Advances in Redox Research 15 (2025) 100132

Contents lists available at ScienceDirect

Advances in Redox Research

journal homepage: www.elsevier.com/locate/arres

Full Length Article

Exogenously added recombinant CLIC proteins provide antioxidant protection to cells in culture

KR Hossain, A Alghalayini, DR Turkewitz, C D'Amario, Catherine A Gorrie, M Wallach, SM Valenzuela *

School of Life Sciences, University of Technology Sydney, Sydney, NSW 2007, Australia

ARTICLE INFO

Keywords:
Chloride intracellular ion channel (CLIC) proteins
CLIC1
CLIC4
Fibroblast cells
Keratinocyte cells
Antioxidant
Oxidoreductase activity
Hydrogen peroxide induced oxidative stress

ABSTRACT

Chloride intracellular ion channels (CLICs) are a family of six human proteins that exist as both soluble and integral membrane proteins and are expressed across a range of different tissues throughout the body. CLIC1 and CLIC4 act as moonlighting proteins, exhibiting oxidoreductase enzymatic activity in addition to their membrane ion channel activity. Transient siRNA knockdown of either CLIC1 or CLIC4 in primary human dermal fibroblast (HDF), human epidermal keratinocyte (HKE) cells and in the stable murine fibroblast cell line, NIH/3T3, showed significant reduction in cell viability. Conversely, NIH/3T3 cells over-expressing CLIC1 or CLIC4 demonstrated that both proteins assist in protecting the cells from hydrogen peroxide (H_2O_2)-induced oxidative damage, resulting in reduced cell death and reduced Reactive Oxygen Species (ROS) generation. While the opposite effect was seen in cells where these proteins had been silenced using siRNA. We have also now demonstrated that by exogenously adding recombinant CLIC (rCLIC) proteins to either HDF or HKE cells in culture, both rCLIC1 and rCLIC4 proteins provided cellular antioxidant protection to the fibroblast and keratinocyte cells against H_2O_2-induced oxidative damage. Our study also demonstrates rCLIC1 and rCLIC4's ability to act as skin cell protective antioxidant agents, arises from their oxidoreductase enzymatic activity. Our findings also showed exogenous addition of rCLIC1 or rCLIC4 to skin cells resulted in similar or greater protection against H_2O_2-induced oxidative damage when compared to other well-known endogenous antioxidants like glutaredoxin (Grx), Glutathione S-transferase-Omega (GST-Ω) and the antioxidant drug, N-acetylcysteine (NAC).

Introduction

To date, seven members of the human CLIC family have been identified: CLIC1 to CLIC4, CLIC5A and 5B (splice variants) and CLIC6. CLICs 1-5A are almost identical in size, consisting of a conserved C-terminal 240 amino acid residue module with one major putative transmembrane domain located in their N-terminus. In contrast, CLIC5B and CLIC6 are two larger variants and consist of an extended N-terminal domain in conjunction with the typical 240 amino acid CLIC module [1–4]. The CLIC proteins are highly conserved across species, with CLIC-like proteins identified in numerous vertebrates such as amphibians, birds, fish, and mammals as well as in invertebrates such as nematodes (EXC-4 and EXL-1) and insects (DmCLIC) [5]. Furthermore, four CLIC-like proteins (DHARs1-4) have also been identified in plants [6] and one in bacteria (SspA) [7]. Based on the primary sequence and 3D crystal structural models [8–11], CLIC proteins were shown to adopt the canonical thioredoxin-like fold and were classified as the newest members of the Glutathione S-transferases (GST) superfamily (Fig. 1) having the topologically conserved glutaredoxin-like motif (Cys-X-X-Cys/X) which has been shown to exhibit glutathione-dependent thiol transferase activity [12–15]. This enzymatic activity catalyses the reduction of intracellular disulfides with reactions that couple together Glutathione (GSH), Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and Glutathione Reductase (GR), a reduction which is considered necessary for the maintenance of a healthy redox environment within the cell [12–15].

A 1.4 Å structure of CLIC1 showed a single GSH molecule covalently bound to the protein through a disulphide bond between Cys24 (that forms part of the thiol site motif) and the GSH thiol group [9]. This GSH-binding site domain can either be monothiol containing one highly reactive cysteine residue as in the cases of CLICs 1, 4, 5 and 6, or dithiol,

---

* Corresponding author.

E-mail addresses: khondker.hossain@uts.edu.au (K. Hossain), amani.alghalayini@uts.edu.au (A. Alghalayini), daniel.r.turkewitz@alumni.uts.edu.au (D. Turkewitz), claudia.damario@alumni.uts.edu.au (C. D'Amario), catherine.gorrie@uts.edu.au (C.A. Gorrie), michael.wallach@uts.edu.au (M. Wallach), stella.valenzuela@uts.edu.au (S. Valenzuela).

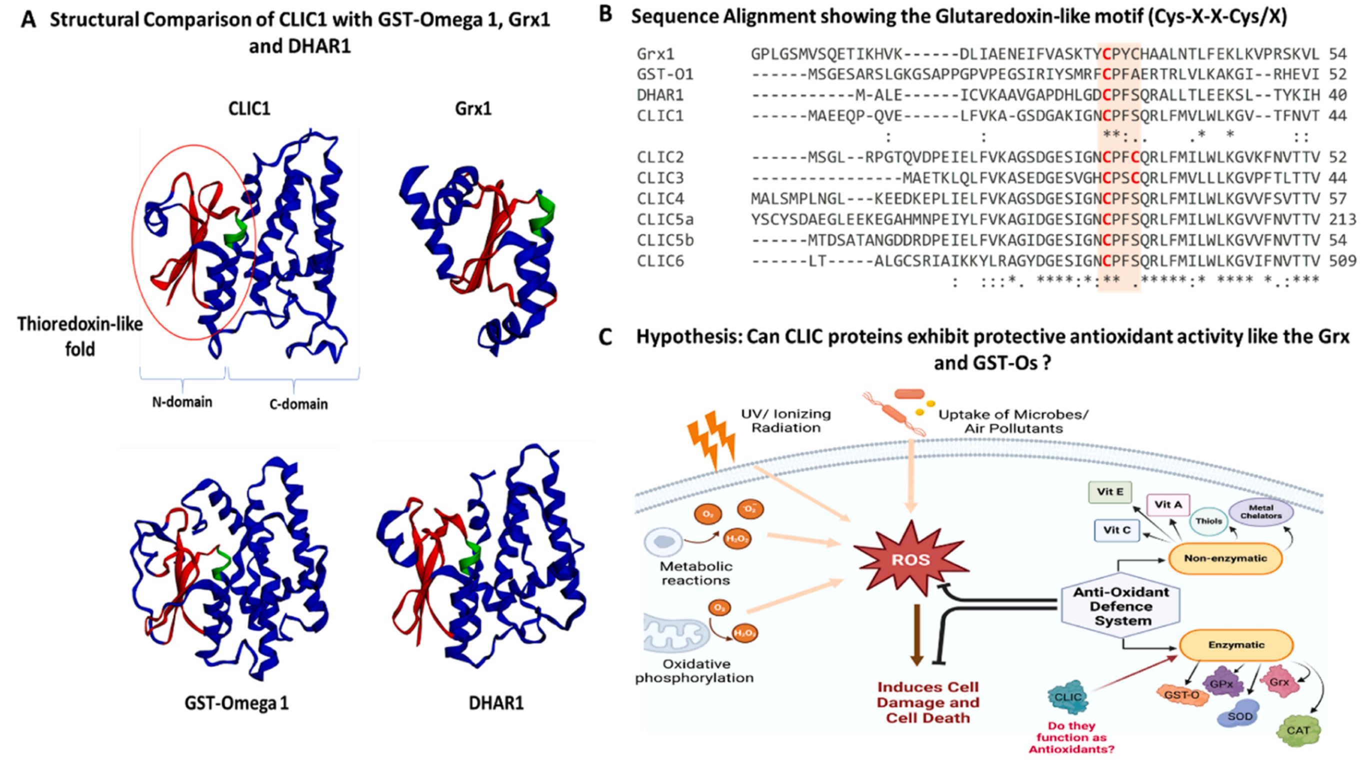
https://doi.org/10.1016/j.arres.2025.100132
Received 7 April 2025; Received in revised form 5 May 2025; Accepted 11 May 2025

having two cysteine residues as seen in CLICs 2 and 3 [16]. Given their structural similarity with the GSTs (as seen in Fig. 1), it was speculated that the soluble form of CLICs can also act as oxidoreductases. Our group has previously demonstrated the oxidoreductase activity of the proteins CLIC 1, 2, and 4 via an *in vitro* HEDS enzyme assay [16], followed by contributions to work on CLIC3, whose glutathione-dependent oxidoreductase activity was shown to drive cancer progression via its regulation of the protein transglutaminase-2 [17].

Several additional *in vitro* studies by our group have further characterised this enzymatic activity [17–19] and there are now a number of other studies implicating CLIC and related CLIC-like proteins in redox cellular processes [20–26]. For example, Ponnalagu et al., 2018 and 2022, have proposed that CLIC4 and CLIC5 are involved in cardio protection from *in vivo* ischemia-reperfusion and respectively aid in maintaining calcium homeostasis and mitochondrial ROS generation [23,24]. A study of CLIC4 in breast cancer cells implicates its involvement in maintaining redox-homeostasis [20]. Plant Dehydroascorbate Reductases (DHARs) are well known to catalyse the reduction of dehydroascorbate (DHA) to ascorbate using reduced GSH as an electron donor, allowing rapid ascorbate re-cycling which in turn affects cell responsiveness and tolerance to environmental ROS [21,26]. Increasingly, evidence appears to be highlighting the roles of CLIC-like proteins in redox biology as well. This ranges from the invertebrate CLIC-like proteins being shown to be associated with maintaining the cellular redox homeostasis within invertebrates [22,25]; knockout of invertebrate *Drosophila melanogaster* ortholog CLIC-like protein (DmCLIC) results in the upregulation of proteins involved in the oxidation–reduction processes. The study demonstrated DmCLIC is involved in the regulation of oxidative stress signalling and this function was shown to overlap with the molecular mechanisms of acute ethanol sensitivity in the fly [25]. Another CLIC homolog, EXL-1 (excretory canal abnormal like-1) expressed by *Caenorhabditis elegans*, responds specifically to heat stress and translocates from the cytoplasm to the nucleus, and depletion of EXL-1 makes the cells more susceptible to heat-stress [22]. This study proposed that *C.elegans* CLICs evolved to achieve different physiological functions through changes in their subcellular localization and they undergo spatial separation in response to external or internal signals [22]. Hence, it is important to further investigate CLIC protein cellular protective ability against external environmental stressors and their function as oxidoreductases via different signalling and regulatory pathways and also based on their subcellular localisation. Thus, taking this experimental evidence, together with their high degree of structural homology to well-studied antioxidant enzymes such as the GSTs, GST-Ωs, Grxs and the DHARs, we postulate that soluble CLICs would also serve as antioxidant proteins in cells. Therefore, we hypothesise that members of the CLIC protein family in their soluble form, provide antioxidant protective functions within cells in order to assist in their defence against exposure to oxidative damage and external environmental stressors. Therefore, this study aims to explore the cellular protective ability of CLIC proteins within primary human fibroblasts and keratinocytes skin cells and the murine fibroblast cell line, NIH/3T3 in culture.

