



# Molecular mechanisms of thalidomide effectiveness on COVID-19 patients explained: ACE2 is a new $\Delta$ Np63 $\alpha$ target gene

Laura Monteonofrio<sup>1</sup> · Ilaria Virdia<sup>1</sup> · Sara Pozzi<sup>2</sup> · Roberto Quadri<sup>2</sup> · Alessandra Amendolare<sup>3</sup> · Flaviana Marzano<sup>3</sup> · Micaela Braile<sup>2</sup> · Virginia Sulfaro<sup>2</sup> · Moira Paroni<sup>2</sup> · Apollonia Tullo<sup>3</sup> · Silvia Soddu<sup>1</sup> · Luisa Guerrini<sup>2</sup>

Received: 26 March 2024 / Revised: 31 August 2024 / Accepted: 4 September 2024 / Published online: 18 September 2024  
© The Author(s) 2024

## Abstract

COVID-19 pandemic is caused by the SARS-CoV-2 virus, whose internalization and infection are mediated by the angiotensin-converting enzyme 2 (ACE2). The identification of novel approaches to tackle this step is instrumental for the development of therapies for the management of COVID-19 and other diseases with a similar mechanism of infection. Thalidomide, a drug sadly known for its teratogenic effects, has potent immunomodulatory and anti-inflammatory properties. Treatment with this drug has been shown to improve the immune functions of COVID-19 patients and proposed for the management of COVID-19 in clinical practice through drug repositioning. Here, we investigated the molecular details linking thalidomide to ACE2 and COVID-19, showing that in conditions mimicking SARS-CoV-2-associated cytokine storm, the transcription factor  $\Delta$ Np63 $\alpha$  and ACE2 are stabilized, and IL-8 production is increased. In such conditions, we found p63 to bind to and regulate the expression of the *ACE2* gene. We previously showed that  $\Delta$ Np63 $\alpha$  is degraded upon thalidomide treatment and now found that treatment with this drug—or with its analogue lenalidomide—downregulates ACE2 in a p63-dependent manner. Finally, we found that thalidomide treatment reduces in vitro infection by pseudo-SARS-CoV-2, a baculovirus pseudotyped with the SARS-CoV-2 spike protein. Overall, we propose the dual effect of thalidomide in reducing SARS-CoV-2 viral re-entry and inflammation through p63 degradation to weaken SARS-CoV-2 entry into host cells and mitigate lung inflammation, making it a valuable option in clinical management of COVID-19.

**Key messages** • Thalidomide treatment results in p63-dependent ACE2 downregulation.

- ACE2 is a p63 transcriptional target.
- Thalidomide reduces the “cytokine storm” associated to COVID-19.
- Thalidomide prevents viral re-entry of SARS-CoV-2 by p63-dependent ACE2 downregulation.
- Thalidomide is a modulator of SARS-CoV-2 or other ACE2-dependent infections.
- ACE2 is modulated by a pharmacological substance.

**Keywords** Thalidomide · SARS-CoV-2 · ACE2 ·  $\Delta$ Np63 $\alpha$  · COVID 19

Laura Monteonofrio, Ilaria Virdia and Sara Pozzi contributed equally to this work.

Silvia Soddu and Luisa Guerrini equally supervised this work.

✉ Luisa Guerrini  
luisa.guerrini@unimi.it

<sup>1</sup> Dipartimento Di Ricerca E Tecnologie Avanzate, Istituto Nazionale Tumori Regina Elena IRCCS, 00144 Rome, Italy

<sup>2</sup> Dipartimento Di Bioscienze, Università Degli Studi Di Milano, Via Celoria 26, 20133 Milan, Italy

<sup>3</sup> Istituto Di Biomembrane, Bioenergetica E Biotecnologie Molecolari (IBIOM), Consiglio Nazionale Delle Ricerche, 70025 Bari, Italy

## Introduction

SARS-CoV-2 infection, mediated by the binding of the viral spike (S) protein to the ACE2 receptor in the host cells [1–3], causes the COVID-19 disease. One of the associated complications is a cytokine storm which involves an uncontrolled release of cytokines and chemokines, leading to a systemic inflammatory response linked to lung failure and acute respiratory distress syndrome. Given the time and cost involved in developing new therapies, repurposing existing drugs with known profiles has become a cost-effective strategy [4]. Therefore, reassessing the efficacy of licensed and

experimental drugs has been a go-on choice by the World Health Organization (WHO) and other health agencies to treat emerging health problems. With the initial lack of vaccines and effective agents against SARS-CoV-2, as well as public health emergencies, WHO had identified promising repositioning therapies, such as the combination of two anti-HIV drugs (i.e., lopinavir and ritonavir) or the experimental antiviral compound, remdesivir [5]. Furthermore, thalidomide (Thal), a small molecule drug with many years of history known to cause misery, became a game changer for its multifaceted pharmacological effects such as immunomodulation, anti-inflammation, anti-angiogenesis, and antiviral effects [6, 7]. Several Thal derivatives have been designed, known as immunomodulatory drugs (IMiDs), among which the most used are lenalidomide (Len) and pomalidomide, that are approved by FDA for the treatment of several severe diseases like multiple myeloma, myelodysplastic syndrome (5q-), mantle cell lymphoma, and follicular lymphoma [8]. IMiDs retain the anti-angiogenic and anti-inflammatory properties of Thal but are devoid of Thal teratogenic activity [6, 8].

Extensive information available on Thal's mechanisms of action and its efficacy and safety in hemophagocytic syndrome-induced cytokine storm, idiopathic pulmonary fibrosis (IPF), or severe H1N1 and paraquat poisoning lung injury argues for the possible action of Thal on COVID-19-induced lung effects and cytokine storm [9–12]. Recent reviews on the COVID-19 treatment endorse the possibility of usage of Thal and its analogues for treating COVID-19 patients. Thal, sadly known for its teratogenic action [13], has been shown to have potent immunomodulatory/anti-inflammatory activities [14], to prevent the development of bleomycin-induced pulmonary fibrosis in mice by blocking the TGF- $\beta$ 1 pathway [9], and to improve respiratory symptoms and life quality in patients with IPF [10, 11] or H1N1 infection [12]. Based on these observations, a repositioning study with Thal, in COVID-19 patients, has been performed in China with positive results [15]. Moreover, Len at very high doses has been shown to induce ACE2 downmodulation through a post-translational mechanism [16].

We have long studied the molecular basis of Thal's teratogenic effects, specifically its role in degrading  $\Delta$ Np63 $\alpha$  and TAp63 $\alpha$  proteins through the ubiquitin ligase CRL4 cereblon (CRBN) [17]. The p63 proteins are members of the p53 family of transcription factors and consist of ten isoforms with distinct and important biological roles during development [18, 19]. In epithelial cells, including lung epithelial cells, the  $\Delta$ Np63 $\alpha$  isoform is the most prominent one in regulating proliferation, apoptosis, and differentiation [20]. In addition,  $\Delta$ Np63 $\alpha$  regulates the transcription of several cytokines, such as IL-1 $\alpha$  [21], IL-31, IL-33 [22], and IFN- $\gamma$  [23], and is expressed in lung epithelium [24].

Based on these observations, we reasoned that the beneficial effects upon Thal treatment observed in COVID-19 patients might be related to  $\Delta$ Np63 $\alpha$  degradation with subsequent modulation of its transcriptional targets. These targets would include already-known factors, such as inflammatory cytokines and novel targets such as the SARS-CoV-2 entry receptor ACE2.

