumin can prevent UBE1L-ISG15 dimerization, which is the first step of ISG15 activation and necessary for ISGylation. Consequently, total ISGylation was downregulated in the presence of curcumin. Since SARS-CoV-2 PLpro is an ISG15-removing enzyme, the daily intake of high amounts of curcumin may enhance SARS-CoV-2 propagation if ISGylation plays a role in prevention of virus propagation.

2. Materials and methods

2.1. Reagents

EDTA, cOmplete EDTA-free protease inhibitor cocktail, Ponceau S, Triton X-100, and Tween-20 were purchased from Merck (Kenilworth, NJ, USA). Can Get Signal™ was purchased from Toyobo (Osaka, Japan). Ni-NTA agarose was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Curcumin (C2302) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Curcumin was dissolved in DMSO at a concentration of 20 mM and added to cell culture medium at a final concentration of 5–10 μ M.

2.2. Plasmid construction

Human *UBE1L*, human *ISG15*, and mouse *Isg15* cDNA were amplified via PCR and subcloned into pcDNA3.1, pFLAG-CMV2 (Merck), or pGEX6p-1 (Merck) [11,13]. Mouse *Isg15* cDNA was subcloned into pCAGGS vector containing a six-His tag [11]. Site-directed point mutation was generated using a QuikChange® XL site-directed mutagenesis kit (Stratagene, San Diego, CA, USA) [33]. These constructs were confirmed via DNA sequencing.

2.3. Cell culture and transduction

HEK293T (American Type Culture Collection, ATCC) and

A549 cells (gift from Dr. Keiichi Nakayama, Kyushu University, Japan) were cultured in Dulbecco's modified essential medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μ g/mL streptomycin. MCF10A cells (gift from Dr. Chin Ha Chung, Seoul National University, Korea) were cultured in DMEM supplemented with 5% FBS, 100 units/mL penicillin G, 100 μ g/mL streptomycin, 10 ng/mL epidermal growth factor (AF-100-15-1mg, PeproTech, Cranbury, NJ, USA), 5 μ g/mL insulin (099–06473, Fujifilm Wako Pure Chemical Corporation), 1 ng/mL cholera toxin (01–511, Bio-Academia, Osaka, Japan), and 1 μ g/mL hydrocortisone (H0533, Tokyo Chemical Industry). HEK293T cells were transfected with expression plasmids using polyethylenimine (MW-25 K; Polysciences, Warrington, PA, USA) at a mass ratio of 1:4.

2.4. IFN stimulation and curcumin treatment

Cells were trypsinized and cultured for 1 d. The next day, cells were stimulated by 1,000 U/mL of human IFN α (#11100-1; R&D systems, Minneapolis, MN, USA) with or without curcumin for 1 d. The next day, the culture medium was changed following culturing for 1 d with or without human IFN α and/or curcumin.

2.5. Antibodies

A rabbit polyclonal antibody against human ISG15 was generated by immunizing rabbits with recombinant human ISG15 (Merck, Tokyo, Japan). The anti-human ISG15 antibody was purified from antisera using glutathione S-transferase-tagged human ISG15 and used for IB (1 μ g/mL in Can Get Signal™). Antibodies against the following proteins were also used: FLAG (1 μ g/mL in PBS; M2; Merck), poly-ubiquitin (1 μ g/mL in Can Get Signal™; FK2; Cosmo Bio, Tokyo, Japan), Herc5 (0.5 μ g/mL in Can Get Signal™; BML-PW0920; Enzo Life Sciences, Farmingdale, NY, USA), His₆ (1 μ g/mL in PBS; MAB050; R&D systems), UBE1L (0.2 μ g/mL in Can Get Signal™; sc-390097; Santa Cruz Biotechnology, Dallas, TX, USA), UBE2L6 (0.5 μ g/mL in Can Get Signal™; ab109086; Abcam, Cambridge, MA, USA), and USP18 (1 μ g/mL in Can Get Signal™; 4813; Cell Signaling Technology, Tokyo, Japan).

2.6. Cell lysis and western blotting

Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.4 mM Na₃VO₄, 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, and cOmplete EDTA-free protease inhibitor cocktail, according to the manufacturer's protocol. Proteins were denatured using SDS-PAGE sample buffer (with or without 2-ME) and boiling, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane (Protran NC 0.1; 10600000; Cytiva, Marlborough, MA, USA). The membrane was placed in 3% skim milk in PBS at room temperature (22–28 °C) for 15 min and then with primary antibodies at 4 °C overnight. The membrane was washed three times with Tris-buffered saline with Tween-20 (TBST) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% tween-20 at room temperature for 15 min, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:8,000-dilution in 3% skim milk in PBS; A4416; Merck) or anti-rabbit IgG (1:8,000-dilution in 3% skim milk in PBS; A6154; Merck) at room temperature for 1 h. The membrane was washed three times with TBST for 15 min. Protein levels were measured using an enhanced chemiluminescence reagent (Luminata Forte, Merck or Super Signal West Pico PLUS, Thermo Fisher Scientific, Waltham, MA, USA) and the SOLO.7S.EDGE (Vilber, France) system. Images were processed using Photoshop; however, they were not

cropped from different parts of the same gel, or from different gels or fields.

2.7. Ni-agarose pull-down

Cell lysates were incubated with 10 μ L of Ni-NTA agarose beads in the presence of 20 mM imidazole at 4 $^{\circ}$ C for 1 h. The beads were washed three times with lysis buffer containing 20 mM

imidazole. His₆-ISG15 was eluted from Ni-NTA agarose beads using SDS-PAGE sample buffer containing 300 mM imidazole and via boiling.