Materials and methods

The following reagents were purchased from ThermoFisher Scientific (Sydney, Australia): TrypLE™ Express Enzyme (1X) no phenol red; DMEM/F-1; GlutaMAX™ supplement; Opti-MEM™ Reduced Serum Medium; DMEM/F-12, HEPES, no phenol red; Human Fibroblast Medium; Low Serum Growth Supplement (LSGS); EpiLife Medium and Human Keratinocyte Growth Supplement (HKGS). Fetal Bovine Serum (FBS) (French Origin) was purchased from Scientifix. NIH/3T3 murine



```
Grx1          GPLGSMVSQETIKHVK------DLIAENEIFVASKTYCPYCHAALNTLFEKLKVPRSKVL 54
GST-O1        ------MSGESARSLGKGSAPPGPVPEGSIRIYSMRFCPFAERTRLVLKAKGI--RHEVI 52
DHAR1         -----------M-ALE------ICVKAAVGAPDHLGDCPFSQRALLTLEEKSL--TYKIH 40
CLIC1         ------MAEEQP-QVE------LFVKA-GSDGAKIGNCPFSQRLFMVLWLKGV--TFNVT 44
                            :         :            **:..    .*  *      ::

CLIC2         ------MSGL--RPGTQVDPEIELFVKAGSDGESIGNCPFCQRLFMILWLKGVKFNVTTV 52
CLIC3         ----------------MAETKLQLFVKASEDGESVGHCPSCQRLFMVLLLKGVPFTLTTV 44
CLIC4         MALSMPLNGL---KEEDKEPLIELFVKAGSDGESIGNCPFSQRLFMILWLKGVVFSVTTV 57
CLIC5a        YSCYSDAEGLEEKEGAHMNPEIYLFVKAGIDGESIGNCPFSQRLFMILWLKGVVFNVTTV 213
CLIC5b        ------MTDSATANGDDRDPEIELFVKAGIDGESIGNCPFSQRLFMILWLKGVVFNVTTV 54
CLIC6         ------LT-----ALGCSRIAIKKYLRAGYDGESIGNCPFSQRLFMILWLKGVIFNVTTV 509
                                   :  :::*. ****:*:** .*****:* **** *.:***
```

Fig. 1. Sequence and Structural Comparison of CLIC1 with GST-Omega 1, Grx1 and DHAR1. A) Crystals structures of CLIC1 (PDB:1K0M), GST-Omega 1 (PDB:1EEM), Grx1 (PDB:6MWS) and DHAR1 (PDB:5LOL) showing the Cys-X-X-Cys/X motif in green, the thioredoxin-fold in red and the α-helixes in blue. The Images were generated using EzMol[2.1] online software. **B)** Sequence alignment using ClustalW shows the glutaredoxin-like motif (Cys-X-X-Cys/X) (with the active cysteine residues in red) conserved in all the different proteins. C) Schematic representation of the anti-oxidant defense system of cells showing the possibility of CLICs being part of that system.

Created in BioRender. Hossain, K. (2025) https://BioRender.com/v05r913.

fibroblast cells were available in-house at UTS. Human Dermal Fibroblasts (HDF) primary cells (C0135C) and Human Epidermal Keratinocytes (HKE) primary cells (C0055C) were purchased from ThermoFisher Scientific (Sydney, Australia). Bacterial streak plates containing CLIC1 in the pIRES2-EGFP Vector (Clonetech) with an amino FLAG (AF-CLIC1) tag and empty construct were generously gifted from the University of New South Wales, Australia. The CLIC4 plasmid (AF-CLIC4), in the same vector, was prepared by Gene Universal as a lyophilized pellet and was transformed into XL1-Blue *E.coli* (Agilent Technologies). The following reagents were all purchased from Sigma Aldrich: glutathione reductase (GR) from yeast, reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), 2-hydroxyethyl disulphide (HEDS), bovine plasma thrombin, kanamycin, isopropyl ß-D-1-thiogalactopyranoside (IPTG), tris(2-carboxyethyl) phosphine (TCEP), indanyloxyacetic acid (IAA-94). All other reagents used were of analytical grade.

Cell culture

HDF cells were grown in Human fibroblast media supplemented with LSGS and HKE cells in EpiLife media supplemented with HKGS. NIH/3T3 cells were grown in DMEM/F12 media supplemented with 5 %FBS. The cells were incubated at 37 °C 5 %CO_2 and passaged twice a week at a 1:5 dilution to maintain healthy growth. The cells were counted using a T20 cell counter (Bio-Rad) before being seeded in either 96/24/6-well cell culture plates and were grown to ~90 % confluency before being subjected to different biochemical analysis and/or collected to prepare cell lysates for further characterisation. Cells were lysed and stored at −80 °C in RIPA lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 0.1 %SDS, 1 %sodium deoxycholate, 1 %NP-40, pH 7.6) and protein concentration was determined using the Pierce ™ BCA Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions.

siRNA knockdown of CLIC1 or CLIC4 in NIH/3T3, HDF and HKE cells

Knockdown of CLIC1 or CLIC4 or Double knockdown of both CLIC1 and CLIC4 were induced in HDF, HKE and NIH/3T3 cells using transient siRNA-based gene knockdowns using either CLIC1(sc-60400) or CLIC4 (sc-105213) siRNAs (Santa Cruz) along with a Scramble siRNA-A (sc-37007) used as a control. For ease of understanding, CLIC1 or CLIC4 knockdown cells are annotated as CLIC1-KD and CLIC4-KD respectively, CLIC1 and CLIC4 double knockdown cells as Double-KD and the scramble siRNA control as Scmb C. Transfection was carried out using Lipofectamine siRNAMAX (ThermoFisher Scientific) according to the manufacturer's instructions with the exception that a 20 pmol concentration of siRNA was used for HDF and HKE cells as concentration above that showed toxicity. For Double-KD cells 10 pmol of each CLIC1 and CLIC4 siRNA was added simultaneously to the cells. Successful knockdown was then assessed via western blot analysis of the cell lysates using rabbit anti-CLIC1 (mAb-53424, Cell Signaling) and mouse monoclonal anti-CLIC4 (sc-135739, Santa Cruz) antibodies with B-Actin (Invitrogen) used as the loading control at a 1:2500 dilution. The day prior to transfection, cells were seeded at a cell density of 2.5×10^4 cells per well in a 6 well plate and incubated over-night at 37 °C 5 %CO_2 in their respective media. siRNA Transfected cells were allowed to grow for a period of 3 days, thrice washed with PBS before they were subjected to different biochemical analysis and/or collected to prepare cell lysates for further characterisation.

Transfection of NIH/3T3 cells for CLIC1 or CLIC4 overexpression

NIH/3T3 cells were harvested and resuspended with 10 %FBS growth medium and transferred to Gene Pulser 0.4 cm Cuvette (Bio-Rad) to which 5 μg of DNA plasmid (either AF-CLIC1, AF-CLIC4 or vector control pIRES2-EGFP) were added to each respective cuvette with the addition of 10 μL 1 M HEPES Buffer, pH 7.2 (Gibco). The cells were electroporated using the GenePulser MXCell Unit (Bio-Rad) at: 240 V and capacitance of 1000μF and then harvested and washed with cold PBS by centrifugation. The cells were then resuspended in sorting buffer (PBS containing 5 %FBS and 5 mM EDTA, pH 7.2), analysed and sorted on the BD Influx (BD Biosciences) into 96 well collection plates containing 200 μL full growth media. Stable EGFP+ cells were grown until confluent and stocks were prepared with FBS containing 10 %DMSO and were stored in liquid nitrogen. For experimental purposes, stable overexpressing NIH/3T3 cells transfected with CLIC1 (AF-CLIC1), CLIC4 (AF-CLIC4) or empty pIRES2-EGFP vector were grown to ~90 % confluency before being subjected to different biochemical analysis and/or collected to prepare cell lysates for further characterisation.

CLIC1, CLIC4 or CLIC3 recombinant protein expression and purification

Glycerol stocks of ClearColi BL21 (DE3) cells transformed with the His-tagged PET28a (+) expression vector (Novagen) containing the coding sequence for either human CLIC1 (NP_001279); CLIC4 (NP_039234) or CLIC3 (NP_004660.2) were used to express either rCLIC1, rCLIC4 or rCLIC3 recombinant proteins for purifications by methods as previously described [16]. Briefly, the recombinant CLIC proteins (rCLIC) were grown in 2xYT medium containing 30 μg/mL kanamycin (Sigma Aldrich) and inducted with 1 mM IPTG (Sigma Aldrich) at 20 °C with overnight shaking at ~180 rpm. Cells were then harvested and the His-tagged rCLIC proteins were purified via affinity chromatography using a Ni^{2+}-NTA (Qiagen) column. The His-tag was removed by in-column thrombin enzymatic cleavage using an overnight incubation of bovine plasma thrombin (Sigma Aldrich) (30 NIH units per 1 L of bacterial culture) at 4 °C. The cleaved rCLIC proteins were then collected in PBS buffer (10 mM phosphate buffer, 2.7 mM KCl, 140 mM NaCl, pH 7.4, and 0.5 mM TCEP) and further purified through size exclusion chromatography (SEC) (AKTA Pure/Amersham Pharmacia Biotech) using a HiPrep™ 16/60 Sephacryl® S-100HR column (Sigma Aldrich) and equilibrated in column sizing buffer (100 mM KCl, 20 mM HEPES, pH 7.2). rCLIC proteins purity was verified via Western blot analysis using their respective anti-CLIC antibodies (Santa Cruz) and anti-His antibody. Protein concentrations were measured using the Pierce ™ BCA Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions and functional activity was assessed using the HEDS assay (details below). The purified samples were then aliquoted and stored at −80 °C for future experiments.

Treatment of HDF and HKE cells with purified rCLIC proteins

Cell Viability assay was carried out to determine the effect of different concentrations of either rCLIC1, rCLIC4 or rCLIC3 on both HDF and HKE cells. Varying concentrations of either rCLIC1 or rCLIC4 ranging from 0 to 0.75 μM were prepared in either HDF or HKE media with no supplement. Cells were seeded in 96 well plates in a concentration of 2×10^4 cells per well and cultivated for 24 h in media (no supplement). Then 100 μL of different concentrations of either rCLIC1, rCLIC4 or rCLIC3 in media (no supplement) were then exogenously added to the cells and incubated for a period of 1 hour. After rCLIC incubation, the cells were again washed thrice and the effect of different concentrations of rCLICs on cell viability was measured using the WST-1 reagent as mentioned below. To indirectly demonstrate that the changes in cellular activity is likely associated with rCLIC protein activity, the drug IAA-94 at a concentration of 1 μM was pre-incubated with the recombinant proteins for an hour on ice before being added to the cells. For each experiment, data was collected from 3 different passages with each passage ran in triplicate for both HDF and HKE cells.

Immunofluorescent staining to determine Intracellular Uptake of recombinant rCLICs by cells in culture: HDF cells were treated with 0.2 μM recombinant proteins, either non-His-tagged or His-tagged rCLIC1 or rCLIC4 and incubated for 1 hour at 37 °C, 5 %CO_2. After which, the cells were washed thrice, fixed with 4 %paraformaldehyde for 10 mins and permeabilized with 0.1 %Triton X-100 in PBS for 10 mins at room temperature. The cells were rinsed with PBS-T (0.1 % Tween 20 in PBS), blocked with 1 % (w/v) Bovine serum albumin (BSA) in PBS-T for 1 h and then incubated with either mouse monoclonal anti-CLIC1 (sc-374202) or anti-CLIC4 (sc-135739) or anti-His-tag (sc-57598) antibodies overnight. After incubation, the cells were washed again with PBS-T and incubated with goat anti-mouse IgG FITC (Sigma Aldrich, 1:200 in 1 %BSA) for 1 h at room temperature. After secondary antibody incubation, cells were washed with PBS-T and nuclear staining was performed using DAPI (4′,6-diamidino-2-phenylindole) mounting medium (Life Technologies), and immunofluorescence images were obtained using the Olympus BX51 Upright Fluorescent Microscope with the cellSens Standard software.

Effects of rCLICs on the viability of human skin cells challenged with hydrogen peroxide

To determine any possible cellular protective and antioxidant roles of CLICs, cells were treated with H_2O_2 to induce oxidative stress. HDF and HKE cells were treated with 0.2 mM H_2O_2 while NIH/3T3 cells were treated with 1 mM H_2O_2 (these H_2O_2 concentrations had been predetermined empirically to cause a reduction in cell viability by no >25 % in control cells in the respective cell types). Cells were first treated with rCLICs in varying concentration range or at optimal dosage in media without supplement for 1 hour and then washed thrice to remove any exogenously added recombinant protein from the well. The cells were then treated with hydrogen peroxide (H_2O_2) for 1 hour to induce oxidative damage. Cells not treated with H_2O_2 is annotated as No H_2O_2 or when treated with H_2O_2 to induce a 25 % cell damage is annotated as + H_2O_2 in Figures later. Cells not treated with rCLICs or subjected to oxidative stress, cells not treated with rCLICs but subjected to oxidative stress and cells treated with rCLICs but not subjected to oxidative stress were used as experimental controls. Following different treatments, cell viability was measured using the WST-1 reagent as mentioned below. For each experiment, data was collected from 3 different passages with each passage ran in triplicate for all the cell types.