## Materials and methods

### Cell culture, treatments, and transfection

A431 (human epidermoid cell line; ATCC# CCL-1555) and A549 (human lung carcinoma cell line; ATCC# CCL-185) were kindly provided by Dr. Ada Sacchi (Istituto Nazionale Tumori Regina Elena, Rome, Italy), H1299 (human lung carcinoma cell line; ATCC# CRL-5803) was kindly provided by Dr. Giovanni Blandino (Istituto Nazionale Tumori Regina Elena, Rome, Italy), U-2 OS (human osteosarcoma cell line; ATCC# HTB-96) was kindly provided by Prof. Francesco Blasi (Università degli Studi di Milano, Milan, Italy), and HaCaT (human keratinocyte cell line) was kindly provided by Prof. Antonio Costanzo (Humanitas Research Hospital, Milan, Italy). All cells were maintained in DMEM enriched with 10% fetal bovine serum (Euroclone #ECS0165L), 1 mM L-glutamine (Euroclone #ECB3000D), 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin (Euroclone #ECB3001D) at 37 °C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub>. Cells were maintained in culture for no more than ten passages and underwent routine testing to ensure that they are mycoplasma-free. For treatments with thalidomide (Tocris #50–35-1) and lenalidomide (LGC products #191,732–72-6),  $5 \times 10^4$  cells were seeded onto 24-well multi-plates and 20 h later incubated with different drug concentrations and times indicated in the figure legends. For transient transfection,  $5 \times 10^4$  A431 cells were plated in 24-well multi-plates and on the next day transfected with Lipofectamine 2000 (Invitrogen #11,668,019) with increasing amount of CEREBLON (CRBN) encoding plasmid or with four different p63-small hairpin RNA (sh-p63) vectors with sequence homology to four different regions of p63 mRNA (OriGene Technologies # TF308688); shRNA-SCRAMBLED (sh-SCRB) was used as a control. For stable transfection of A431 cells, shp63#3/shp63#4 and shSCRB plasmids were transfected with Lipofectamine 2000 (Invitrogen #11668019), and after 2 days, 1  $\mu$ g/mL puromycin was added to select stable transfectants. The cells were maintained as polyclonal populations under puromycin at 0.25  $\mu$ g/mL. For U-2 OS,  $5 \times 10^4$  cells were plated in 24-well multi-plates and the next day transfected with Lipofectamine 2000 with increasing amount of  $\Delta$ Np63 $\alpha$  encoding plasmid.

## Western blot (WB) analysis

At the indicated times, cells were lysed in 100  $\mu$ L of Loading Buffer 2X (2% sodium dodecyl sulfate, 30% glycerol, 144 mM  $\beta$ -mercaptoethanol, 100 mM Tris–HCl pH 6.8, and 0.1% bromophenol blue). Samples were incubated at 98 °C for 10 min and resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Amersham #GEH10600001). The blots were incubated with the following antibodies (Abs): anti-p63 4A4 (Santa Cruz Biotechnology sc-8431), anti-ACE2 (Abcam ab15348), anti-CRBN (Cell Signaling Technology D8H3S), and anti-actin (Santa Cruz Biotechnology sc-8432). The following secondary Abs were used: goat anti-mouse IgG-HRP (Santa Cruz Biotechnology sc-2005) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology sc-2030). Proteins were visualized by an enhanced chemiluminescence method (GeneSpin #STSE500) according to the manufacturer's instructions using a ChemiDoc Touch (BioRad).

## Plasmids

The plasmids carrying  $\Delta$ Np63 $\alpha$ , CRBN, or shCRBN were previously described [17].

## RNA purification, reverse transcriptase RT-PCR, and quantitative real-time qPCR analyses

Total mRNA from cells was isolated using the RNeasy mini-Kit (Qiagen #74,104). cDNA was synthesized by M-MLV RTase and amplified with GoTaq DNA polymerase (Promega #M3001). For quantitative PCR analysis, mRNA expression level was evaluated using the Power SYBR Green PCR Master Mix with ABI Prism 7500HT Fast Real-Time PCR System Detector (Applied Biosystems). Relative mRNA expression levels were determined by using the  $2^{-\Delta\Delta CT}$  method, employing GAPDH gene expression for data normalization. All reactions were performed in triplicate. Primer sequences are as follows:

ACE2 forward 5'-CAT TGG AGC AAG TGT TGG ATC TT-3';

ACE2 reverse 5'-GAG CTA ATG CAT GCC ATT CTC A-3';

GAPDH forward 5'-TCC CTG AGC TGA ACG GGA AG-3';

GAPDH reverse 5'-GGA GGA GTG GGT GTC GCT GT-3'.

## ELISA assay

HaCaT cells,  $1.8 \times 10^5$ , were plated in 35-mm culture dishes and treated with recombinant human TNF- $\alpha$  (rhTNF- $\alpha$ ;

BioLegend # BMS301) 5 ng/mL for 7 or 17 h or with 0.5  $\mu$ g/mL LPS from *E. coli* (SIGMA #O111:B4) for 4, 6, or 17 h. Quantification of IL-8 in the supernatants of treated HaCaT cells was performed by ELISA (hIL-8; ImmunoTools #31,670,089) according to the manufacturer's instructions. The ELISA plates were read by a microplate reader (SAFAS MP96).

## Chromatin immunoprecipitation (ChIP)

A431 cells,  $4 \times 10^6$ , were plated in 15-cm culture dishes and treated with LPS 0.5  $\mu$ g/mL for 2 h. Then, proteins were cross-linked to DNA in living nuclei and the ChIP assay was performed using the MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher #492,024), as described by the manufacturer. The following quantities of Abs were used to immunoprecipitate the DNA–protein complexes: 2  $\mu$ g of anti-p63 $\alpha$  (D2K8X) XP® Rabbit mAb (Cell Signaling Technology #131,095), 2  $\mu$ g of anti-acetylated H4-histone Ab (BioRad #AHP148), and 1  $\mu$ g of an unrelated, negative control Ab. DNA fragments obtained by the ChIP assay were analyzed by qPCR using specific primers spanning the responsive elements (RE) found in the first intron of the *ACE2* gene. Primers specific for exon 8 of the *ACE2* gene were used as the negative control. As a positive control for PCR and as normalizer, DNA prepared from samples prior to immunoprecipitation (whole cell lysates) was used as total or input DNA. Primer sequences are as follows:

First intron of the *ACE2* gene (+ 1067; + 1087):

Forward: 5'-ACGACGCGTGTGGAGAAGTCCATC AGA-3';

Reverse: 5'-ACGAGATCTGGTCAACCACACATA CCA-3';

Exon 8 *ACE2* gene (+ 19,634; + 19,730):

Forward: 5'-GGGATGCACAGAGAATATTCAAGG-3';

Reverse: 5'-AGACTGCTTTCTGAACATTTCCTG-3'.