2.8. Statistical analysis

Data are expressed as the mean \pm standard deviation of three independent experiments. Means of different groups were

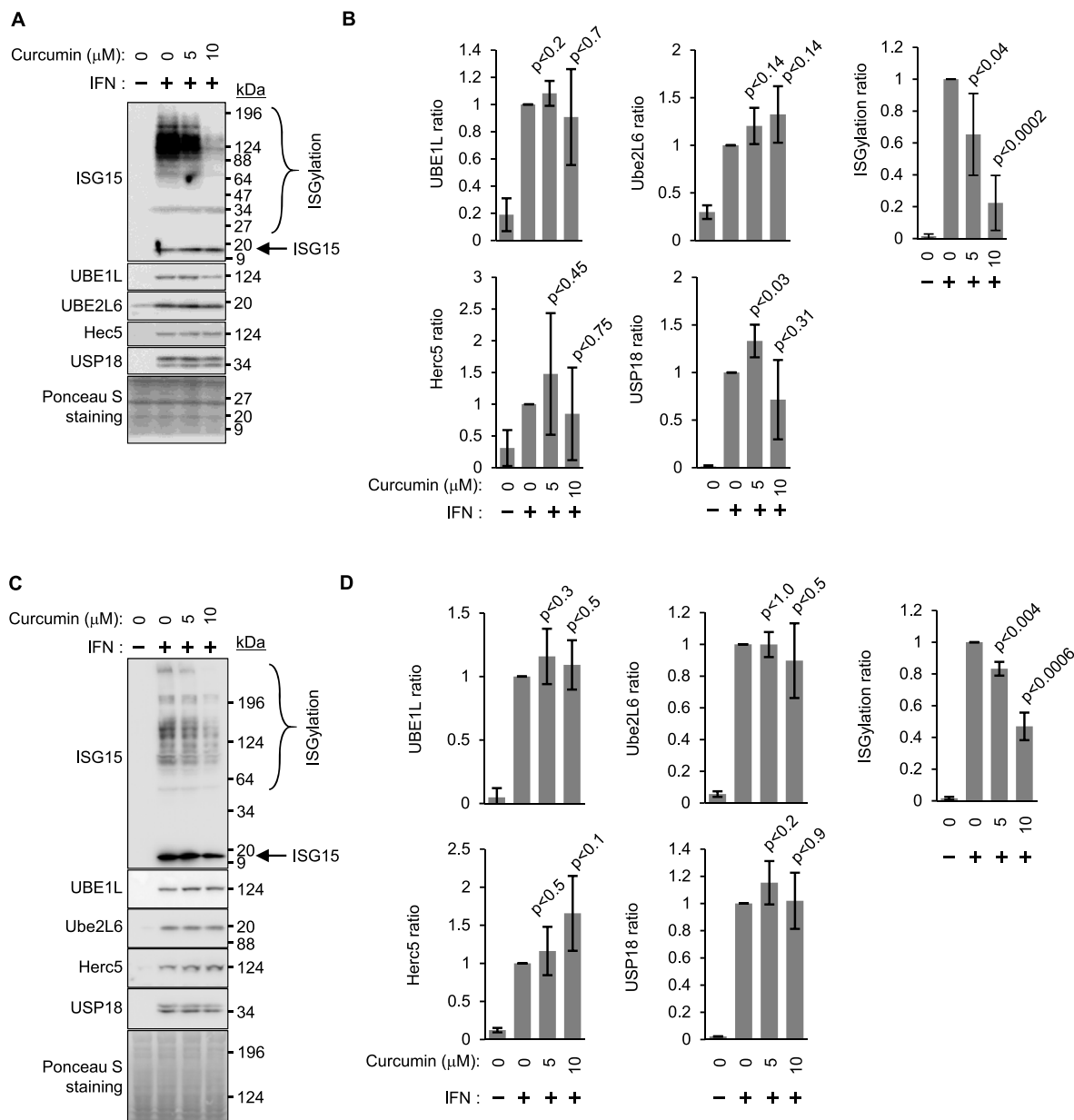


Fig. 1. Downregulation of ISGylation but not of UBE1L, UBE2L6, Herc5, and USP18 via curcumin treatment. (A) Curcumin partly prevents ISGylation in MCF10A cells. MCF10A cells were stimulated by human IFN α with or without curcumin (5 or 10 μ M) for 2 d. The cell lysates were subjected to immunoblotting with an anti-ISG15, anti-UBE1L, anti-UBE2L6, anti-Herc5, or anti-USP18 antibody. Ponceau S staining was used as loading control. Representative data of three independent experiments. (B) Quantification of UBE1L, UBE2L6, Herc5, and USP18 expression levels, as well as of ISGylation in (A). Each signal was normalized to that of Ponceau S staining. The signal intensity of cells stimulated by human IFN α was set as 1. Data are expressed as the mean \pm standard deviation of three independent experiments. Means of different groups were compared using Student's *t*-test. A P value < 0.05 was considered statistically significant. (C) Curcumin partly prevents ISGylation in A549 cells. A549 cells were stimulated by human IFN α with or without curcumin (5 or 10 μ M) for 2 d. The cell lysates were subjected to immunoblotting with an anti-ISG15, anti-UBE1L, anti-UBE2L6, anti-Herc5, or anti-USP18 antibody. Ponceau S staining was used as loading control. Representative data of three independent experiments. (D) Quantification of UBE1L, UBE2L6, Herc5, and USP18 expression levels, as well as of ISGylation in (C). Each signal was normalized to that of Ponceau S staining. The signal intensity of cells stimulated by human IFN α was set as 1. Data are expressed as the mean \pm standard deviation of three independent experiments. IFN α , interferon alpha; ISG15, interferon-stimulated gene, 15 kDa; ISGylation, ISG15 modification; UBE1L, ubiquitin-activating enzyme E1-like protein; UBE2L6, ubiquitin-conjugating enzyme E2 L6; Herc5, probable E3 ubiquitin-protein ligase; USP18, ubiquitin-specific peptidase 18. Means of different groups were compared using Student's *t*-test. A P value < 0.05 was considered statistically significant.

compared using Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Curcumin downregulated ISGylation

MCF10A human mammary gland epithelial cell line shows strong ISGylation in response to human IFN α . Therefore, MCF10A cells were cultured in the presence or absence of IFN α and curcumin for 2 d. The cell lysates were subjected to SDS-PAGE followed by immunoblotting (IB) with anti-ISG15, anti-UBE1L, anti-UBE2L6, anti-probable E3 ubiquitin-protein ligase (Herc5), and anti-USP18 antibody. IFN α induced ISG15 expression at approximately 15 kDa, as well as ISGylation at 34–200 kDa (Fig. 1A and B) as reported previously [33]. IFN α also induced UBE1L, UBE2L6, Herc5, and USP18 expression as reported previously (Fig. 1A and B) [10–12,34–36]. Curcumin treatment reduced ISGylation in a dose-dependent manner; however, it did not reduce UBE1L, UBE2L6, Herc5, and USP18 expression. A549 human lung adenocarcinoma cell line also shows strong ISGylation in response to type I IFN [37]. Therefore, A549 cells were stimulated by human IFN α with or without curcumin for 2 d. The cell lysates were subjected to SDS-PAGE followed by IB with anti-ISG15, anti-UBE1L, anti-UBE2L6, anti-Herc5, or anti-USP18 antibodies. ISGylation, and not UBE1L, UBE2L6, Herc5, and USP18 expression, was downregulated via curcumin treatment in a dose-dependent manner (Fig. 1C and D). These results suggest that the downregulation of ISGylation by curcumin was not cell type-specific.