Cell viability assay

Cell viability was measured using a colorimetric assay for 96/6-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoliummonosodium salt (WST-1) reagent according to the manufacturer's instructions. Briefly, all the different cells (including siRNA CLIC knockdown cells or stable overexpressing CLIC cells) following different treatments, were washed with PBS thrice and incubated with 10 %WST-1 reagent in media (without phenol red and no supplement) for a period of 4 h. Cell viability was measured using a TECAN-Infinite M1000 microplate reader at 440 nm with reference wavelength at 600-nm. Data was analysed using GraphPad Prism 10 and represented as percentage cell viability in comparison to that of the non-treated cells (cells not subjected to any sort of treatment) and as percentage cell protection where the difference in cell viability is compared between the rCLIC treated and non-treated cells in the absence or presence of oxidative stress. For each experiment, data was collected from 3 different passages with each passage run in triplicate for all the cell types.

HEDS assays using whole cell lysates of HDF, HKE and NIH/3T3 cells

In order to determine changes in the oxidoreductase activity, cell lysates collected from the different cells (including siRNA CLIC knockdown cells or stable overexpressing CLIC NIH/3T3 cells) following different treatments and their respective controls were subjected to HEDS assay. All HEDS enzyme assays were performed in a flat 96-well plate containing 10 μg final protein concentration added to a potassium phosphate buffer (5mM/pH 7) that contained 1 mM EDTA, 250 μM NADPH, 1 mM HEDS, and 0.5μg/mL GR. The mixture was incubated for 5 min at 37 °C, and the enzymatic reaction was initiated by the addition of GSH to a final concentration of 1 mM, to a final total volume of 200 μL / well. The consumption of NADPH was measured at A340nm using the TECAN-Infinite M1000 microplate reader. Statistical analysis was performed using either One-way ANOVA or Two-way Anova with Turkey's comparison and are presented as the Mean ± SD. For each HEDS assay, cell lysates were collected from 3 different passages with each passage run in triplicate for each of the different types of cells. To indirectly demonstrate that the change in oxidoreductase activity is likely associated with CLIC protein activity, 10 μM of IAA-94 was pre-incubated with the whole cell lysates for an hour on ice before being added to the HEDS assay mix. Buffer with or without 10 μM IAA-94 was used as control to ensure that the drug itself does not interfere with the assay.

Measuring reactive oxygen species (ROS) levels in different types of cells in culture

To detect the levels of reactive oxygen species (ROS), the cellular reactive oxygen species detection assay kit (Red Fluorescence) (Abcam) was used according to the manufacturer's instructions. Cells were seeded at a density of 2×10^4 cells per well in 96 flat black well plates (Corning) and incubated at 37 °C, 5 %CO_2 overnight. The following day the different types of cells were subjected to different treatments, after which the cells were washed thrice and the fluorescent stain applied to the cells. The samples were immediately read using the TECAN-Infinite M1000 microplate reader at Ex/m=520/605 nm. For NIH/3T3 cells, ROS levels were measured in NIH/3T3 cells overexpressing either CLIC1 or CLIC4 (AF-CLIC1/AF-CLIC4) or in siRNA knockdown (CLIC1-KD/CLIC4-KD) cells and their respective controls: Scmb C, pIRES2-EGFP and NIH/3T3 cell control either No H_2O_2 or treated with 1 mM H_2O_2. For HDF and HKE cells, the cells were treated with either rCLIC1 or rCLIC4 for 1 hour followed by either No H_2O_2 or 0.2 mM H_2O_2 treatment for 1 hour. HDF and HKE Cells not treated with rCLICs were used as Control. For each experiment, data was collected from 3 different passages with each passage run in triplicate.

Characterisation of antioxidant activity of exogenously added rCLICs and rCLIC1 mutants in comparison to well-known antioxidants

The following annotations will be used to refer to each mutant, with each containing a single amino acid substitution to alanine: C24A and K37A. The cDNA encoding the wild-type His-CLIC1 fusion protein (NP_001279), cloned into the pET-28a vector, was used to generate the point mutations using the QuikChange site-directed mutagenesis kit (Stratagen, La Jolla, USA) according to the manufacturer's instruction, sequenced at Macrogen Inc. (Seoul, Korea) to confirm the incorporation of the correct mutations. Following the conformation of the correct point mutation, each plasmid encoding a particular CLIC1 mutant was used to transform *E.coli* BL21 (DE3) pLysS strains for expression of the recombinant CLIC1 mutant proteins as previously described above. HDF cells were plated and treated as described above with the exception that the cells were treated with either rCLICs or different rCLIC1 mutants before being subjected to oxidative stress. Cells were also treated with equimolar concentration of enzymatic antioxidants like Glutaredoxin (Grx), GST-Omega (GST-Ω) and 4 mM of N-acetylcysteine (NAC) as positive controls.

Effect of rCLIC treatment on either CLIC1 or CLIC4 siRNA knockdown HDF cells

To better understand the effects of rCLICs treatment, knockdown of CLIC1 or CLIC4 were induced in HDF cells using transient siRNA-based gene knockdowns as described above. siRNA CLIC1-KD/CLIC4-KD cells were incubated with either rCLIC1 or rCLIC4 in HDF media (no supplement) for 1 hour followed by either no treatment or treatment with

0.2 mM H_2O_2 for 1 hour. Cell viability was determined using the WST-1 reagent (Sigma Aldrich) according to the manufacture's instruction.

Immunofluorescence (IF) imaging of CLIC1, CLIC3, and CLIC4 in human skin tissue sections and in varying aged murine skin sections

Commercially prepared Human skin paraffin sections were purchased from Banksia Scientific/Zyagen (HP-101) and consisted of 17 (male), 55 (female), and 96 (female)-year-old donor tissue as a custom order. Whole murine skin sections were generously donated by Fiona Ryan (Facilities Manager, Technical Services Research at the Ernst Facility, University of Technology Sydney) using previously culled murine. Approval from the UTS Animal Care and Ethics Committee (ETH2017-1402). The murine consisted of TLR4/Rage strains of varying age groups from date of birth: 1.5 (male), 4 (female), and 8 (female) months. IF was used to observe the expression patterns of CLIC1, CLIC3, and CLIC4 in the Human and Murine Skin Paraffin Sections using mouse monoclonal anti-CLIC1 (SAB1403677-100UG, Sigma Aldrich), mouse monoclonal anti-CLIC3 (WH009022M2-100UG, Sigma Aldrich) and rabbit anti-CLIC4, N-terminal (SAB4501098-100UG, Sigma Aldrich) antibodies. Sections were de-paraffinized in absolute Xylene followed by washes in decreasing alcohol gradients and then water and PBS-T. Afterwards, the samples were blocked for 1 hour with 5 % normal goat serum (NGS) in PBS-T. The primary antibodies were added respectively at a 1:100 dilution on each tissue section in PBG (PBS pH 7.4, 5 % normal serum, 0.2 %Triton-X, 0.04 %BSA) and incubated overnight with their respective antibodies at 4 °C. Primary antibody was omitted from the negative controls. The following day, the samples were incubated for 2 h at room temperature with the following secondary antibodies: Alexa Fluor® (AF)-488 Goat anti Mouse (Life Technologies-A11001) or AF-488 Goat anti Rabbit (Invitrogen-A11008) in a 1:200 dilution in PBG and the nucleus was stained using a 1:5000 Hoechst solution (Invitrogen-33342). Samples were imaged using the Olympus BX51 Upright Fluorescent Microscope with the cellSens Standard software. The images were further processed in Fiji/ImageJ to isolate the epidermal network for area of fluorescence and integrated density (Area times Mean Gray Value).

Results and discussion

In vitro cell studies of CLIC1, CLIC4 or double CLIC1&CLIC4 knockdowns

To better understand the role of CLICs in fibroblast and keratinocyte cells, transient siRNA-gene knockdown of either CLIC1 (CLIC1-KD), CLIC4 (CLIC4-KD) or double knockdown (Double-KD) was induced in different cell types using Lipofectamine siRNAMAX (ThermoFisher) according to the manufacturer's instructions. Fig. 2 shows the western blot analysis and their corresponding densitometry to confirm knockdown or down-regulation of CLIC protein expression, along with cell viability studies, in the different cell types. Loading controls and original gels are included in supplementary material see Supplementary Figure S1.

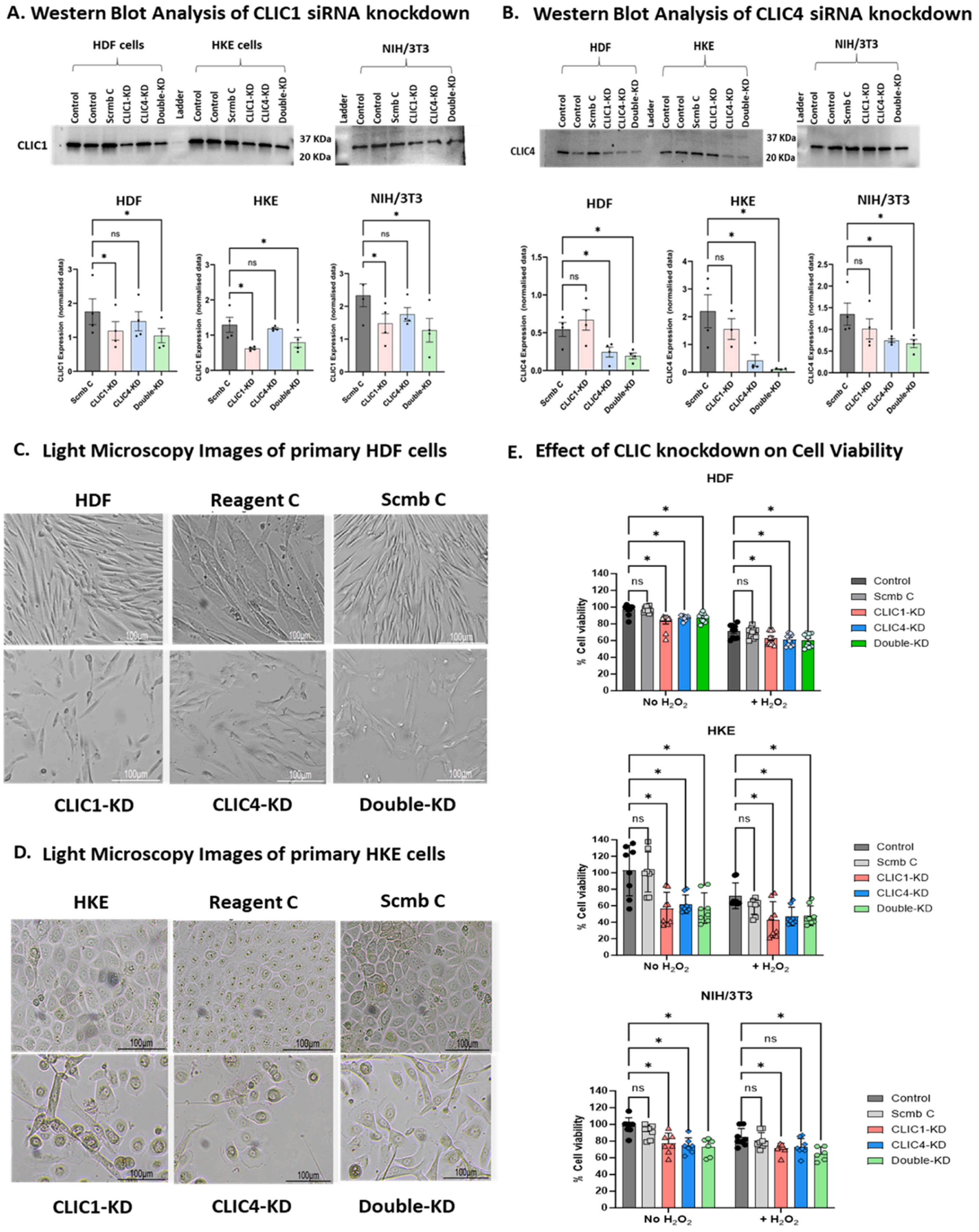
As seen in Fig. 2A, the blots clearly show successful knockdown of CLIC1 which is evident from the low intensity bands and the corresponding CLIC1 densitometry results showing a significant (p value < 0.05) reduction in CLIC1 protein expression by both CLIC1-KD and Double-KD cells in comparison to the Control and Scmb C in all the different cell types. There was no significant difference in protein expression between the non-treated (Control), lipofectamine reagent control (LipF) and Scmb C cells for all the different cell types (see Supplementary Figure S2). Similar results were also obtained with CLIC4 siRNA treatment as seen in Fig. 2B, low intensity bands are clearly visible in CLIC4-KD and Double-KD cells in all the three blots shown for the HDF, HKE and NIH/3T3 cells showing significant reduction in CLIC4 expression in comparison to the controls and this was further verified from the densitometry analysis. HDF and HKE cells showed greater down-regulation of CLIC4 expression in comparison to the murine NIH/3T3 cells. Despite not achieving a 100 % knockdown, these results clearly indicate down-regulation of either CLIC1 or CLIC4 or both the proteins in Double-KD cells in all the three different cell types. Furthermore, as seen in Fig. 2A and 2B, immunoblotting for CLIC4 indicated that CLIC1 siRNA did not target CLIC4 expression in CLIC1-KD cells and similarly, CLIC1 expression levels were not affected by CLIC4 siRNA across all the different cell types. Our results are comparable with numerous other studies, which have also shown siRNA treatment leads to reduced or down-regulation of CLIC1 or CLIC4 expression, but could not achieve complete knockdown of these proteins without inducing severe cell death [20,27–32].