## In vitro SARS-CoV-2 spike protein pseudovirus infection

A baculovirus expressing the green fluorescent protein (GFP) and pseudotyped with SARS-CoV-2 spike protein (Montana Molecular #C1110G) was employed to evaluate its entry into cells through ACE2 receptor. To assess Thal-induced effect,  $5 \times 10^3$  A431 cells were plated in triplicates in 96-well plates. Thal was added 17 h post cell seeding; after 24 h of treatments, 50  $\mu$ L of pseudo-SARS-CoV-2 suspension (viral titer:  $2 \times 10^{10}$  viral genes (VG) per mL) was added to each well following the manufacturer's instructions. At 30 h post-infection, cells were washed with PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature, incubated for 10 min with the reagent containing 4,6-diamidino-2-phenylindole (DAPI), and washed again

with PBS. Images were acquired using a Nikon CSU-W1 microscope in widefield mode, using a  $\times 20$  objective and analyzed with FIJI [25].

## FACS analysis

For FACS analysis, shp63 and shSCRB stable transfectants were plated and infected with the SARS-CoV-2 spike protein pseudovirus as above, and after 24 h, the FACS analysis was performed by a BD (Franklin Lakes, NJ, USA) FACSCanto II flow cytometer. Data were analyzed with FlowJo software (version 10.4.2; BD, Franklin Lakes, NJ, USA).

## Results

### Thalidomide and lenalidomide at clinically relevant concentrations reduce ACE2 expression

We first investigated whether Thal or Len could modulate the expression of ACE2 receptor in association with  $\Delta$ Np63 $\alpha$  degradation in human cells, such as HaCaT keratinocytes, A431 epidermoid cells, and A549 lung carcinoma cells, which express both  $\Delta$ Np63 $\alpha$  and ACE2 proteins. Cells were treated for 24 h with 10 or 100  $\mu$ M Thal or 1 or 5  $\mu$ M Len. As expected [17],  $\Delta$ Np63 $\alpha$  protein was degraded by the pharmacological treatments, with the ACE2 levels decreasing with the reduction of  $\Delta$ Np63 $\alpha$  levels in the three cell lines (Fig. 1A). We then verified whether Thal or Len could modulate ACE2 expression in p63-null cells in a dose-dependent manner, since it has been reported that Len at high concentrations induces ACE2 downmodulation by a post-translational mechanism [16]. At the highest concentrations of Thal, we did not observe ACE2 downmodulation in the p63-null U-2 OS human osteosarcoma cells. In contrast, the treatment with Len at the high concentrations reported to modulate ACE2 expression [16] (i.e., 80–100  $\mu$ M) resulted to be toxic, as it can be inferred by the concomitant reduction of the actin levels (Fig. 1B). Next, we assessed whether modulation of ACE2 in response to Thal or Len treatment is regulated also at the transcription level. HaCaT, A431, and A549 cells were treated for 24 h with 100  $\mu$ M Thal or 5  $\mu$ M Len and ACE2 mRNA levels measured by qRT-PCR. Human lung carcinoma H1299 cells that do not express any of the p63 isoforms were used as further negative control. In  $\Delta$ Np63 $\alpha$  proficient cells,  $\Delta$ Np63 $\alpha$  protein was degraded by both treatments with a concomitant decrease of ACE2 mRNA levels, whereas we did not observe ACE2 mRNA decrease in the p63-null cells (Fig. 1C).

Taken together, these results suggest that ACE2 could be a  $\Delta$ Np63 $\alpha$  target gene with Thal or Len leading to CRBN-mediated  $\Delta$ Np63 $\alpha$  degradation (CRBN is part of the E3 ubiquitin ligase complex that targets  $\Delta$ Np63 $\alpha$  for

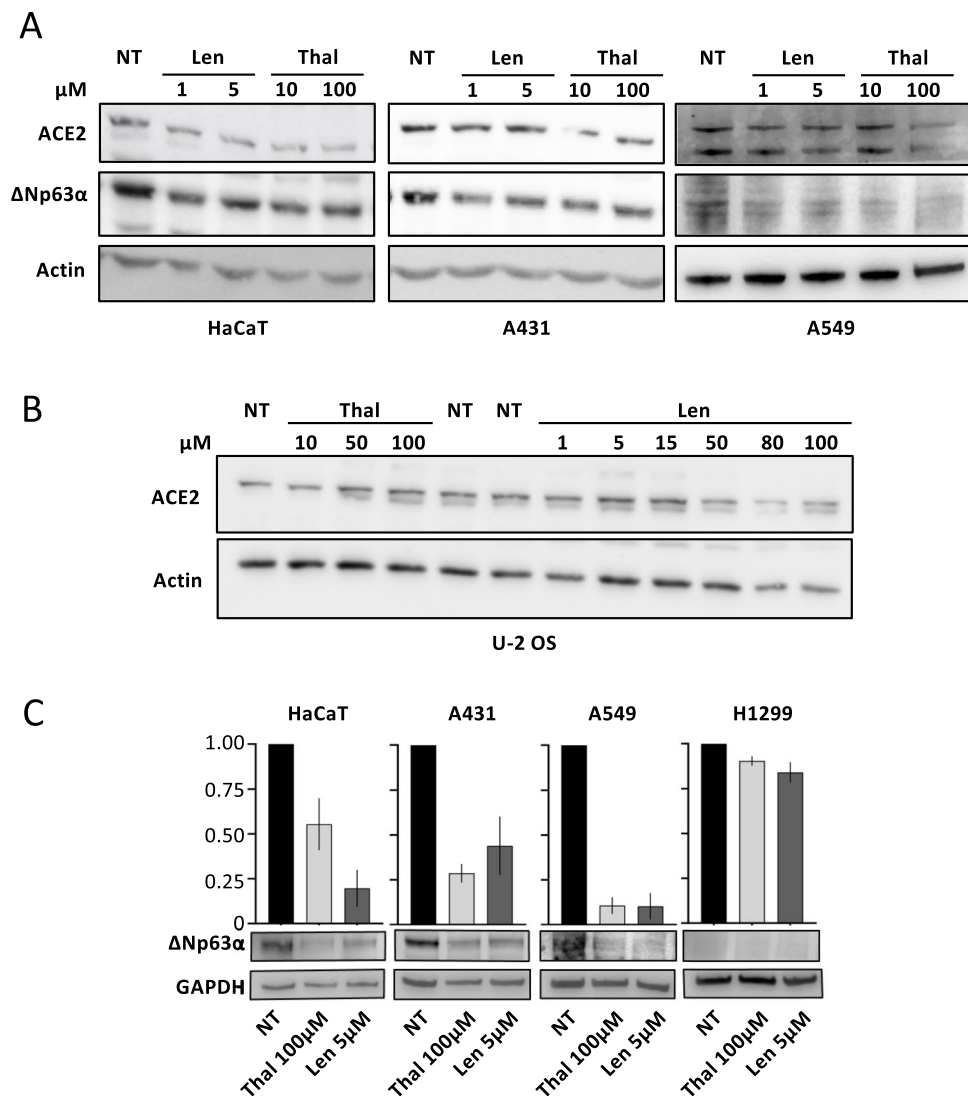
degradation upon Thal treatment) [17, 26] that in turn would give rise to reduced ACE2 transcription.