3.2. Curcumin downregulated ISGylation and not ubiquitin modification (ubiquitylation)

ISG15 is a ubiquitin-like protein, and the ISG15 modification system is similar to the ubiquitylation system [3–6,38]. Therefore, we evaluated the effect of curcumin on ubiquitylation. Ubiquitylation has been found to be increased by curcumin treatment [39,40]. Mechanistically, proteasome activity is downregulated by curcumin [41]. In contrast, ubiquitylation in the muscles of diabetes mellitus mice is downregulated by curcumin [42]. These data suggest that the effect of curcumin on ubiquitylation may vary under different conditions. Therefore, we determined the effect of curcumin on ubiquitylation in MCF10A cells. MCF10A cells were stimulated by human IFN α for 2 d with or without curcumin. The cell lysates were subjected to SDS-PAGE followed by IB with anti-poly ubiquitin or ISG15 antibody (Fig. 3A). Curcumin treatment did not downregulate ubiquitylation (Fig. 2A and B). Therefore, curcumin may selectively downregulate ISGylation in MCF10A cells.

3.3. Curcumin treatment did not affect ISG15 homodimerization

ISG15 molecules interact with each other and form non-covalent homodimers [33]. The minor fraction of ISG15 covalently dimerizes via disulfide bonds of cysteine residues [33]. This covalent dimerization reduces the levels of ISG15 monomers required for ISGylation [33]. Thus, curcumin may affect non-covalent or covalent homodimerization of ISG15 and downregulate ISGylation. The human embryonic kidney cell line HEK293T was used to examine this possibility owing to high transfection efficiency. FLAG-tagged ISG15 (FLAG-ISG15) was transiently expressed with or without His₆-tagged ISG15 (His₆-ISG15) in HEK293T cells. The cell lysates were subjected to pull-down assays using nickel-nitrilotriacetic acid (Ni-NTA) resin to evaluate the interaction between FLAG-ISG15 and His₆-ISG15 (Fig. 2C). ISG15 molecules

interacted with each other; however, curcumin did not affect the homodimerization of ISG15 (Fig. 2C and D). This assay cannot distinguish disulfide bond-mediated covalent binding and non-covalent bond-mediated dimerization. Next, the effect of curcumin on the ISG15 disulfide bond formation was examined as reported previously [33]. Given that mouse ISG15 contains two cysteine residues at 76 and 143 positions, wild-type (WT) ISG15 and mutant ISG15(C76/143S), in which cysteine residues were mutated to serine, were used (Fig. 3A). FLAG-ISG15 (WT or C76/143S) was transiently expressed in HEK293T cells, followed by curcumin treatment for 1 d. The cell lysates containing FLAG-ISG15 (WT or C76/143S) were subjected to either non-reducing or reducing SDS-PAGE, followed by anti-FLAG IB. Disulfide bond-mediated ISG15 dimerization, which represented a minor fraction, was detected only in the non-reducing gel, and the cysteine mutation to serine almost completely prevented dimerization. We hypothesized that curcumin treatment would accelerate covalent ISG15 homodimerization, thereby reducing the levels of available monomer ISG15 required for ISGylation. However, curcumin treatment reduced covalent ISG15 homodimerization (Fig. 3A and B). This data suggests that curcumin treatment may increase monomer ISG15 levels by partially preventing disulfide bond formation.

3.4. Curcumin downregulated UBE1L-ISG15 formation

The downregulation of ISGylation by curcumin, despite the increase in level of monomer ISG15, was not understood (Fig. 3). ISGylation begins with the activation of ISG15 [43]. In short, a thioester bond is formed between UBE1L and ISG15, and ISG15 is activated [43]. Therefore, we examined the effect of curcumin on the thiol-ester bond formation between UBE1L and ISG15. UBE1L was expressed with or without FLAG-ISG15 in HEK293T cells, followed by curcumin treatment for 1 d. The cell lysates were subjected to reducing SDS-PAGE, followed by anti-UBE1L or FLAG IB (Fig. 4A). Additional two major and minor UBE1L bands (approximately 140 kDa) were observed when FLAG-ISG15 was co-expressed (Fig. 4A). Both bands were also detected using anti-FLAG antibody, suggesting that they were a UBE1L-ISG15 heterodimer covalently bound by thioester. Notably, UBE1L-ISG15 was downregulated by curcumin treatment, indicating that curcumin partly prevents the activation of ISG15 by UBE1L (Fig. 4A and B). Altogether, curcumin may downregulate ISGylation by partly preventing the activation of ISG15 by UBE1L.

4. Discussion

In total, 434 proteins have been identified to be ISGylated after *Listeria monocytogenes* infection [7]. Several ISGylated proteins have been individually analyzed, and ISGylation has been found to activate or inhibit few biological pathways, such as autophagy, bacterial infection, cancer progression, cytoskeleton dynamics, DNA translesion synthesis, exosome secretion, hypoxia and ischemic response, and viral infection [1]. However, whether ISGylation has a common effect on the substrate, as ubiquitylation usually destabilizes its substrate, has not been determined. It is necessary to analyze additional ISGylated substrates to collect basic data to understand the physiological role of ISGylation. In contrast, PLpro of SARS-CoV-2 has been shown to be able to remove ISG15 from substrates [25–29]. PLpro inhibitors have been sought after to overcome SARS-CoV-2 infection [44–48]. This indicates that relatively higher ISGylation may enhance the defense against SARS-CoV-2; however, the substrate that is ISGylated remains unknown. Therefore, strong ISGylation may indicate the ISGylation of key substrates. IFNs can regulate the ISG15 modification system,