As can be seen from the light microscopy images (Figs. 2C and 2D) there are clear observable differences in cell appearance, where knockdown or down-regulation of either CLIC1/CLIC4 and both the CLICs in Double-KD cells showed greater cell damage whereas there was no visible difference between the Control and Scmb C cells. Also, the reagent control (without any siRNA) showed no impact on cell viability thereby suggesting that the cellular damages seen in the knockdown cells is likely induced by changes in CLIC expression. Changes in cell viability was determined using WST-1 reagent and results are shown in Fig. 2E. CLIC1-KD resulted in a percentage reduction in cell viability of approximately 20 ± 7.5 %, 41 ± 10.4 % and 22 ± 14 % in HDF, HKE and NIH/3T3 cells respectively. Likewise, CLIC4-KD also caused percentage reductions in cell viability of approximately 23 ± 6 %, 38 ± 9.4 % and 28 ± 10.3 % in HDF, HKE and NIH/3T3 cells respectively. Both the murine and human fibroblast cells (NIH/3T3 and HDF) showed similar results, whereas the human keratinocyte cells were clearly more sensitive to CLIC knockdowns. A study by Suh KS et.al. (2007) showed that knockdown of CLIC4 protein by antisense or shRNA prevents Ca^{2+}-induced keratin 1, keratin 10 and filaggrin expression and causes cell cycle arrest in both human and murine keratinocytes undergoing differentiation [30]. This might explain the significant reduction in cell viability that was observed for the keratinocyte cells as seen in Fig. 2. To better understand the possible role of CLICs as antioxidants, like similar to that of GST-Omega and Grx, single or double siRNA knockdown cells were treated with H_2O_2 to induce oxidative stress (Fig. 2E). Both CLIC1-KD or CLIC4-KD cells showed significant reduction in cell viability in comparison to the Control and Scmb C cells in the presence of H_2O_2, thereby suggesting that down-regulating CLIC1 or CLIC4 expression makes these human skin cells more susceptible to H_2O_2-induced oxidative stress leading to greater damage.

Interestingly, simultaneously down regulating both CLIC1 and CLIC4 protein expression in Double-KD cells also showed significant reduction in cell viability both in the absence and presence of H_2O_2 in all the three different cell types. However, there was no significant difference between the Double-KD and the CLIC1-KD or CLIC4-KD cells indicating that there is no apparent additive effect of double knockdown of both CLIC1 and CLIC4 on the cell viability of these cells. A study by Uretmen Kagiali et.al. (2019) showed knockdown of CLIC1 or CLIC4 caused regression of the cleavage furrow at late cytokinesis leading to multinucleated HeLa cells, however no difference was observed between the single and double knockdown cells which led them to suggest that CLIC1 and CLIC4 do not act independently from each other and are most likely involved in the same pathway during cell division [33]. Also, the same study showed that mutating two different glutathione S-transferase activity–related residues in CLIC4 diminished its nuclear translocation resulting in incomplete completion of cytokinesis in mammalian cells [33].

Effects of CLIC1, CLIC4 or double knockdown on total cell oxidoreductase activity

Total cell oxidoreductase enzymatic activity in the whole cell lysates was measured using the HEDS enzyme assay. This assay has been used extensively in the characterisation of the glutaredoxin family



(caption on next page)

Fig. 2. Western Blot and Densitometry Analysis of CLIC1, CLIC4 or Double siRNA knockdown of HDF, HKE and NIH/3T3 whole cell lysates and cell viability assay. A) Western blots and the corresponding densitometry analysis of Scramble Control (Scmb C, silver), CLIC1-KD (red), CLIC4-KD (blue) and Double-KD (green) whole cell lysates probed for CLIC1, with β-actin used as loading control. **B)** Western blots and the corresponding densitometry analysis of Scmb C (silver), CLIC1-KD (red), CLIC4-KD (blue) and Double-KD (green) whole cell lysates probed for CLIC4, with β-actin used as loading control. Samples were collected from 4 different passages for each cell type, data shown as mean ± SEM, analysed using multiple unpaired *t*-test, $^*P < 0.05$. **C)** Light Microscopy Images of primary HDF cells after single or double siRNA knockdown of CLIC1 and/or CLIC4 **D)** Light Microscopy Images of primary HKE cells after single or double siRNA knockdown of CLIC1 and/or CLIC4. Images were taken using an Olympus BX51 Upright Fluorescent Microscope with a 20x magnification. **E)** Percentage Cell Viability of HDF, HKE and NIH/3T3 cells following single or double siRNA knockdown of CLIC1 and/or CLIC4 compared to cells not treated (Control, black) as control in the absence or presence of 0.2 mM H_2O_2. Percentage Cell Viability is expressed as Mean ± SEM. One-way ANOVA with Tukey's multiple comparisons test was done. $^*P < 0.05$, Data was collected in triplicate from each of the 3 different passages of each cell type. Full-length blots for 2A and 2B are shown in supplementary figures S1B and S1C respectively.

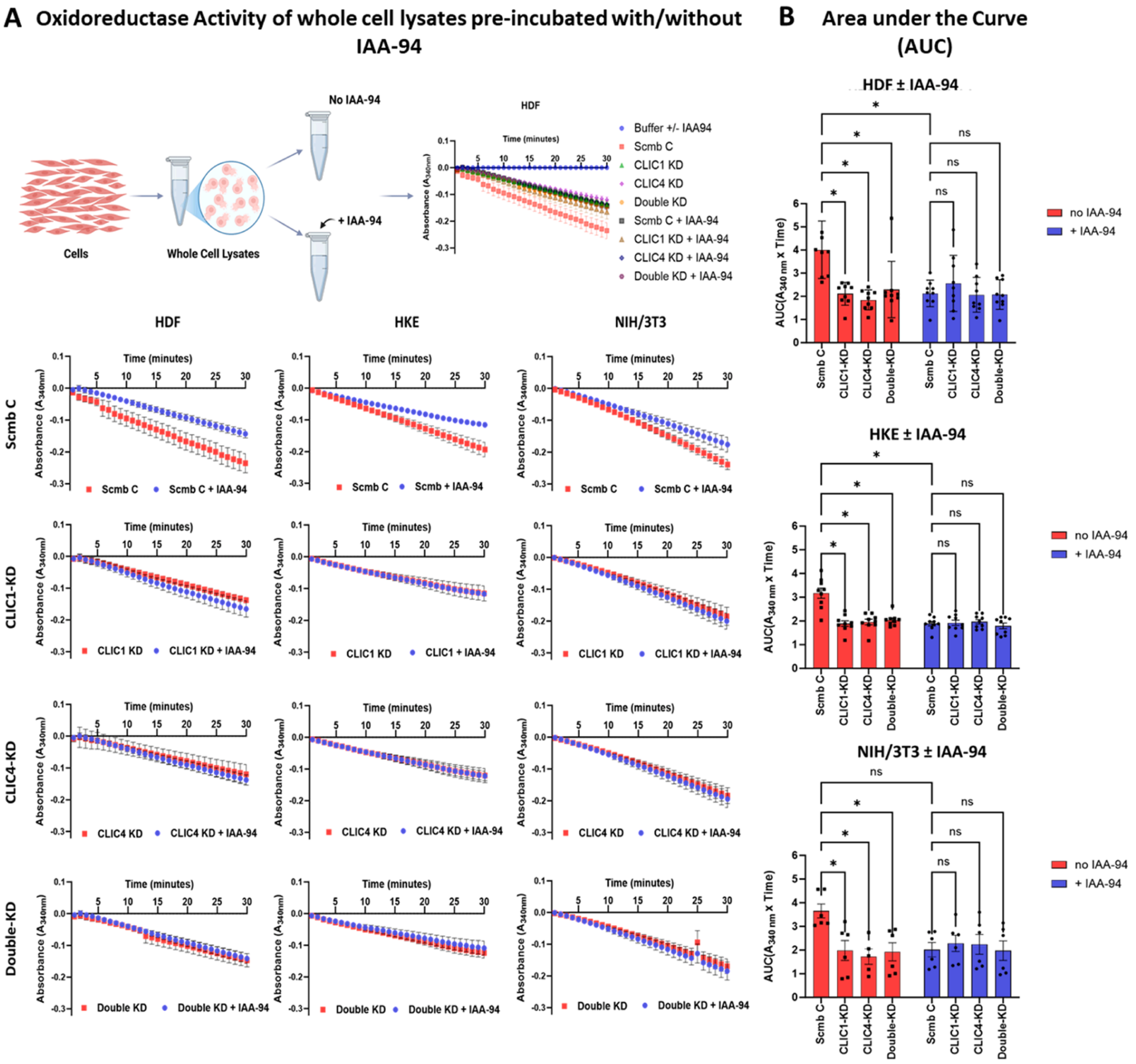


Fig. 3. Comparing Changes in Oxidoreductase Activity of CLIC1-KD, CLIC4-KD or Double-KD HDF, HKE or NIH/3T3 cells in the absence or presence of IAA-94 ion channel blocker. A) XY plot showing the oxidoreductase activity of whole cell lysates of Scmb C, CLIC1-KD, CLIC4-KD and Double-KD for HDF (left), HKE (middle) and NIH/3T3 (right) cells in the absence (red) or presence of IAA-94 (blue). **B)** Comparison of the AUC in the absence (red) or presence (blue) of the drug IAA-94 in HDF (top), HKE (middle) and NIH/3T3 (bottom) cells. Two-way ANOVA with Tukey's multiple comparisons test was done. All Data are expressed as Mean ± SD. $^*P < 0.05$, ns= not significant. Data was collected in triplicate from each of the 3 different passages for each cell type.
Schematic Image was created in BioRender. Hossain, K. (2025) https://BioRender.com/v05r913.

oxidoreductase activity, thus it measures all Grx-like oxidoreductase enzymatic activity in the cells [16,34]. Fig. 3 shows the oxidoreductase activity of the whole cell lysates collected from single or double CLIC siRNA knockdown cells of HDF, HKE and NIH/3T3 cells and the corresponding area under the curve calculations. Lysates were also assayed following pre-incubation with the blocker drug IAA-94. As seen in Fig. 3A, lysates from CLIC1-KD, CLIC4-KD and Double-KD cells all showed significant decrease in their oxidoreductase activity in comparison to the control, Scmb C, in all the three different cell types in culture, strongly suggesting CLIC1 and CLIC4 are contributing to the total oxidoreductase activity of the cells. To further show specific activity related to CLIC proteins, the whole cell lysates were also pre-incubated with the inhibitor drug IAA-94, known to block both the ion channel [35,36] and enzymatic activity of the CLIC proteins [16,18]. A decrease in enzymatic activity was seen for all samples following pre-incubation with IAA94 (Fig. 3B), albeit the reduction in the control sample did not reach significance. These findings further validate that the CLIC proteins contribute significantly to the total oxidoreductase activity in cells. This is most clearly demonstrated in the control Scmb C cells, which express normal levels of CLIC1 and CLIC4 proteins.