### Thalidomide reduces ACE2 expression through CRBN-mediated $\Delta$ Np63 $\alpha$ degradation

To verify this hypothesis, we reduced  $\Delta$ Np63 $\alpha$  protein levels by transfecting HaCaT and A431 cells with the CRBN encoding plasmid. As expected [17], CRBN overexpression led to a dose-dependent  $\Delta$ Np63 $\alpha$  degradation in both cell lines (Fig. 2A) that was paralleled, also in this case, by ACE2 downmodulation, supporting the idea that ACE2 levels are correlated with  $\Delta$ Np63 $\alpha$  levels. Next, we transiently transfected the p63-null U-2 OS cells with a  $\Delta$ Np63 $\alpha$  encoding plasmid and observed a positive correlation between expression levels of ACE2 and  $\Delta$ Np63 $\alpha$  (Fig. 2B). Moreover, evaluation of ACE2 mRNA by qRT-PCR in parallel samples showed increased ACE2 mRNA levels in the samples transfected with the  $\Delta$ Np63 $\alpha$  plasmid, thus indicating that ACE2 might be a  $\Delta$ Np63 $\alpha$  transcriptional target (Fig. 2C). To evaluate this point, we performed p63 silencing in HaCaT and A431 cells by transfection with small hairpin RNA (shRNA) plasmids targeting the p63 mRNA. For this type of experiment, we used four different p63 shRNA vectors (OriGene) with sequence homology to four different regions of the p63 mRNA, with the shp63#3 and the shp63#4 vectors resulting in the strongest effect on  $\Delta$ Np63 $\alpha$  silencing and a concomitant decrease of ACE2 protein levels, in both cell lines (Fig. 2D). Since we did not find a wholly consistent matching between  $\Delta$ Np63 $\alpha$  downregulation and reduction of ACE2 protein levels with all shp63 RNAs, we generated stable transfected polyclonal populations with the shp63#3 and shp63#4 and the relative control with the shSCRB vector. Reduced level of  $\Delta$ Np63 $\alpha$  protein was associated with the reduction of ACE2 protein and mRNA levels (Fig. 2E) further supporting the  $\Delta$ Np63 $\alpha$  role in ACE2 regulation.

### ACE2 is a new $\Delta$ Np63 $\alpha$ target gene

One of the main problems with SARS-CoV-2 infection is the triggering of a “cytokine storm” [27], a hyper-inflammatory state characterized by the production of extremely high levels of proinflammatory cytokines that eventually leads to patient death. In order to mimic the hyper-inflammatory state in vitro, HaCaT cells were treated with LPS or the proinflammatory cytokine TNF- $\alpha$ , both known to stabilize  $\Delta$ Np63 $\alpha$  protein levels and to stimulate cytokine production, possibly by stabilized  $\Delta$ Np63 $\alpha$  acting on the promoter of several cytokine genes [28–30]. Upon TNF- $\alpha$  and LPS treatments, both  $\Delta$ Np63 $\alpha$  and ACE2 expression levels were induced in a dose-dependent and time-dependent manner (Fig. 3A, lower panel). The levels of IL-8, known to



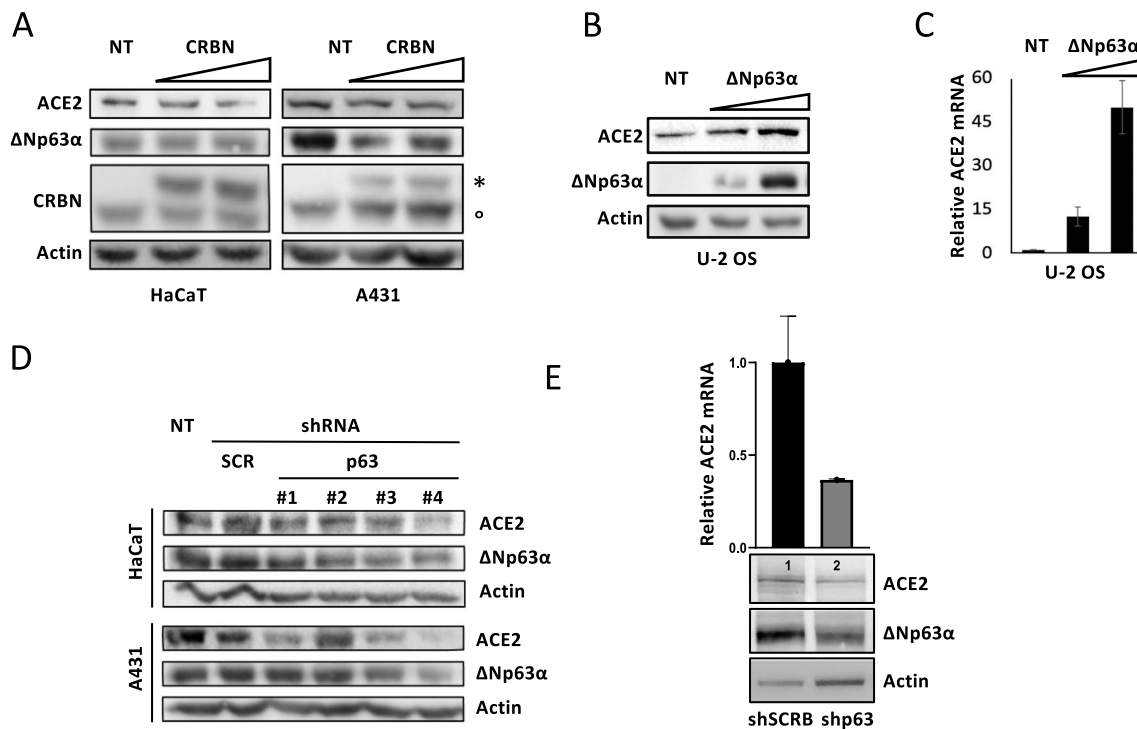
**Fig. 1** Thal and Len at clinically relevant concentrations reduce ACE2 expression. **A** HaCaT, A431, and A549 cells were treated for 24 h with Len (1 or 5 μM) or Thal (10 or 100 μM). Cell extracts were prepared and analyzed by WB with anti-ACE2 or anti-p63 Abs. Actin was used as the loading control, and one representative experiment is shown. **B** p63-null U-2 OS cells were treated for 24 h with increasing concentrations of Thal (10, 50, 100 μM) or Len (1, 5, 15, 50, 80, 100 μM). Cell extracts were analyzed by WB with anti-ACE2 Ab. Actin was used as the loading control, and one representative experiment is shown. **C** HaCaT, A431, A549, and H1299 cells were treated

with Thal 100 μM or Len 5 μM for 8 h; DMSO was used as the control (NT). Lower panel: cell extracts were prepared and analyzed by WB with anti-p63 Ab; GAPDH was used as the loading control, and one representative experiment is shown. Upper panel: compared to the relative Ctrl, ACE2 mRNA levels decreased in p63-proficient HaCaT, and A431 and A549 cells were treated with Thal or Len but not in p63-deficient H1299 cells. ACE2 mRNA was evaluated by qRT-PCR, mRNA levels were normalized using GAPDH, and the means  $\pm$  standard errors (SE) of three replicates are shown

be overproduced in COVID-19 patients and expressed in HaCaT cells [31, 32], were also increased by our treatments (Fig. 3A, upper panel), likely by stabilized ΔNp63α.