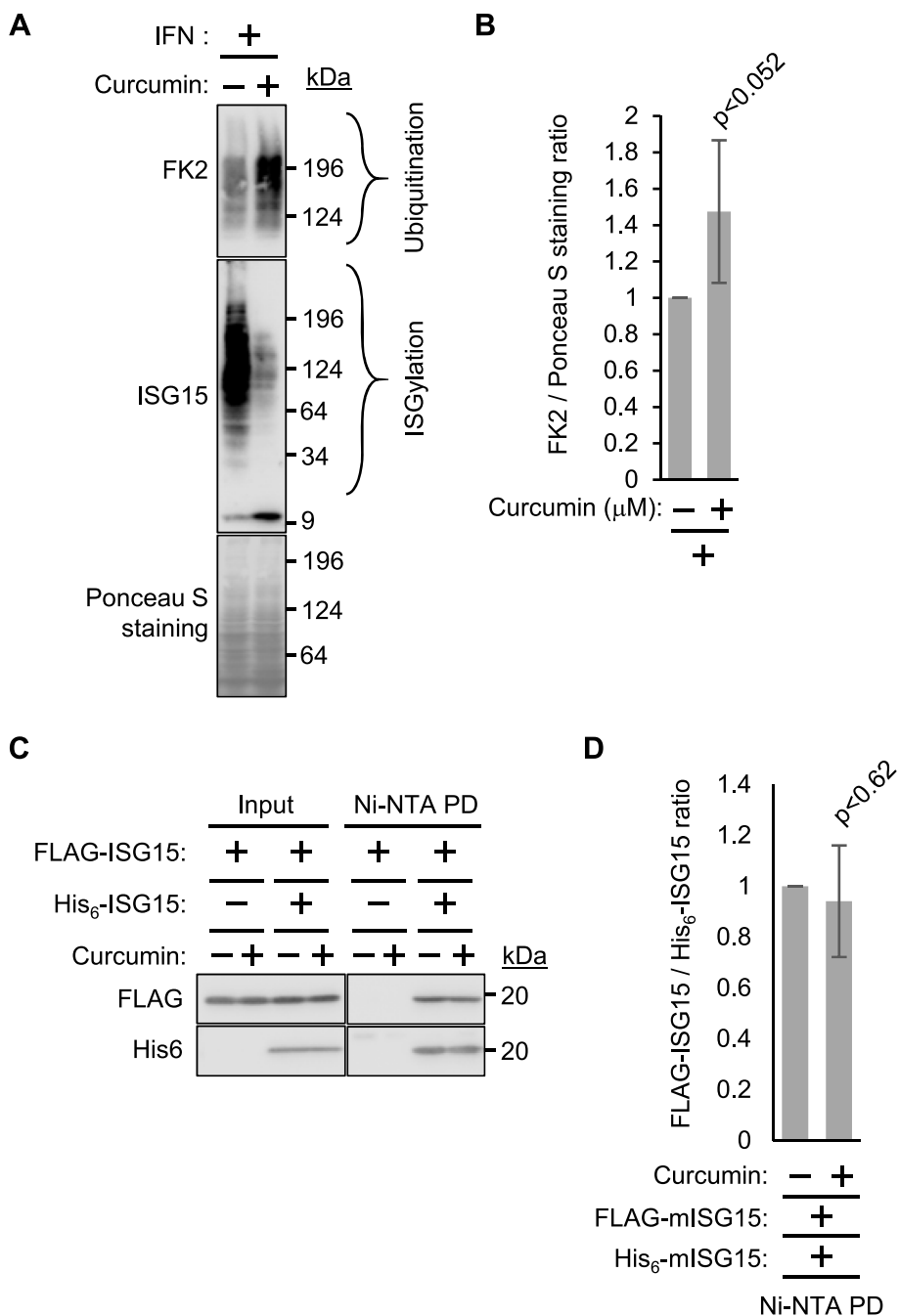


Fig. 2. No effect of curcumin on ubiquitylation and ISG15 homodimerization. (A) Curcumin downregulates ISGylation but not ubiquitylation. MCF10A cells were stimulated by human IFN α with or without curcumin (5 or 10 μ M) for 2 d. The cell lysates were subjected to immunoblotting with an anti-ISG15 or polyubiquitin antibody (FK2). Ponceau S staining was used as loading control. Representative data of three independent experiments. (B) Quantification of ubiquitylation and ISGylation in (A). The signal of ubiquitylation was normalized to that of Ponceau S staining. The signal intensity of cells without curcumin treatment was set as 1. Data are expressed as the mean \pm standard deviation of three independent experiments. Means of different groups were compared using Student's *t*-test. A *P* value < 0.05 was considered statistically significant. (C) No effect of curcumin on ISG15 homodimerization. HEK293T cells cultured in a 6 cm dish were transfected with plasmid encoding FLAG-ISG15 with or without His₆-ISG15 as indicated and cultured for 1 d. Next day, each sample was subcultured into two 6 cm dishes, followed by incubation for 1 d with or without 10 μ M curcumin. His₆-ISG15 was pulled-down using Ni-NTA agarose and immunoblotted using an anti-FLAG or His₆ antibody. Representative data of three independent experiments. (D) Quantification of FLAG-ISG15 bound to His₆-ISG15 in (C). The signal of FLAG-ISG15 was normalized to that of His₆-ISG15. The signal intensity of cells without curcumin treatment was set as 1. Data are expressed as the mean \pm standard deviation of three independent experiments. Means of different groups were compared using Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

but factors that enhance or decrease ISGylation have not been identified, except for nitrogen monoxide (NO) which enhances ISGylation by increasing monomeric ISG15 levels [33]. However, NO is relatively unstable and high levels of NO induces necrosis and apoptosis [49]. Moreover, neurons are sensitive to NO-induced excitotoxicity, which may cause neuronal death [49].