These findings strongly suggest that down-regulation of CLIC1 and CLIC4 in fibroblast and keratinocyte cells reduces their oxidoreductase activity, and also impacts their cell viability. It can be speculated that in the knockdown cells, their reduced oxidoreductase activity, likely contributes to their increased susceptibility to oxidative damage, while the control Scmb C cells expressing CLICs, were less susceptible to H_2O_2-induced oxidative damage (Fig. 2E). However, whether this decrease in activity is directly associated with the reduction seen in cell viability warrants further investigation. Also, it is important to note that the cell lysates contain a plethora of other oxidoreductase enzymes (most

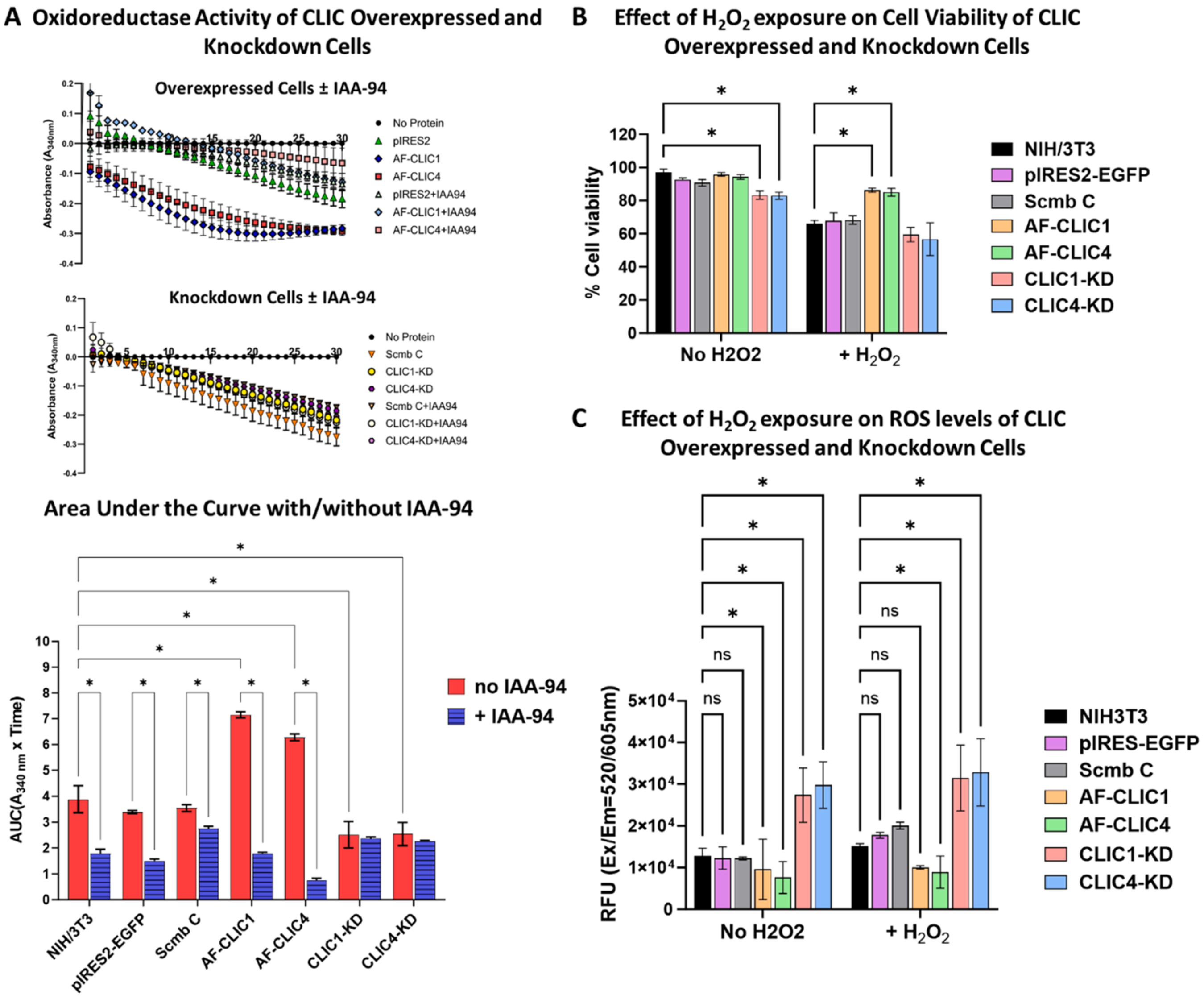


Fig. 4. Oxidoreductase activity, Cell Viability and ROS Levels for NIH/3T3 Cells Overexpressing or Knockdown for CLIC1 or CLIC4. A) Comparing changes in Oxidoreductase Activity of CLIC1 or CLIC4 Over-expressing or knockdown in NIH/3T3 cells ±IAA-94. XY plot showing the oxidoreductase activity of whole cell lysates either overexpressing (top) or knockdown (middle) CLIC1 or CLIC4 in the presence or absence of IAA-94 over a period of 30 min. Comparison of the Area under the Curve (bottom) of the over-expressed and knockdown cell lysates along with NIH/3T3 cells, pIRES-EGFP and Scmb C controls in the presence (stripped bar) or absence (solid bar) of IAA-94. **B)** Examining Changes in Cell Viability after Hydrogen Peroxide treatment. **C)** Examining changes in cellular ROS Levels after Hydrogen Peroxide treatment. In each figure, the results are shown for overexpressing cells: AF-CLIC1 (orange) and AF-CLIC4 (olive); siRNA knockdown cells: CLIC1-KD (red) and CLIC4-KD (blue) and the three control cell lines: NIH/3T3 (black), empty vector pIRES2-EGFP (purple) and Scmb C (silver). Data expressed as Mean ± SD. Two-way ANOVA with Tukey's multiple comparisons test was done. $^*P < 0.05$, Data was collected from three different passages with each passage run in triplicate.

notably Grxs and Trxs) and specifically the Grx enzymes can also be detected by the HEDS assay, which likely also contributed to the overall oxidoreductase activity measured in the whole cell lysates. Furthermore, IAA-94 may also be inhibiting other non-CLIC oxidoreductase proteins, therefore these results need to be interpreted accordingly and warrant further investigation.

Hence, to better delineate the role of CLIC1 and CLIC4 in these cells, we also explored the effects of increased levels of CLIC1 or CLIC4 proteins on the various cells. As such, we generated stable CLIC1 or CLIC4 over-expressing NIH/3T3 cells and subjected these to further analysis. A number of attempts to overexpress either protein in the primary human skin cells (HDF and HKE) were not successful, therefore only data for murine NIH/3T3 cells were obtained and shown below.

Effect of CLIC1 or CLIC4 overexpression in NIH/3T3 cells

Stable overexpressing CLIC1 (AF-CLIC1) or CLIC4 (AF-CLIC4) murine fibroblast cell lines, along with an empty vector control NIH/3T3 cell (pIRES2-EGFP) were generated. Western blot analysis of AF-CLIC1, AF-CLIC4 and pIRES2-EGFP control and their corresponding densitometry and the effect of overexpressing either CLIC1 or CLIC4 on the percentage cell viability is shown in Supplementary Figure S3. Overexpression of CLIC1 and CLIC4 was confirmed from blots probed with anti-Flag antibody which showed distinct bands for FLAG-tagged-CLIC1/4 and with anti-CLIC1 or anti-CLIC4 antibodies which confirmed overexpression. Cell viability analysis showed no significant difference between the overexpressing cells and the empty vector control (Supplementary Figure S3B), thereby suggesting that, unlike down-regulation, overexpression of either CLIC1 or CLIC4 has no obvious deleterious effects on the cells. To explore the role of CLICs in fibroblast cells, these stable AF-CLIC1 and AF-CLIC4 overexpressing cell lines, along with the knockdown cells (CLIC1-KD and CLIC4-KD) and their respective control (pIRES2-EGFP, Scmb C and NIH/3T3 cells), were subjected to further analysis to determine their oxidoreductase activity, cell viability and ROS levels (Fig. 4).

In Fig. 4A, AF-CLIC1 or AF-CLIC4 overexpressing cells showed significant increase in their oxidoreductase activity in comparison to the controls and when whole cell lysates were pre-incubated with the drug IAA-94, there was a significant decrease in this activity. On the other hand, CLIC1-KD and CLIC4-KD knockdown cells showed significantly reduced activity in comparison to the controls and there was no difference in activity upon treatment with IAA-94 (as previously seen in Fig. 3). There was also no significant difference in the oxidoreductase activity seen between the controls: NIH/3T3 cells, Scmb C and pIRES2-EGFP, with all the controls showing significantly reduced activity when pre-incubated with IAA-94. These findings therefore support the idea that the increase or decrease in oxidoreductase activity, as seen for AF-CLIC1/AF-CLIC4 or CLIC1-KD/CLIC4-KD cells respectively, is attributed to the overexpression or down-regulation of either CLIC1 or CLIC4 proteins by the cells respectively.

In order to corroborate the protective roles of CLIC proteins, cells were treated with pre-determined concentration of H_2O_2 for 1 hour, to induce oxidative damage resulting in no greater than a 25 % reduction in total cell viability. Changes in cell viability between the overexpressing or knockdown cells and the controls were measured and data shown in Fig. 4B H_2O_2 concentration of 1 mM were detrimental to the CLIC1-KD and CLIC4-KD cells, resulting in greater than 40 ± 4 % cell damage in comparison to only 13 ± 2.8 % and 15 ± 6 % cell damage shown by AF-CLIC1 and AF-CLIC4 cells respectively and 30 ± 2.5 % cell damage shown by the Control cell lines. This clearly shows that cells overexpressing either CLIC1 or CLIC4 proteins are less susceptible to H_2O_2 induced oxidative damage in comparison to their counterpart knockdown and control cell lines. This was also evident from Fig. 4C, which clearly shows AF-CLIC1 and AF-CLIC4 cells display significantly lower levels of ROS following treatment with H_2O_2 in comparison to the controls. On the other hand, CLIC1-KD and CLIC4-KD cells show a significant 2-fold increase in the ROS levels and hence were more susceptible to H_2O_2-induced oxidative damage. In the absence of hydrogen peroxide treatment, the overexpressing cells also had lower native ROS levels while the knockdown cells had higher basal ROS levels. These results further support CLIC1 and CLIC4 playing a significant role in maintaining cellular ROS levels.