We then verified whether the p53 family responsive elements (RE) were present in the regulatory regions of the *ACE2* gene. For this purpose, we queried the p53Fam-Tag database [33] and identified one strong putative p53/p63-RE composed by three decamers in the first intron of the *ACE2* gene (Fig. 3B). To evaluate the in vivo recruitment of

ΔNp63α on the identified p53/p63-RE, a chromatin immunoprecipitation assay (ChIP) was performed. Cross-linked chromatin from A431 cells treated with LPS 0.5 μg/mL for 2 h was immunoprecipitated with anti-acetylated H4-histone or anti-p63α Abs. In the presence of LPS (i.e., 2 h treatment), but not in the untreated control cells, ΔNp63α was consistently recruited on the p53/p63-RE of the *ACE2* gene (Fig. 3D). The increased p63 occupancy was accompanied by an increase in histone H4 acetylation (Fig. 3D) and,



**Fig. 2**  $\Delta$ Np63 $\alpha$  and ACE2 parallel modulation at both protein and mRNA levels. **A** HaCaT and A431 cells were transiently transfected with 100 and 250 ng of CRBN encoding plasmid. After 24 h from transfection, cell extracts were prepared and analyzed by WB with anti-ACE2, anti-p63, and anti-CRBN Abs. Actin was used as the loading control; one representative experiment is shown. Two bands of the CRBN protein are evident: °stays for endogenous CRBN, \*for transfected CRBN that has higher molecular weight due to an HA tag. **B** p63-null U-2 OS cells were transiently transfected with 10 and 25 ng of  $\Delta$ Np63 $\alpha$  expression vector. After 24 h from transfection, cell extracts were prepared and analyzed by WB with anti-ACE2 or anti-p63 Abs. Actin was used as the loading control, and one representative experiment is shown. **C** ACE2 mRNA levels were evaluated by qRT-PCR, and mRNA levels were normalized using GAPDH as housekeeping gene. Means  $\pm$  SE of three replicates are shown. **D** HaCaT and A431 cells were transiently transfected with 100 ng of

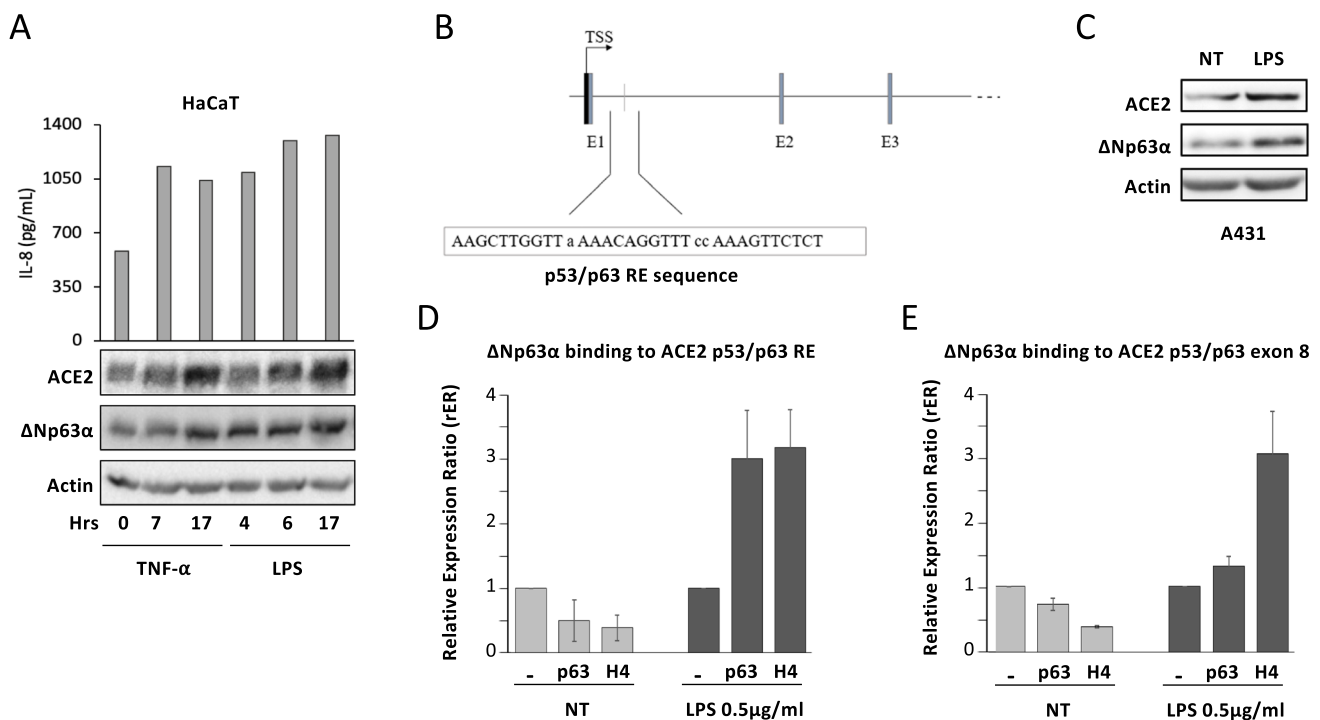
small hairpin RNA (shRNA) plasmids targeting p63 mRNA; four different p63 shRNA vectors with sequence homology to four different regions of p63 mRNA were used (#1, #2, #3, #4); the cells were also transfected with a control (100 ng), shRNA-SCR, that encodes for an RNA that is not complementary to any mRNA sequence. After 24 h from transfection, cell extracts were prepared and analyzed by WB with anti-ACE2 and anti-p63 Abs. Actin was used as the loading control; one representative experiment is shown. **E** A431 cells were stably transfected with both p63shRNA#3 and p63shRNA#4, and polyclonal populations were isolated by puromycin selection. As the control, cells were also transfected with a shRNA-SCR vector. Cell extracts were prepared and analyzed by WB with anti-ACE2 and anti-p63 Abs, and parallel samples were used for ACE2 mRNA level evaluation by qRT-PCR; mRNA levels were normalized using GAPDH as housekeeping gene. Means  $\pm$  SE of three replicates of three cultures are shown

consistently, in ACE2 protein levels (Fig. 3C). As negative control, the exon 8 of the *ACE2* gene, not containing any p53/p63-RE, was not amplified in the same samples (Fig. 3E). Taken together, these results clearly indicate that ACE2 is a new target gene of  $\Delta$ Np63 $\alpha$ .

### Thalidomide weakens in vitro infection by pseudo-SARS-CoV-2

It has been reported that COVID-19 patients treated with Thal had a faster recovery in respect to untreated patients [15]. From the data obtained, we hypothesized that the observed protection might be due to the diminished viral re-entry due to ACE2 downmodulation upon Thal treatment

as a consequence of  $\Delta$ Np63 $\alpha$  degradation. To verify this hypothesis, we pretreated A431 cells with 100  $\mu$ M Thal for 24 h before adding, for additional 24 h, the pseudo-SARS-CoV-2, a GFP-expressing baculovirus pseudotyped with the SARS-CoV-2 spike protein (Fig. 4A). We found that Thal pretreatment impairs pseudoviral infection in vitro, as evidenced by reduced GFP signals in the Thal-pretreated samples compared with the controls (Fig. 4B and C). Next, we assessed whether  $\Delta$ Np63 $\alpha$  downregulation is sufficient to reduce pseudo-SARS-CoV-2 infectivity by employing the stable shp63#3/shp63#4 transfected cells. No difference was observed between control shSCR and  $\Delta$ Np63 $\alpha$  downregulated cells suggesting that Thal triggers additional events to  $\Delta$ Np63 $\alpha$  degradation to reach its effects (Fig. 4D).