Initially, we examined several chemicals that are easy to ingest and are associated with less side effects. During the experiment, curcumin was unexpectedly found to reduce ISGylation. This is important information for the regulation of ISGylation. If ISGylation prevents SARS-CoV-2 amplification and/or propagation directly or indirectly, then the intake of excess curcumin should be avoided

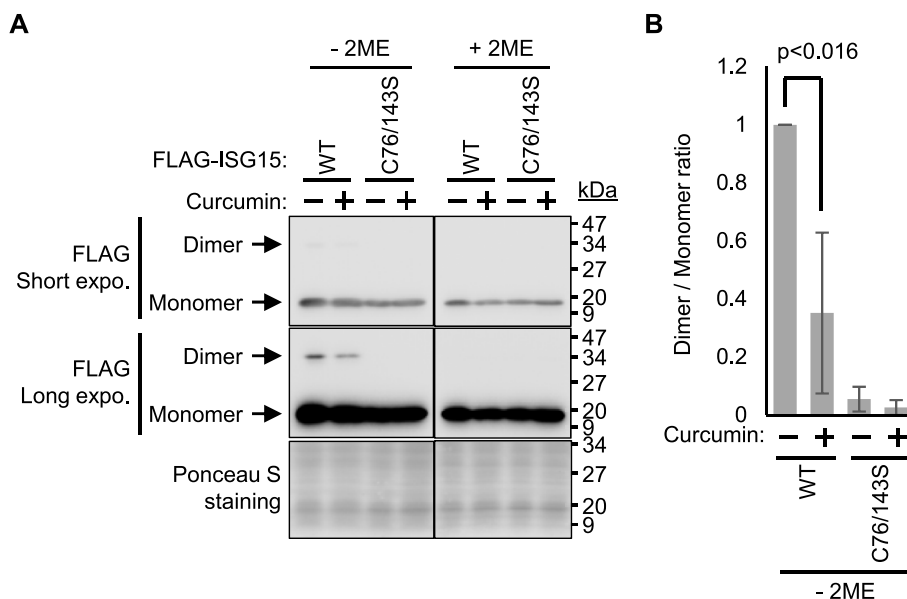


Fig. 3. Downregulation of disulfide bond-mediated ISG15 dimerization by curcumin. (A) HEK293T cells cultured in a 6 cm dish were transfected with plasmid encoding wild-type (WT) FLAG-ISG15 or mutant ISG15 in which cysteine residues at 76 and 143 positions were mutated to serine residues following culturing for 1 d. Next day, each sample was subcultured into two 6 cm dishes, followed by incubation for 1 d with or without 10 μM curcumin. The cell lysates were boiled in SDS-PAGE sample buffer with or without 2-mercaptoethanol and subjected to immunoblotting using an anti-FLAG antibody. Short and long exposures are shown to get appropriate signals. Ponceau S staining was used as loading control. Representative data of three independent experiments. (B) Quantification of dimerized FLAG-mouse ISG15 in (A). The signal of dimerized FLAG-ISG15 in the non-reducing gel was normalized to that of monomer ISG15. The signal intensity of ISG15 (WT) without curcumin treatment was set as 1. Data are expressed as the mean ± standard deviation of three independent experiments. Means of different groups were compared using Student's *t*-test. A P value < 0.05 was considered statistically significant.

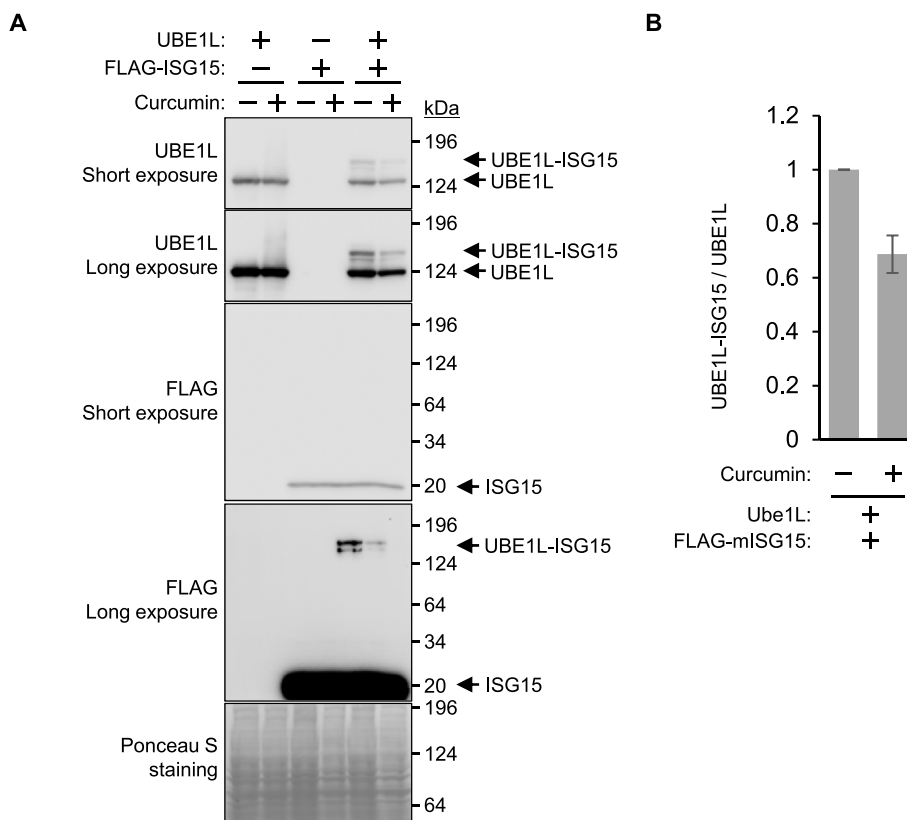


Fig. 4. Downregulation of ISG15 activation by UBE1L in the presence of curcumin. (A) HEK293T cells cultured in a 6 cm dish were transfected with plasmid encoding UBE1L and/or FLAG-ISG15 as indicated and cultured for 1 d. Next day, each sample was subcultured in two 6 cm dishes, followed by incubation for 1 d with or without 10 μM curcumin. The cell lysates were separated via SDS-PAGE under reducing condition and subjected to immunoblotting with an anti-UBE1L or FLAG antibody. Short and long exposure are shown to get appropriate signals. Free or ISG15-bound UBE1L are indicated by arrows. Ponceau S staining was used as loading control. Representative data of three independent experiments. (B) Quantification of ISG15-bound UBE1L in (A). The signal of ISG15-bound UBE1L was normalized to that of monomer UBE1L. The signal intensity of ISG15-bound UBE1L without curcumin treatment was set as 1. Data are expressed as the mean ± standard deviation of three independent experiments. Means of different groups were compared using Student's *t*-test. A P value < 0.05 was considered statistically significant.