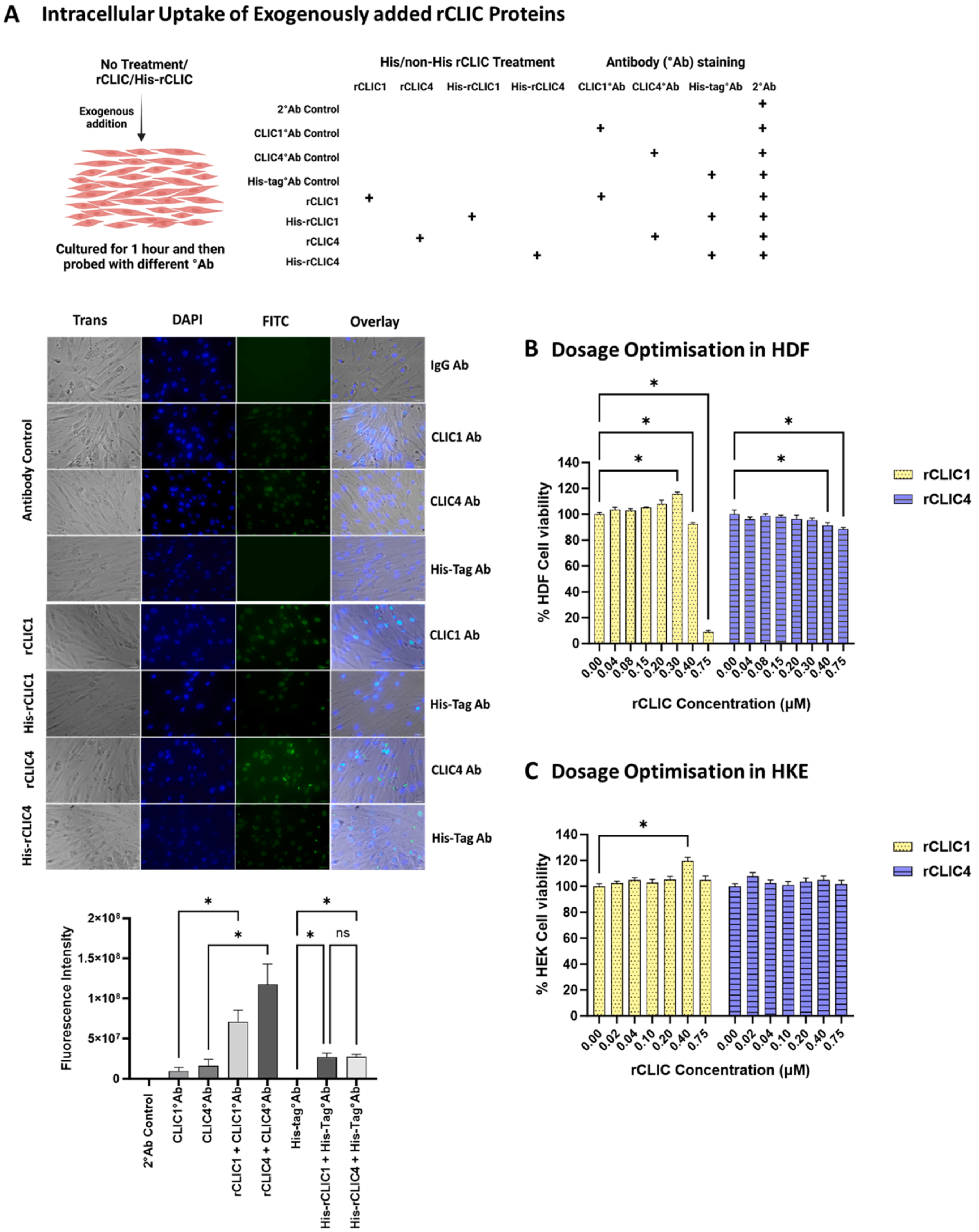
A recent study by Al Khamici H et al. (2023) revealed that deletion of CLIC4 from murine 6DT1 breast tumor cells enhanced the accumulation of ROS, increased superoxide generation, and the cells were more susceptible to oxidative damage [20]. Upon treatment with H_2O_2, cells lacking CLIC4 were sensitised to apoptosis, on the other hand, in control 6DT1 cells (expressing CLIC4) H_2O_2 treatment increased the expression of both CLIC4 mRNA and protein and showed protection against oxidative damage. They concluded that CLIC4 helps maintain the redox-homeostasis and mitochondrial functions in 6DT1 cells [20]. Similarly, CLIC1 has also been shown to function as a negative regulator of ROS in other tumors, with the depletion of CLIC1 by siRNA in human esophageal squamous cell carcinoma, induced apoptosis via the JNK pathway, strongly associated with excessive ROS production [27,28]. Hence, we propose that CLIC1 and CLIC4 regulate cellular ROS levels, via their oxidoreductase activity, and form part of the cellular antioxidant defence system involved in maintaining redox homeostasis.

Exogenous addition of recombinant CLIC proteins to primary skin cells in culture

(A) Intracellular Uptake of Purified rCLIC Proteins and Dosage Optimisation

Previous studies have shown exogenous addition of well-known enzyme antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidase (Gpx) and Grx to cells, were able to protect cells from oxidative damage [37–43]. In order to determine if addition of purified rCLIC proteins could endow similar protective activity to primary skin cells in culture, it was first important to confirm both the optimal concentration to avoid toxic cell effects, and to demonstrate their uptake by the cells. Cellular uptake and absorption of exogenously added recombinant rCLIC proteins were confirmed by fluorescent microscopy as shown in Fig. 5A. HDF cells treated with His-tagged rCLIC1 or His-tagged rCLIC4 shows fluorescent staining with anti-His antibody confirming the intracellular uptake of the His-tag proteins by the cells. Since the His-tag proteins are not naturally expressed by the cells, any fluorescent staining with anti-His antibody indicates the intracellular uptake of the exogenously added proteins by the cells. This is also validated from the absence of FITC fluorescence in cells probed with anti-His antibody in the absence of His-tag rCLICs. The was approximately a 6-fold increase in the fluorescence staining seen in cells treated with rCLIC1 or rCLIC4 protein (non-His-tagged), when probed with anti-CLIC1 or anti-CLIC4 antibody, which also confirms the intracellular uptake of the exogenously added proteins. Cells not treated with rCLICs when stained with primary CLIC1 and CLIC4 antibody fluoresce, as expected, given both CLIC proteins are constitutively expressed in these cells. Absence of FITC fluorescence intensity in cells stained with secondary antibody only, confirms primary antibody, anti-CLIC specific staining.

The primary human skin cells were treated with either rCLIC1 or rCLIC4 at varying concentrations ranging from 0 to 0.75 μM for 1 hour, after which cell viability was measured using WST-1 reagent. Addition of rCLIC1 to HDF cells at concentrations ranging from 0 to 0.2 μM showed no significant change in cell viability compared to control cells (not treated with rCLIC), while concentrations > 0.3 μM showed significant changes in cell viability with 0.3 μM rCLIC1 showing 15.8 ± 3.4 % increase whereas concentrations of 0.4 and 0.75 μM, resulted in a decrease in cell viability by approximately 8 ± 2.06 % and a dramatic 90 ± 3.3 % respectively. Similar to rCLIC1, addition of rCLIC4 at higher concentrations of 0.4 and 0.75 μM also resulted in a decrease in cell

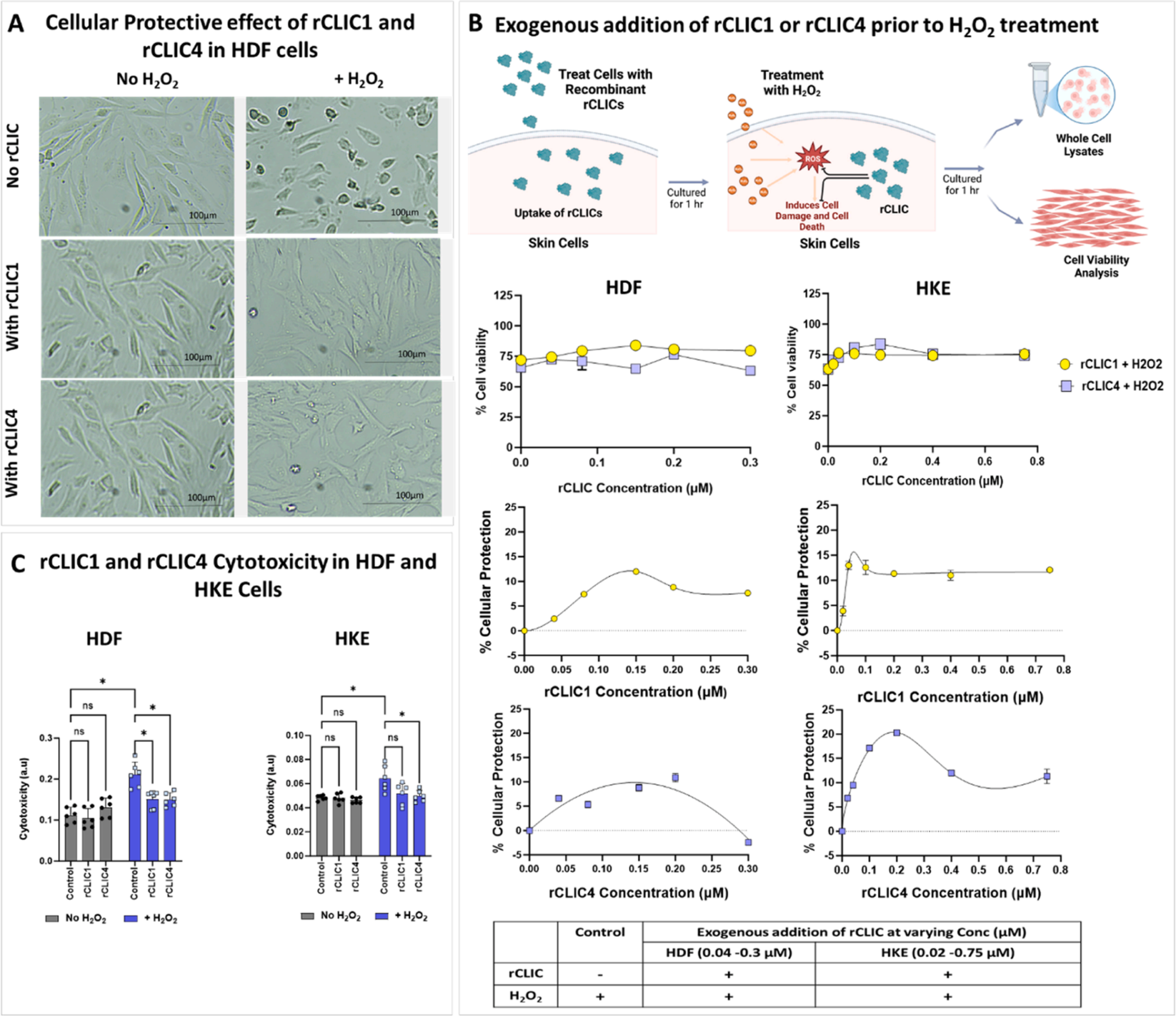


	rCLIC1	rCLIC4	His-rCLIC1	His-rCLIC4	CLIC1°Ab	CLIC4°Ab	His-tag°Ab	2°Ab
2°Ab Control								+
CLIC1°Ab Control					+			+
CLIC4°Ab Control						+		+
His-tag°Ab Control							+	+
rCLIC1	+				+			+
His-rCLIC1			+				+	+
rCLIC4		+				+		+
His-rCLIC4				+			+	+

(caption on next page)

Fig. 5. Intracellular Uptake and Dosage optimisation of rCLIC1 and rCLIC4 added to Human Dermal Fibroblast (HDF) and Human Epidermal Keratinocyte (HKE) cells. A) Intracellular uptake of exogenously added non-His tagged or His-tagged rCLIC1 and rCLIC4 proteins determined by fluorescence staining in HDF cells ($n = 3$). Images were further processed in Fiji/ImageJ to determine the fluorescence intensity and graphically presented using Prism10. Statistical analysis was done using One-way ANOVA with Tukey's multiple comparisons test. **B)** Effect of adding 0, 0.04, 0.08, 0.15, 0.2, 0.3, 0.4 and 0.75 μM of: rCLIC1 (yellow, dotted) or rCLIC4 (purple, stripped) to HDF cells. **C)** Effect of adding varying concentrations of 0, 0.04, 0.08, 0.15, 0.2, 0.3, 0.4 and 0.75 μM of either rCLIC1 (yellow, dotted) or rCLIC4 (purple, stripped) to HKE cells. Cell viability was measured using WST-1 reagent and represented as percentage cell viability compared to non-treated cells (no rCLIC added). One-way ANOVA with Tukey's multiple comparisons test was done. $^*P < 0.05$, Data represented as Mean ± SD. For each dosage optimisation experiment, data was collected from 3 different passages with each passage run in triplicate for both HDF and HKE cells.
Schematic image was created in BioRender. Hossain, K. (2025) https://BioRender.com/v05r913.

viability by approximately 10 ± 7.1 % and 20 ± 4.5 % respectively, with 0.75 μM rCLIC1 being more toxic to HDF cells in comparison to that seen for rCLIC4. On the other hand, addition of rCLICs to primary human keratinocyte cells showed no cellular toxicity and greater tolerance to higher concentrations tested, unlike the HDF cells (Fig. 5B). Only exogenous addition of rCLIC1 at 0.4 μM concentration showed significant increase of 19 ± 5.2 % in HKE cell viability. From these results, we determined the optimal working concentration of both rCLIC1 and



	Control	Exogenous addition of rCLIC at varying Conc (μM)	
		HDF (0.04 -0.3 μM)	HKE (0.02 -0.75 μM)
rCLIC	-	+	+
H_2O_2	+	+	+

Fig. 6. Exogenous addition of rCLIC1 and rCLIC4 to HDF and HKE cells shows protection against H_2O_2-induced oxidative damage. A) Light Microscopy images of HDF cells treated with/without rCLIC1/rCLIC4 for 1 hour prior to treatment with/without 0.2 mM H_2O_2. **B)** The percentage cell viability (top) and the percentage cellular protection (bottom two graphs) in comparison to Control shown by the different concentrations of rCLIC1 or rCLIC4 in HDF (left) and HKE (right) cells following H_2O_2 treatment. Cell viability was measured using WST-1 reagent. Data was further analysed to determine the percentage cellular protection shown by rCLIC1 (yellow) and rCLIC4 (purple) in comparison to the Control Cells (not treated with rCLICs but H_2O_2). Schematic representation of the experimental design was created in BioRender (https://BioRender.com/v05r913). **C)** The extent of cellular damage in HDF (left) and HKE (right) cells treatment with rCLICs prior to No H_2O_2 treatment (black) or following H_2O_2 treatment (blue). Two-way ANOVA with Tukey's multiple comparisons test was done. $^*P < 0.05$, ns= not significant. Data represented as Mean ± SD. For each experiment, data was collected from 3 different passages with each passage run in triplicate for both HDF and HKE cells.
Schematic representation of the methodology was created in BioRender. Hossain, K. (2025) https://BioRender.com/v05r913.

rCLIC4, when added to the cells, that neither impacted negatively on cell viability, nor induced cell proliferation. This was determined to be equal to, or less than, 0.3 μM for application to HDF cells; while the working concentration of the recombinant proteins for use with HKE cells, could be as high as 0.75 μM.