**Fig. 3** ACE2 is a new  $\Delta$ Np63 $\alpha$  target gene. **A** HaCaT cells were treated with 5 ng/mL of TNF- $\alpha$  or with 0.5  $\mu$ g/mL of LPS for the indicated times. Cell extracts were prepared and analyzed by WB with an anti-ACE2 and anti-p63 Abs. Actin was used as the loading control, and one representative experiment is shown. The cell supernatants were collected, and IL-8 levels were quantified by the ELISA assay. **B** The schematic representation and sequence of the p53/

p63RE were identified in the first intron of ACE2. **C** A431 cells were treated or not with LPS 0.5  $\mu$ g/mL for 2 h. Cell extracts were prepared and analyzed by WB using anti-ACE2 and anti-p63 Abs. Actin was used as the loading control, and one representative experiment is shown. **D**, **E** A431 cells were treated with LPS for 2 h and then collected for chromatin immunoprecipitation (ChIP) analysis

Altogether, these data offer a mechanistic explanation of the protective effect from severe COVID-19 observed in patients treated with Thal [15].

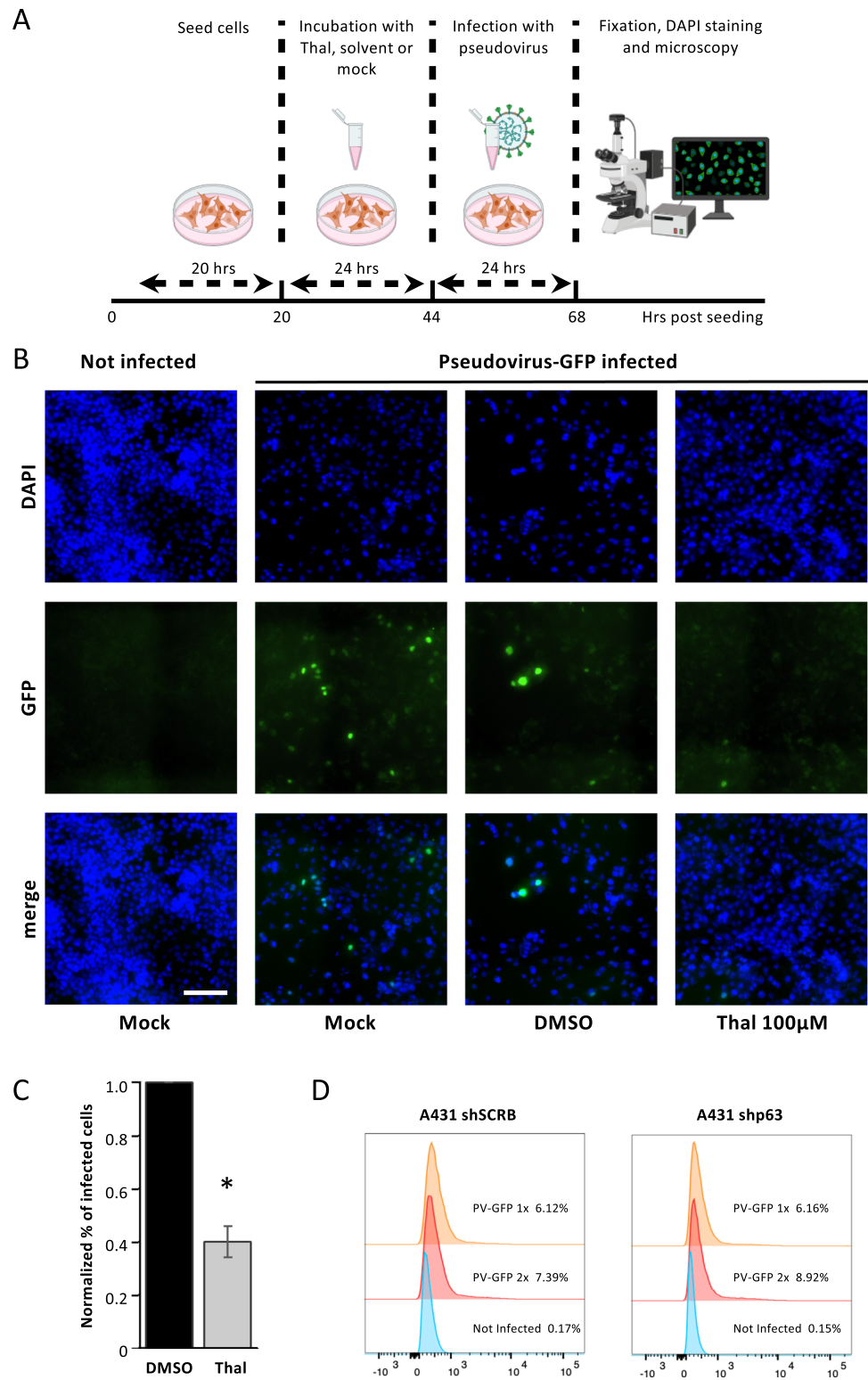
## Discussion

COVID-19 is an infectious disease caused by SARS-CoV-2 that started to spread at the end of 2019. The impellent need for effective therapies at the beginning of the pandemic drove several drug repositioning approaches to tackle the severe adverse effects of the virus infection [5]. Among these, the usage of the immunomodulatory drug Thal was proposed, and its administration proved effective in fastening patient recovery and reducing the levels of inflammatory cytokines in the serum of the patients [15].

Thal was originally used as anti-emetic compound to treat morning sickness of pregnant women and was withdrawn from the market in 1961 due to its teratogenic effects [6, 13]. This adverse effect was recently shown to depend on the Thal-induced degradation of  $\Delta$ Np63 $\alpha$  and TAp63a, two isoforms of the p63 transcription factor known to have essential roles in skin and limb development [17]. Nonetheless, this

drug has found usage in clinical practice due to its anti-tumoral, immunomodulatory, and anti-inflammatory effects [7, 34]. In addition, it has been observed that the SARS-CoV-2 virus induced lower mortality in Len-treated multiple myeloma patients compared to Len-naïve counterparts [35], supporting that Thal and its analogues might be useful in the management of COVID-19 patients. However, the underlying molecular mechanism is still unclear. Starting from these evidences, we hypothesized that the beneficial effects observed in COVID-19 patients treated with Thal could stem from  $\Delta$ Np63 $\alpha$  degradation, with p63 eventually playing a role on the regulation of ACE2. Noteworthy, this p63-mediated mechanism of protection by SARS-CoV-2 infection has been observed in studies exploiting other known inducers of p63 degradation, such as metformin [36, 37]. Supporting this hypothesis, we identified in silico a p53/p63-RE in the ACE2 gene and demonstrated that, in conditions mimicking the well-known COVID-19-associated cytokine storm, p63 does indeed bind to this element. On top of this, ACE2 mRNA and protein levels both scale with p63 abundance in the cell, as shown by p63 overexpression or silencing further proving that p63 is indeed active at the ACE2 locus to promote ACE2 expression. We speculated that the effects observed