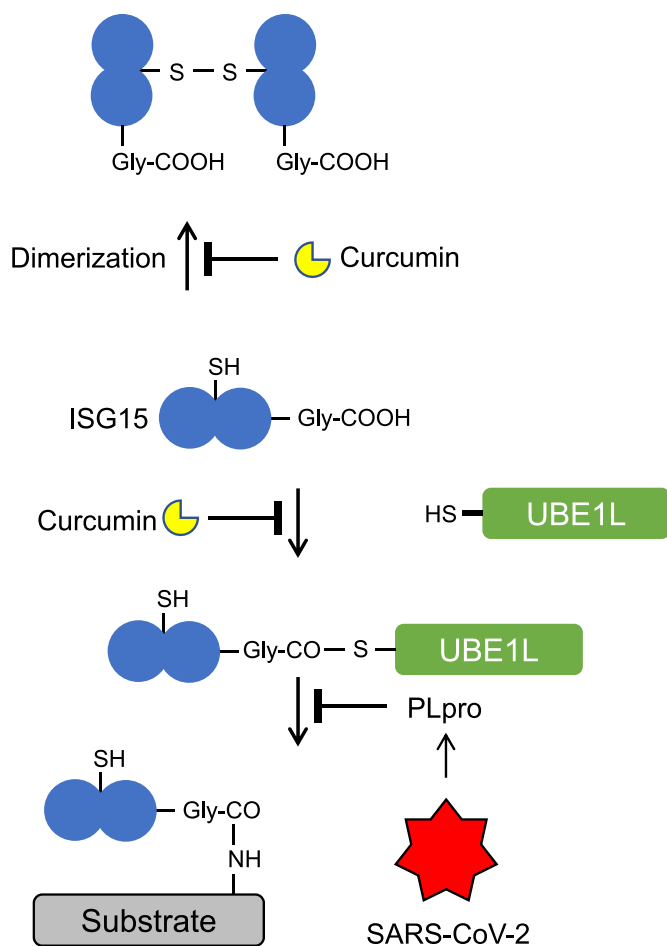


Fig. 5. Hypothetical model of the mechanism via which curcumin downregulates ISGylation. ISG15 forms homodimers via disulfide bond formation as reported previously. Curcumin may react with the thiol group of ISG15 directly or modify it indirectly, which results in the increase in level of monomer ISG15. ISG15 forms thioester bonds with UBE1L, which is the first step of ISGylation. Curcumin may also react with the thiol group of UBE1L directly or modify it indirectly, which results in the downregulation of ISG15 activation, thereby downregulating ISGylation. In contrast, SARS-CoV-2 PLpro prevents ISGylation by removing ISG15 from the substrate, which may accelerate virus propagation. ISG15, interferon-stimulated gene, 15 kDa; ISGylation, ISG15 modification; UBE1L, ubiquitin-activating enzyme E1-like protein; SARS-CoV-2 PLpro, severe acute respiratory syndrome coronavirus 2-derived papain-like protease.

which may downregulate ISGylation, although curcumin has antiviral activity under certain conditions [30].

Altogether, it is suggested that curcumin partly prevents disulfide bond-mediated ISG15 dimerization directly or indirectly, thereby increasing monomer ISG15 levels (Figs. 3 and 5). The C-terminal carboxyl group of ISG15 is activated by UBE1L in an ATP-dependent manner. Consequently, a thiol-ester bond is formed between UBE1L and monomer ISG15, and ISG15 is activated [43,50]. Active ISG15 is then transferred to UBE2L6 and further transferred to a substrate protein, which is recognized by E3 enzymes [43]. Curcumin partly prevents UBE1L-ISG15 thioester bond formation probably by blocking the thiol group directly or indirectly (Figs. 4 and 5). Therefore, curcumin treatment reduces ISGylation. It is unclear whether ISGylation has anti-SARS-CoV-2 activity; however, ISG15-deficient mice are relatively more susceptible to influenza A/WSN/33 and influenza B/Lee/40 virus infections [51]. These mice also exhibit increased susceptibility to herpes simplex virus type 1, murine gammaherpesvirus 68, and Sindbis virus infections [51]. The increased susceptibility of ISG15-

deficient mice to Sindbis virus infection is rescued by expressing WT ISG15 and not a mutant form of ISG15 that cannot form conjugates [51], which suggests that ISGylation is important in the resistance against Sindbis virus infection. The amount of curcumin required for the most effective anti-virus activity remains unclear.

This study had a few limitations. Since anti-curcumin antibodies are unavailable, it is unclear whether curcumin directly binds to the thiol group of ISG15. However, curcumin has been shown to inhibit protein and DNA oxidation [30,52,53], suggesting that curcumin may prevent oxidation of ISG15 and UBE1L thiol group indirectly. We showed that treatment with at least 10 μ M curcumin partly prevents ISGylation in two cell lines MCF10A and A549 (Fig. 1). However, the amount of curcumin required to obtain benefits without side effects, such as reduced ISGylation, remains unknown. Since human diet contains various nutrients, it is difficult to determine the exact effect of other anti-oxidant compounds on curcumin activity. It is also unknown whether long-term or short-term intake of curcumin affects its impact on ISGylation *in vivo*. Although SARS-CoV-2 PLpro inhibits ISGylation, the key substrate which is ISGylated remains unknown, and the role of PLpro in virus propagation has not been studied.

Author contributions

Conceptualization, N.O., and F.O.; Methodology, Validation, Formal Analysis, and Investigation, N.O., S.Y., T.T., H.F., and F.O.; Data Curation, N.O., S.Y., T.T., H.F., S.H., and F.O.; Writing – Original Draft, F.O.; Writing – Review & Editing, N.O., S.Y., T.T., H.F., S.H., and F.O.; Supervision, F.O.; Project Administration, F.O.; Funding Acquisition, F.O.