(B) Exogenous addition of either rCLIC1 or rCLIC4 to HDF and HKE cells shows protection against Hydrogen Peroxide induced oxidative damage:

In order to assess the rCLIC proteins' effectiveness as cellular antioxidant and protective agents when added exogenously, varying concentrations of rCLIC1 or rCLIC4 were added to HDF (0 - 0.3 μM) and HKE (0 - 0.75 μM) cells one hour prior to the cells being subjected to oxidative stress via treatment with 0.2 mM H_2O_2. As can be seen from the light microscopy images of HDF cells (Figs. 6A) there are clear observable differences in cell appearance, with the cells pre-treated with either rCLIC1 or rCLIC4 showing fewer damaged cells following H_2O_2 exposure for 1 hour, compared to equivalently exposed cells that had not received the rCLIC1 or rCLIC4 pre-treatment. This difference was quantified via use of WST-1 reagent and graphically displayed in Figs. 6B as the percentage cell viability and percentage cellular protection against H_2O_2-induced oxidative damage afforded by the different concentrations of rCLIC1 or rCLIC4 on HDF and HKE cells.

In Fig. 6B, HDF cells pre-treated with rCLIC1 at varying concentrations all demonstrate significantly higher tolerance to hydrogen peroxide exposure in comparison to control cells that had not been pre-treated with rCLIC proteins. There was a steady increase in cellular protection with increase in rCLIC1 concentration followed by gradual decrease in protection, with 0.15 μM rCLIC1 showing the greatest level of cell protection at approximately 12.5 %. Similarly, rCLIC4 also showed a gradual increase in protection followed by a decrease, with 0.2 μM rCLIC4 showing the greatest amount of protection of approximately 11 % in HDF cells. Interestingly, in HKE cells, rCLIC1 showed an initial steep increase in protection of approximately 15 % at lower concentrations of 0.1 μM followed by a stable cell protection of around 10 ± 2 % at concentrations ranging from 0.2 to 0.75 μM. On the other hand, rCLIC4 showed a similar pattern to that observed for rCLIC1 in HDF cells, where an increase in cellular protection was observed with increase in concentration from 0.02 to 0.2 μM then followed by a slight decrease at protein concentrations above 0.2 μM; with the greatest protection seen at 0.2 μM rCLIC4 which was similar to that observed for HDF cells. We also looked into the role of CLIC3 exogenously added to the skin cells, however, exogenous addition of CLIC3 at varying concentrations had no significant effect on the cell viability of skin cells both in the absence or presence of H_2O_2 (see Supplementary Figure S4).

Furthermore, to determine the extent of cellular damage in HDF and HKE cells following treatment with hydrogen peroxide in the absence and presence of rCLIC proteins, the CyQUANT™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific) was used in accordance with the manufacturer's instructions, with the results shown in Fig. 6C. Lactate dehydrogenase (LDH) is a stable enzyme whose release is often indicative of cellular cytotoxicity within cells that are undergoing different forms of cellular damage. The amount of formazan produced has been shown to correspond to the number of dead or damaged cells [44]. As seen in Fig. 6C, in the absence of oxidative stress (No H_2O_2), both HDF and HKE cells treated with rCLIC1 or rCLIC4 show no significant difference in cell cytotoxicity when compared to the Control cells (not pre-treated with rCLICs), thus further validating that exogenously added rCLIC1 or rCLIC4, do not induce cellular damage and are non-toxic at the optimal dosage concentrations used. Upon treatment with H_2O_2, both HDF and HKE control cells showed significant increase in their cytotoxicity levels as expected but when pre-treated with rCLICs showed significantly reduced cellular damage in comparison to the Control cells; with the exception that HKE cells pre-treated with rCLIC1, although reduced cellular damage was seen, this did not reach significance. This further corroborates the ability of CLIC1 and CLIC4 to protect the cells from H_2O_2 induced oxidative damage thereby resulting in reduced cellular damages.

(C) Measuring Oxidoreductase Activity, Reactive Oxygen Species (ROS) Levels and Cell Viability following rCLIC treatments in HDF and HKE cells:

Whole cell lysates were collected from HDF or HKE cells that had been pre-treated with rCLIC1 or rCLIC4 for 1 hour. The cell lysates were measured for Grx-like oxidoreductase activity via the HEDS assay, including lysates pre-treated with the blocker drug IAA94 for 1 hour prior to being assayed. Fig. 7A shows the oxidoreductase activity of the whole cell lysates of HDF and HKE cells and the corresponding area under the curve calculation in the absence or presence of IAA-94. In the absence of IAA-94, HDF cells pre-treated with either rCLIC1 or rCLIC4 showed significant increase in their oxidoreductase activity in comparison to the Control cells (no rCLICs). This increase in oxidoreductase activity can be attributed to the exogenously added rCLIC proteins following their intracellular uptake by the cells. This was further confirmed when the whole cell lysates were pre-incubated with the drug IAA-94, which showed significant decrease in their activity in comparison to that seen in the absence of IAA-94. Interestingly, in the presence of IAA-94, there were no significant differences in the oxidoreductase activity seen between the Control, rCLIC1 and rCLIC4 cells. Correspondingly, whole cell lysates collected from HKE cells also showed an increase in their activity, with the exception that only rCLIC1 treated HKE cells showed significant increase but not rCLIC4 treated cells. When pre-incubated with IAA-94, all the different treatments showed a significant decrease in their activity, again with no significant difference seen between the Control, rCLIC1 and rCLIC4 HKE cells. Previously (as seen in Fig. 4), changes in the oxidoreductase activity seen for CLIC overexpressing AF-CLIC1/AF-CLIC4 or knockdown CLIC1-KD/CLIC4-KD NIH/3T3 cells was clearly attributed to the overexpression or downregulation of either CLIC1 or CLIC4 proteins by the cells respectively. These findings also thereby strongly suggest that the increase in oxidoreductase activity is most likely attributed to the uptake of the exogenously added rCLICs by the cells, which in turn, is inhibited when pre-incubated with the blocker drug IAA-94.

It is evident from earlier results that exogenous addition of rCLIC1 or rCLIC4 to both HDF and HKE cells imparts cellular protection against H_2O_2-induced oxidative damage. Whether this protection is associated with changes in the cellular ROS levels was also determined and results shown in Fig. 7B HDF and HKE cells, not subjected to any oxidative assault (No H_2O_2) but treated with either rCLIC1 or rCLIC4, all showed a combined significant decrease of approximately 15–20 % in the basal ROS levels in comparison to the Control cells (not pre-treated with rCLICs). Pre-treating the skin cells with rCLICs prior to exposure to H_2O_2, all showed significant reduction in their cellular ROS levels whereas the Control cells (both HDF and HKE cells) showed significant increase in their ROS levels when exposed to H_2O_2 treatment as expected. These results complement our earlier findings (Fig. 4) which showed that NIH/3T3 cells overexpressing either CLIC1 or CLIC4 have significantly reduced basal ROS production, whereas there was a 2-fold increase in the ROS levels in CLIC1 or CLIC4 NIH/3T3 knockdown cells. This study demonstrates for the first time that recombinant CLIC1 and CLIC4 proteins exogenously added to primary human fibroblast and keratinocyte cells, can act as antioxidants, protecting cells against H_2O_2 induced oxidative damage and likely help regulate the redox environment of skin cells.

To further confirm the cellular protective roles of CLIC1 and CLIC4, purified rCLIC proteins at optimal dosage concentration of 0.2 μM were pre-incubated with 1 μM IAA-94 for 1 hour before being exogenously added to HDF or HKE cells and then exposed to H_2O_2 treatment (Fig. 7C). HDF/HKE cells treated with/without equimolar IAA-94 but not treated with rCLICs and not exposed to H_2O_2 treatment; and cells