**Fig. 4** Thal impairs in vitro infection by GFP-expressing pseudo-SARS-CoV-2. **A** The schematic representation of the experiment is shown in **B**. **B** Immunofluorescent staining of A431 cells treated with Thal 100  $\mu$ M; after 24-h treatment, 50  $\mu$ L of pseudo-SARS-CoV-2 suspensions was added to the cells following the manufacturer's instructions; the viral titer was  $2 \times 10^{10}$  viral genes (VG) per mL. After 24 h post-infection, cells were washed with PBS and fixed with 4% formaldehyde in 1X PBS for 10 min at room temperature. Nuclei were stained with DAPI. Images were acquired using a Nikon CSU-W1 microscope in widefield mode, using a  $\times 20$  objective and analyzed with FIJI [24], and representative images are shown. Scale bar 150  $\mu$ m. **C** Quantification of GFP reduction in Thal-pretreated samples. **D** FACS analysis was performed 24 h after pseudo-SARS-CoV-2 infection of shp63#3/shp63#4 and shSCRB stable transfectants



in COVID-19 patients treated with Thal or Len could likely be caused by a p63 degradation-dependent reduction of ACE2 levels, and that this downmodulation could reduce the SARS-CoV-2 viral infection and, in COVID-19 patients, viral re-entry. This hypothesis holds true as we show that

Thal induces ACE2 reduction only in cells expressing p63 and reduces infection by a pseudo-SARS-CoV-2 virus. However, additional factors such as TMPRSS2 [1, 38] have been recently shown to be required, together with ACE2, to allow SARS-CoV-2 infection. The observation that Thal



does reduce pseudo-SARS-CoV-2 infectivity, while the sole  $\Delta Np63\alpha$  downregulation does not, suggests that Thal might regulate multiple factors, warranting further investigations.

Overall, our work sheds new light onto current comprehension of Thal and p63 molecular details at various levels. First, our data envisage new roles of p63 on the regulation of cell protein expression and support the general concept that Thal may efficiently modify the expression of genes through p63 regulation, among which is *ACE2*. Second, they support a protective role for Thal against SARS-CoV-2 viral infection and explain the molecular details of how this compound exerts its functions, potentially making it a valuable option in the management of SARS-CoV-2 or other ACE2-dependent infections. Third, this work represents a proof of concept of a pharmacological substance that may play an effect on ACE2. This effect opens to new consideration on possible roles that the modulation of ACE2 cellular levels may offer in the clinical practice. Indeed, modulation of ACE2 levels with Thal may prevent or effectively contrast SARS-CoV-2 infection if properly given in early phases of the virus attack. While these data suggest a potential new preventive approach to SARS-CoV-2, they also pose some important potential problems related to the use of Thal, the major one being the need to consider the negative effect of ACE2 blockage on the renin–angiotensin–aldosterone system and specifically on hypertension and its effects on heart and renal function. It seems that specific anti-angiotensin II therapies such as ARBs should follow and/or be given in concomitance with Thal. Clinical trials should be designed to resolve this aspect.

**Acknowledgements** The authors would like to thank Maria Pia Gentileschi, Giulia Bon, Davide Valente (Regina Elena National Cancer Institute IRCCS) and Claudia Bazzini (Flow cytometry facility – Department of Biosciences, University of Milan) for technical support.

**Author contribution** LG, AT, and SS conceived and designed the study. SP, LM, RQ, IV, MB, VS, AA, FM, and MP performed experiments and analyzed the data. LG, AT, and SS wrote and revised the manuscript. LG, AT, and SS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

**Funding** Open access funding provided by Università degli Studi di Milano within the CRUI-CARE Agreement.

**Data availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate** Not applicable.

**Patient consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

- Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A et al (2020) SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181(2):271–280. <https://doi.org/10.1016/j.cell.2020.02.052>
- Liu M, Wang T, Zhou Y, Zhao Y, Zhang Y, Li J (2020) Potential role of ACE2 in coronavirus disease 2019 (COVID-19) prevention and management. *J Transl Int Med* 8(1):9–19. <https://doi.org/10.2478/jtim-2020-0003>
- Zou X, Chen K, Zou J, Han P, Hao J, Han Z (2020) Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med* 14(2):185–192. <https://doi.org/10.1007/s11684-020-0754-0>
- Hughes JP, Rees S, Kalindjian SB, Philpott KL (2011) Principles of early drug discovery. *Br J Pharmacol* 162(6):1239–1249. <https://doi.org/10.1111/j.1476-5381.2010.01127.x>
- Singh TU, Parida S, Lingaraju MC, Kesavan M, Kumar D, Singh RK (2020) Drug repurposing approach to fight COVID-19. *Pharmacol Rep* 72(6):1479–1508. <https://doi.org/10.1007/s43440-020-00155-6>
- Vargesson N (2009) Thalidomide-induced limb defects: resolving a 50-year-old puzzle. *BioEssays* 31(12):1327–1336. <https://doi.org/10.1002/bies.200900103>
- Rehman W, Arfons LM, Lazarus HM (2011) The rise, fall and subsequent triumph of thalidomide: lessons learned in drug development. *Ther Adv Hematol* 2(5):291–308. <https://doi.org/10.1177/2040620711413165>
- Ito T, Handa H (2020) Molecular mechanisms of thalidomide and its derivatives. *Proc Jpn Acad Ser B Phys Biol Sci* 96(6):189–203. <https://doi.org/10.2183/pjab.96.016>
- Dong X, Li X, Li M, Chen M, Fan Q, Wei W (2017) Inhibitory effects of thalidomide on bleomycin-induced pulmonary fibrosis in rats via regulation of thioredoxin reductase and inflammations. *Am J Transl Res* 9(10):4390–4401
- Horton MR, Santopietro V, Mathew L, Horton KM, Polito AJ, Liu MC, Danoff SK, Lechtzin N (2012) Thalidomide for the treatment of cough in idiopathic pulmonary fibrosis: a randomized trial. *Ann Intern Med* 157(6):398–406. <https://doi.org/10.7326/0003-4819-157-6-201209180-00003>
- Knobloch J, Jungck D, Koch A (2011) Apoptosis induction by thalidomide: critical for limb teratogenicity but therapeutic potential in idiopathic pulmonary fibrosis? *Curr Mol Pharmacol* 4(1):26–61. <https://doi.org/10.2174/1874467211104010026>
- Zhu H, Shi X, Ju D, Huang H, Wei W, Dong X (2014) Anti-inflammatory effect of thalidomide on H1N1 influenza virus-induced