Data availability

No datasets were generated during the current study.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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References

- [1] C. Villarroya-Beltri, S. Guerra, F. Sanchez-Madrid, ISGylation – a key to lock the cell gates for preventing the spread of threats, *J. Cell Sci.* 130 (2017) 2961–2969.
- [2] T. Kaisho, S. Akira, Toll-like receptor function and signaling, *J. Allergy Clin. Immunol.* 117 (2006) 979–987, quiz 988.
- [3] D. Jimenez Fernandez, S. Hess, K.P. Knobloch, Strategies to target ISG15 and USP18 toward therapeutic applications, *Front. Chem.* 7 (2019) 923.
- [4] Y.C. Perng, D.J. Lenschow, ISG15 in antiviral immunity and beyond, *Nat. Rev. Microbiol.* 16 (2018) 423–439.
- [5] D. Bogunovic, S. Boisson-Dupuis, J.L. Casanova, ISG15: leading a double life as a secreted molecule, *Exp. Mol. Med.* 45 (2013) e18.
- [6] P.J. Farrell, R.J. Broeze, P. Lengyel, Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells, *Nature* 279 (1979) 523–525.
- [7] Y. Zhang, F. Thery, N.C. Wu, E.K. Luhmann, O. Dussurget, M. Foecke, C. Bredow,