- pulmonary injury in mice. *Inflammation* 37(6):2091–2098. <https://doi.org/10.1007/s10753-014-9943-9>
13. Vargesson N (2015) Thalidomide-induced teratogenesis: history and mechanisms. *Birth Defects Res C Embryo Today* 105(2):140–156. <https://doi.org/10.1002/bdrc.21096>
  14. Paravar T, Lee DJ (2008) Thalidomide: mechanisms of action. *Int Rev Immunol* 27(3):111–135. <https://doi.org/10.1080/08830180801911339>
  15. Chen C, Qi F, Shi K, Li Y, Li J, Chen Y, Pan J, Zhou T, Lin X, Zhang J et al (2020) Thalidomide combined with low-dose short-term glucocorticoid in the treatment of critical coronavirus disease 2019. *Clin Transl Med* 10(2):e35. <https://doi.org/10.1002/ctm2.35>
  16. Su S, Chen J, Wang Y, Wong LM, Zhu Z, Jiang G, Liu P (2021) Lenalidomide downregulates ACE2 protein abundance to alleviate infection by SARS-CoV-2 spike protein conditioned pseudoviruses. *Signal Transduct Target Ther* 6(1):182. <https://doi.org/10.1038/s41392-021-00608-1>
  17. Asatsuma-Okumura T, Ando H, De Simone M, Yamamoto J, Sato T, Shimizu N, Asakawa K, Yamaguchi Y, Ito T, Guerrini L et al (2019) p63 is a cereblon substrate involved in thalidomide teratogenicity. *Nat Chem Biol* 15(11):1077–1084. <https://doi.org/10.1038/s41589-019-0366-7>
  18. Ghioni P, Bolognese F, Duijff PH, Van Bokhoven H, Mantovani R, Guerrini L (2002) Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains. *Mol Cell Biol* 22(24):8659–8668. <https://doi.org/10.1128/MCB.22.24.8659-8668.2002>
  19. Mangiulli M, Valletti A, Caratozzolo MF, Tullo A, Sbisà E, Pesole G, D'Erchia AM (2009) Identification and functional characterization of two new transcriptional variants of the human p63 gene. *Nucleic Acids Res* 37(18):6092–6104. <https://doi.org/10.1093/nar/gkp674>
  20. Guerrini L, Costanzo A, Merlo GR (2011) A symphony of regulations centered on p63 to control development of ectoderm-derived structures. *J Biomed Biotechnol* 2011:864904. <https://doi.org/10.1155/2011/864904>
  21. Barton CE, Johnson KN, Mays DM, Boehnke K, Shyr Y, Boukamp P, Pietenpol JA (2010) Novel p63 target genes involved in paracrine signaling and keratinocyte differentiation. *Cell Death Dis* 1(9):e74. <https://doi.org/10.1038/cddis.2010.49>
  22. Rizzo JM, Oyelakin A, Min S, Smalley K, Bard J, Luo W, Nyquist J, Guttman-Yassky E, Yoshida T, De Benedetto A et al (2016)  $\Delta$ Np63 regulates IL-33 and IL-31 signaling in atopic dermatitis. *Cell Death Differ* 23(6):1073–1085. <https://doi.org/10.1038/cdd.2015.162>
  23. Mehta SY, Morten BC, Antony J, Henderson L, Lasham A, Campbell H, Cunliffe H, Horsfield JA, Reddel RR, Avery-Kiejda KA et al (2018) Regulation of the interferon-gamma (IFN- $\gamma$ ) pathway by p63 and  $\Delta$ 133p53 isoform in different breast cancer subtypes. *Oncotarget* 9(49):29146–29161. <https://doi.org/10.18632/oncotarget.25635>
  24. Wang BY, Gil J, Kaufman D, Gan L, Kohtz DS, Burstein DE (2002) P63 in pulmonary epithelium, pulmonary squamous neoplasms, and other pulmonary tumors. *Hum Pathol* 33(9):921–926. <https://doi.org/10.1053/hupa.2002.126878.PMID:12378518>
  25. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B et al (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9(7):676–682. <https://doi.org/10.1038/nmeth.2019>
  26. Ito T, Ando H, Suzuki T, Ogura T, Hotta K, Imamura Y, Yamaguchi Y, Handa H (2010) Identification of a primary target of thalidomide teratogenicity. *Science* 327(5971):1345–1350. <https://doi.org/10.1126/science.1177319>
  27. Ragab D, Salah Eldin H, Taimah M, Khattab R, Salem R (2020) The COVID-19 cytokine storm; what we know so far. *Front Immunol* 11:1446. <https://doi.org/10.3389/fimmu.2020.01446>
  28. Si H, Lu H, Yang X, Mattox A, Jang M, Bian Y, Sano E, Viadiu H, Yan B, Yau C et al (2016) TNF- $\alpha$  modulates genome-wide redistribution of  $\Delta$ Np63 $\alpha$ /TAP73 and NF- $\kappa$ B cREL interactive binding on TP53 and AP-1 motifs to promote an oncogenic gene program in squamous cancer. *Oncogene* 35(44):5781–5794. <https://doi.org/10.1038/onc.2016.112>
  29. Liu X, Yin S, Chen Y, Wu Y, Zheng W, Dong H, Bai Y, Qin Y, Li J, Feng S et al (2018) LPS-induced proinflammatory cytokine expression in human airway epithelial cells and macrophages via NF- $\kappa$ B, STAT3 or AP-1 activation. *Mol Med Rep* 17(4):5484–5491. <https://doi.org/10.3892/mmr.2018.8542>
  30. Choi DH, Hwang HS (2019) Anti-inflammation activity of brazilin in TNF- $\alpha$  induced human psoriasis dermatitis skin model. *Appl Biol Chem* 62:46. <https://doi.org/10.1186/s13765-019-0455-z>
  31. Takada K, Komine-Aizawa S, Hirohata N, Trinh QD, Nishina A, Kimura H, Hayakawa S (2017) Poly I: C induces collective migration of HaCaT keratinocytes via IL-8. *BMC Immunol* 18(1):19. <https://doi.org/10.1186/s12865-017-0202-3>
  32. Yang S, Jiang Y, Yu X, Zhu L, Wang L, Mao J, Wang M, Zhou N, Yang Z, Liu Y et al (2021) Polyphyllin I inhibits propionibacterium acnes-induced IL-8 secretion in HaCaT cells by down-regulating the CD36/NOX1/ROS/NLRP3/IL-1 $\beta$  pathway. *Evid Based Complement Alternat Med* 2021:1821220. <https://doi.org/10.1155/2021/1821220>
  33. Sbisà E, Catalano D, Grillo G, Licciulli F, Turi A, Liuni S, Pesole G, De Grassi A, Caratozzolo MF, D'Erchia AM, et al: p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data. *BMC Bioinformatics* 8 Suppl 1: S20, 2007. <https://doi.org/10.1186/1471-2105-8-S1-S20>
  34. Brigle K, Rogers B (2017) Pathobiology and diagnosis of multiple myeloma. *Semin Oncol Nurs* 33(3):225–236. <https://doi.org/10.1016/j.soncn.2017.05.012>
  35. Tailor IK, Alshehry NF, Zaidi SZ, Marei MA, Motabi IH, Alfayez M, Altaf SY (2023) Outcome of myeloma patients with COVID-19 on active lenalidomide-based therapy: does lenalidomide protect from severe COVID-19? *Hematol Oncol Stem Cell Ther* 16(1):88–90. <https://doi.org/10.1016/j.hemonc.2020.08.002>
  36. Ojeda-Fernández L, Foresta A, Macaluso G, Colacioppo P, Tettamanti M, Zambon A, Genovese S, Fortino I, Leoni O, Roncaglioni MC et al (2022) Metformin use is associated with a decrease in the risk of hospitalization and mortality in COVID-19 patients with diabetes: a population-based study in Lombardy. *Diabetes Obes Metab* 24(5):891–898. <https://doi.org/10.1111/dom.14648>
  37. Yi Y, Chen D, Ao J, Sun S, Wu M, Li X, Bergholz J, Zhang Y, Xiao ZX (2017) Metformin promotes AMP-activated protein kinase-independent suppression of  $\Delta$ Np63 $\alpha$  protein expression and inhibits cancer cell viability. *J Biol Chem* 292(13):5253–5261. <https://doi.org/10.1074/jbc.M116.769141>
  38. Saunders N, Fernandez I, Planchais C, Michel V, Rajah MM, Baquero Salazar E, Postal J, Porrot F, Guivel-Benhassine F, Blanc C et al (2023) Tmprss2 is a functional receptor for human coronavirus HKU1. *Nature* 624(7990):207–214. <https://doi.org/10.1038/s41586-023-06761-7>

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.