- D. Jimenez-Fernandez, K. Leandro, A. Beling, K.P. Knobloch, F. Impens, P. Cossart, L. Radoshevich, The in vivo ISGylome links ISG15 to metabolic pathways and autophagy upon *Listeria monocytogenes* infection, *Nat. Commun.* 10 (2019) 5383.
- [8] C.M. Pickart, Back to the future with ubiquitin, *Cell* 116 (2004) 181–190.
- [9] K. Kok, R. Hofstra, A. Pilz, A. van den Berg, P. Terpstra, C.H. Buys, B. Carritt, A gene in the chromosomal region 3p21 with greatly reduced expression in lung cancer is similar to the gene for ubiquitin-activating enzyme, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 6071–6075.
- [10] C. Zhao, S.L. Beaudenon, M.L. Kelley, M.B. Waddell, W. Yuan, B.A. Schulman, J.M. Huijbregtse, R.M. Krug, The Ubch8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN- α /beta-induced ubiquitin-like protein, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 7578–7582.
- [11] K.I. Kim, N.V. Giannakopoulos, H.W. Virgin, D.E. Zhang, Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation, *Mol. Cell Biol.* 24 (2004) 9592–9600.
- [12] A. Dastur, S. Beaudenon, M. Kelley, R.M. Krug, J.M. Huijbregtse, Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells, *J. Biol. Chem.* 281 (2006) 4334–4338.
- [13] W. Zou, D.E. Zhang, The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase, *J. Biol. Chem.* 281 (2006) 3989–3994.
- [14] F. Okumura, W. Zou, D.E. Zhang, ISG15 modification of the eIF4E cognate 4EHP enhances cap structure-binding activity of 4EHP, *Genes Dev.* 21 (2007) 255–260.
- [15] L. Ketscher, A. Basters, M. Prinz, K.P. Knobloch, mHERC6 is the essential ISG15 E3 ligase in the murine system, *Biochem. Biophys. Res. Commun.* 417 (2012) 135–140.
- [16] K.R. Loeb, A.L. Haas, The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins, *J. Biol. Chem.* 267 (1992) 7806–7813.
- [17] L.A. Durfee, N. Lyon, K. Seo, J.M. Huijbregtse, The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15, *Mol. Cell* 38 (2010) 722–732.
- [18] A. Garcia-Sastre, C.A. Biron, Type 1 interferons and the virus-host relationship: a lesson in detente, *Science* 312 (2006) 879–882.
- [19] T. Kawai, S. Akira, Innate immune recognition of viral infection, *Nat. Immunol.* 7 (2006) 131–137.
- [20] N. Honke, N. Shaabani, D.E. Zhang, C. Hardt, K.S. Lang, Multiple functions of USP18, *Cell Death Dis.* 7 (2016) e2444.
- [21] L.Q. Liu, R. Ilaria Jr., P.D. Kingsley, A. Iwama, R.A. van Etten, J. Palis, D.E. Zhang, A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation, *Mol. Cell Biol.* 19 (1999) 3029–3038.
- [22] M.P. Malakhov, O.A. Malakhova, K.I. Kim, K.J. Ritchie, D.E. Zhang, UBP43 (USP18) specifically removes ISG15 from conjugated proteins, *J. Biol. Chem.* 277 (2002) 9976–9981.
- [23] H. Schwer, L.Q. Liu, L. Zhou, M.T. Little, Z. Pan, C.J. Hetherington, D.E. Zhang, Cloning and characterization of a novel human ubiquitin-specific protease, a homologue of murine UBP43 (Usp18), *Genomics* 65 (2000) 44–52.
- [24] G. Eriani, F. Martin, Viral and Cellular Translation during SARS-CoV-2 Infection, *FEBS Open Bio*, 2022.
- [25] B.T. Freitas, I.A. Durie, J. Murray, J.E. Longo, H.C. Miller, D. Crich, R.J. Hogan, R.A. Tripp, S.D. Pegan, Characterization and noncovalent inhibition of the deubiquitinase and deISGylase activity of SARS-CoV-2 papain-like protease, *ACS Infect. Dis.* 6 (2020) 2099–2109.
- [26] V. Clemente, P. D'Arcy, M. Bazzaro, Deubiquitinating enzymes in coronaviruses and possible therapeutic opportunities for COVID-19, *Int. J. Mol. Sci.* 21 (2020).
- [27] J.R. Clasman, R.K. Everett, K. Srinivasan, A.D. Mesecar, Decoupling deISGylating and deubiquitinating activities of the MERS virus papain-like protease, *Antivir. Res.* 174 (2020), 104661.
- [28] T. Klemm, G. Ebert, D.J. Calleja, C.C. Allison, L.W. Richardson, J.P. Bernardini, B.G. Lu, N.W. Kuchel, C. Grohmann, Y. Shibata, Z.Y. Gan, J.P. Cooney, M. Doerflinger, A.E. Au, T.R. Blackmore, G.J. van der Heden van Noort, P.P. Geurink, H. Ovaa, J. Newman, A. Riboldi-Tunnicliffe, P.E. Czabotar, J.P. Mitchell, R. Feltham, B.C. Lechtenberg, K.N. Lowes, G. Dewson, M. Pellegrini, G. Lessene, D. Komander, Mechanism and inhibition of the papain-like protease, PLpro, of SARS-CoV-2, *EMBO J.* 39 (2020), e106275.
- [29] D. Shin, R. Mukherjee, D. Grewé, D. Bojkova, K. Baek, A. Bhattacharya, L. Schulz, M. Widera, A.R. Mehdipour, G. Tascher, P.P. Geurink, A. Wilhelm, G.J. van der Heden van Noort, H. Ovaa, S. Müller, K.P. Knobloch, K. Rajalingam, B.A. Schulman, J. Cinatl, G. Hummer, S. Ciesek, I. Dikic, Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity, *Nature* 587 (2020) 657–662.
- [30] G. Kuttan, K.B. Kumar, C. Guruvayoorappan, R. Kuttan, Antitumor, anti-invasion, and antimetastatic effects of curcumin, *Adv. Exp. Med. Biol.* 595 (2007) 173–184.
- [31] P.B. Luis, W.E. Boeglin, C. Schneider, Thiol reactivity of curcumin and its oxidation products, *Chem. Res. Toxicol.* 31 (2018) 269–276.
- [32] N. Jurrmann, R. Brigelius-Flohe, G.F. Bol, Curcumin blocks interleukin-1 (IL-1) signaling by inhibiting the recruitment of the IL-1 receptor-associated kinase IRAK in murine thymoma EL-4 cells, *J. Nutr.* 135 (2005) 1859–1864.
- [33] F. Okumura, D.J. Lenschow, D.E. Zhang, Nitrosylation of ISG15 prevents the disulfide bond-mediated dimerization of ISG15 and contributes to effective ISGylation, *J. Biol. Chem.* 283 (2008) 24484–24488.
- [34] M. Liu, X.L. Li, B.A. Hassel, Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15, *J. Biol. Chem.* 278 (2003) 1594–1602.
- [35] J.J. Wong, Y.F. Pung, N.S. Sze, K.C. Chin, HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 10735–10740.
- [36] X.L. Li, J.A. Blackford, C.S. Judge, M. Liu, W. Xiao, D.V. Kalvakolanu, B.A. Hassel, RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response, *J. Biol. Chem.* 275 (2000) 8880–8888.
- [37] R. Gonzalez-Sanz, M. Mata, J. Bermejo-Martín, A. Alvarez, J. Cortijo, J.A. Melero, I. Martínez, ISG15 is upregulated in respiratory syncytial virus infection and reduces virus growth through protein ISGylation, *J. Virol.* 90 (2016) 3428–3438.
- [38] X. Huang, V.M. Dixit, Drugging the undruggables: exploring the ubiquitin system for drug development, *Cell Res.* 26 (2016) 484–498.
- [39] X. Si, Y. Wang, J. Wong, J. Zhang, B.M. McManus, H. Luo, Dysregulation of the ubiquitin-proteasome system by curcumin suppresses coxsackievirus B3 replication, *J. Virol.* 81 (2007) 3142–3150.
- [40] Y. Jung, W. Xu, H. Kim, N. Ha, L. Neckers, Curcumin-induced degradation of ErbB2: a role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, *Biochim. Biophys. Acta* 1773 (2007) 383–390.
- [41] N.R. Jana, P. Dikshit, A. Goswami, N. Nukina, Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway, *J. Biol. Chem.* 279 (2004) 11680–11685.
- [42] T. Ono, S. Takada, S. Kinugawa, H. Tsutsui, Curcumin ameliorates skeletal muscle atrophy in type 1 diabetic mice by inhibiting protein ubiquitination, *Exp. Physiol.* 100 (2015) 1052–1063.
- [43] C.T. Dao, D.E. Zhang, ISG15: a ubiquitin-like enigma, *Front. Biosci.* 10 (2005) 2701–2722.
- [44] S.A. Amin, S. Banerjee, K. Ghosh, S. Gayen, T. Jha, Protease targeted COVID-19 drug discovery and its challenges: insight into viral main protease (Mpro) and papain-like protease (PLpro) inhibitors, *Bioorg. Med. Chem.* 29 (2021), 115860.
- [45] C. Mouffouk, S. Mouffouk, S. Mouffouk, L. Hambaba, H. Haba, Flavonols as potential antiviral drugs targeting SARS-CoV-2 proteases (3CL(pro) and PL(pro)), spike protein, RNA-dependent RNA polymerase (RdRp) and angiotensin-converting enzyme II receptor (ACE2), *Eur. J. Pharmacol.* 891 (2021), 173759.
- [46] Z. Fu, B. Huang, J. Tang, S. Liu, M. Liu, Y. Ye, Z. Liu, Y. Xiong, W. Zhu, D. Cao, J. Li, X. Niu, H. Zhou, Y.J. Zhao, G. Zhang, H. Huang, The complex structure of GRL0617 and SARS-CoV-2 PLpro reveals a hot spot for antiviral drug discovery, *Nat. Commun.* 12 (2021) 488.
- [47] Y. Zhao, X. Du, Y. Duan, X. Pan, Y. Sun, T. You, L. Han, Z. Jin, W. Shang, J. Yu, H. Guo, Q. Liu, Y. Wu, C. Peng, J. Wang, C. Zhu, X. Yang, K. Yang, Y. Lei, L.W. Guddat, W. Xu, G. Xiao, L. Sun, L. Zhang, Z. Rao, H. Yang, High-throughput screening identifies established drugs as SARS-CoV-2 PLpro inhibitors, *Protein Cell* 12 (2021) 877–888.
- [48] F. Yan, F. Gao, An overview of potential inhibitors targeting non-structural proteins 3 (PL(pro) and Mac1) and 5 (3CL(pro)/M(pro)) of SARS-CoV-2, *Comput. Struct. Biotechnol. J.* 19 (2021) 4868–4883.
- [49] G.C. Brown, Nitric oxide and neuronal death, *Nitric Oxide* 23 (2010) 153–165.
- [50] L.A. Durfee, M.L. Kelley, J.M. Huijbregtse, The basis for selective E1-E2 interactions in the ISG15 conjugation system, *J. Biol. Chem.* 283 (2008) 23895–23902.
- [51] D.J. Lenschow, C. Lai, N. Frias-Staheli, N.V. Giannakopoulos, A. Lutz, T. Wolff, A. Osiak, B. Levine, R.E. Schmidt, A. Garcia-Sastre, D.A. Leib, A. Pekosz, K.P. Knobloch, I. Horak, H.W.t. Virgin, IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 1371–1376.
- [52] Q.Y. Wei, W.F. Chen, B. Zhou, L. Yang, Z.L. Liu, Inhibition of lipid peroxidation and protein oxidation in rat liver mitochondria by curcumin and its analogues, *Biochim. Biophys. Acta* 1760 (2006) 70–77.
- [53] S.K. Borra, J. Mahendra, P. Gurumurthy, Jayamathi, S.S. Iqbal, L. Mahendra, Effect of curcumin against oxidation of biomolecules by hydroxyl radicals, *J. Clin. Diagn. Res.* 8 (2014), CC01-05.