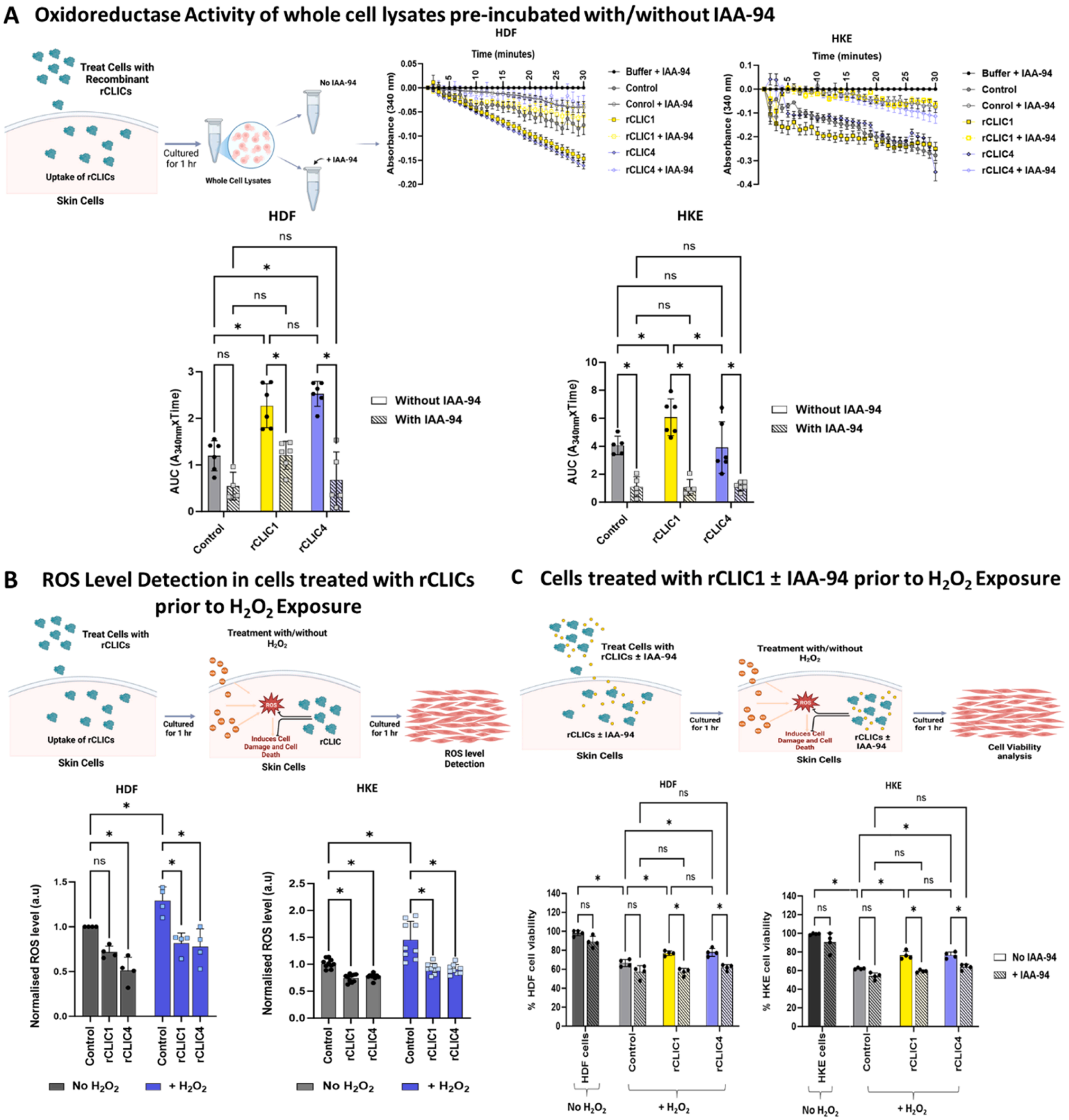


Fig. 7. Oxidoreductase Activity, Reactive Oxygen Species (ROS) Levels and Cell Viability following rCLIC treatments in HDF and HKE cells. A) Oxidoreductase activity and the corresponding Area under the Curve (AUC) of whole cell lysates collected from HDF (left) or HKE (right) cells pre-treated with rCLICs for 1 hour and then the cell lysates were either not pre-incubated or pre-incubated with the drug IAA-94 for 1 hour before being subjected to HEDS assay. B) Cellular ROS Level detection in HDF (left) and HKE (right) cells pre-treated with rCLICs prior to exposure to No H_2O_2 (black) or H_2O_2 (blue) treatment. C) Percentage Cell Viability of HDF (left) and HKE (right) cells pre-treated with rCLICs with/without IAA-94 for 1 hour prior to exposure to No H_2O_2 or H_2O_2 treatment. Control cells not treated with rCLICs are shown in silver and cells treated with rCLIC1 or CLIC4 are shown in yellow or purple respectively in all the graphs. Cells without or with IAA-94 are represented as solid or stripped bars respectively. HDF/HKE cells control shows the effect of IAA-94 only on cell viability in the absence of any treatment. Two-way ANOVA with Tukey's multiple comparisons test was done. $*P < 0.05$, ns= not significant. Data represented as Mean ± SD. For each experiment, data was collected from 3 different passages with each passage run in triplicate for both HDF and HKE cells.
Schematic representation of the methodology was created in BioRender. Hossain, K. (2025) https://BioRender.com/v05r913.

treated with/without equimolar IAA-94 and exposed to H_2O_2 but not treated with rCLICs (Control) were used as controls to determine any effect the 1 μM IAA-94 may have on cell viability. As seen in Fig. 7C, in the absence of pre-incubation with IAA-94, rCLIC1 or rCLIC4 exogenously added to HDF/HKE cells, prior to treatment with H_2O_2, showed a significant increase in cell viability in comparison to the respective Control cells thereby showing protection against H_2O_2-induced oxidative damage as expected. Whereas, rCLICs pre-incubated with IAA-94 and then added to either HDF or HKE cells prior to treatment with H_2O_2, showed no difference in cell viability compared to the Control, thus showing no protection. Addition of 1 µM IAA-94 only to HDF or HKE cells also had little to no effect on the cell viability of these cells in the presence or absence of H_2O_2. These findings strongly suggest that IAA-94 had no obvious effect on the skin cells, and pre-incubation of the rCLIC proteins with the drug IAA-94, failed to provide cellular protection against H_2O_2 treatment, likely due to inhibition of their enzymatic activity by IAA-94. This further corroborates the ability of CLIC1 or CLIC4 proteins to protect skin cells against H_2O_2-induced oxidative damage, contributing to the cell's overall health and viability. It can therefore be postulated that CLIC1 and CLIC4 proteins act as enzymatic antioxidants and help in regulating cellular ROS levels and thus cellular redox homeostasis within skin cells.

(D) Characterisation of antioxidant activity of exogenously added rCLIC proteins:

To further characterise the role of CLICs as antioxidants, their activity was compared to that of known endogenous protein antioxidants including Grx and GST-Omega 1 and with the small molecule antioxidant drug, N-acetyl cysteine (NAC), a precursor of L-cysteine known to act as a direct ROS scavenger due to its sulfhydryl group [45–47]. HDF cells were treated with equimolar amounts of rCLIC1, rCLIC4, rGrx (abcam, ab86987) or rGST-Ω (Merck, GS75) proteins or 4 mM NAC (optimal antioxidant concentration previously published by others [45–47]) for 1 hour and then cells were challenged with H_2O_2. The difference in cell viability between the Control cells (not treated with any antioxidants) and the treated cells after being exposed to H_2O_2 were calculated to determine the percentage cellular protection imparted by the different treatments, with data shown in Fig. 8A.

As seen in Fig. 8A, treatment with rCLIC1 or rCLIC4 protected HDF cells against H_2O_2-induced oxidative damage resulting in cellular protection of approximately 6.7 ± 0.6 % and 9 ± 0.6 % respectively. These results were comparable to that seen by the other well-known antioxidants like the Grx and GST-Ω proteins showing 10.6 ± 0.5 % and 8.6 ± 0.7 % cellular protection respectively, while NAC showed the greatest amount of protection of around 18.2 ± 1.5 %. Comparison between the different endogenous protein antioxidants showed that cells treated with rCLIC1 or rGST-Ω showed similar percentage cellular protection which was relatively less than that shown by equivalent amounts of rCLIC4 and rGrx, with no significant difference seen between rCLIC4 and rGrx. This strongly suggests that equimolar amounts of rCLIC1 showed antioxidant activity similar to that of rGST-Ω protein while rCLIC4 antioxidant activity was similar to that of rGrx. This further validates an antioxidant role for CLIC1 and CLIC4 proteins, regulating the redox environment of skin cells; likely via similar mechanism to the Grx and GST-Ω proteins.

Furthermore, cells were also treated with two CLIC1 mutants, C24A and K37A, added exogenously, and the percentage cell viability and the percentage cellular protection against H_2O_2 challenge determined. As seen in Fig. 8B, there was no significant difference between the Control cells and those treated with either of the rCLIC1 mutants: C24A or K37A, whereas, HDF cells treated with either rCLIC1 or rCLIC4 showed significant protection against H_2O_2-induced damage. These results indicate a crucial role for the two residues; cysteine24 and lysine37 in CLIC1, and their impact on the protein's cellular protective ability and/or the antioxidant activity. In CLIC proteins, glutathione was shown covalently bound to the glutathione-binding site via a conserved CLIC cysteine residue, which in CLIC1 is Cys-24 [9]. Mutation of this cysteine residue to alanine (C24A) has shown diminished oxidoreductase activity in the HEDS assay [18,48] whereas other studies have shown this Cys24 residue is not essential for CLIC1's ion channel function [35,48]. However, Cys24 does appear important for optimal ion channel activity [35,48]. On the other hand, lysine 37 (K37) is one of the two charged residues

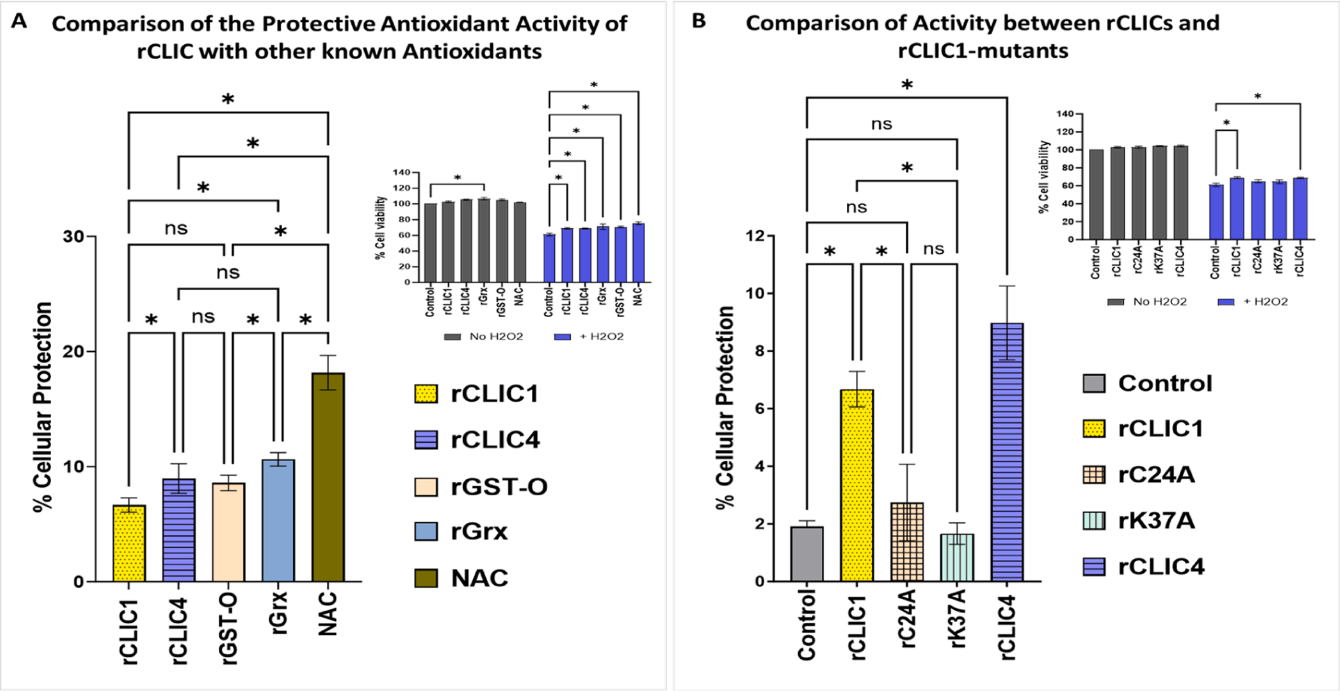


Fig. 8. Comparison of the percentage cellular protection shown by different rCLIC treatments in HDF cells against the following known antioxidants: Grx, GST-O, and NAC. A) Comparison of the percentage cellular protection shown by the different antioxidant treatments against H_2O_2-induced oxidative damage in HDF cells. **B)** Comparison of the percentage cellular protection against H_2O_2-induced damage shown by rCLICs and rCLIC1-mutants: C24A and K37A. Cell viability was measured using WST-1 reagent and the percentage cell viability in the absence (black) or presence (blue) of oxidative stress is shown at the top right. Data was further analysed to determine the percentage cellular protection. Cells not treated with any antioxidant agents but H_2O_2 was used as the Control. One-Way ANOVA with Tukey's multiple comparisons test was done. Data represented as Mean ± SD. Data was collected from 3 different passages with each passage run in triplicate. $^*p < 0.05$, ns= non-significant.

along with arginine 29 found in the putative transmembrane region of CLIC1 [9]. As charged residues are likely to have a key role in ion channel function, a study using the CLIC1-K37A mutant showed that K37A mutation in CLIC1 increased the single channel conductance of the protein [49]. Whereas, the same mutant, K37A, significantly reduced the oxidoreductase activity of CLIC1 as determined via the HEDS assay [18]. These results indicate that the antioxidant and cellular protective ability of CLIC1 is attributed entirely to its oxidoreductase activity and likely does not involve its ion channel activity, given that both C24A and K37A mutants provided no protection to the cells from oxidative damage by H_2O_2.

(E) Exogenously added rCLIC protein protects siRNA knockdown HDF cells from Cellular Damage:

Earlier we have shown that down-regulation of either or both CLIC1 or CLIC4 protein expression had detrimental effects on the cell viability of both human and murine fibroblast cells and human keratinocyte cells in comparison to the control cells. We have also showed that both CLIC1 or CLIC4 knockdown increases cell susceptibility to oxidative damage, whereas cells overexpressing CLIC1 or CLIC4, showed enhanced protection. To this end, we next investigated the effect of exogenously adding rCLICs to CLIC1-KD and CLIC4-KD HDF cells prior to H_2O_2 treatment. Cells treated with scrambled siRNA (Scmb C), CLIC1-KD and CLIC4-KD cells, were used as the controls. Fig. 9 shows the percentage cell viability of the siRNA knockdown cells treated with/without rCLIC proteins in the absence or presence of oxidative stress.

Fig. 9 shows significant reduction in cell viability in CLIC1-KD and CLIC4-KD cells in the absence or presence of H_2O_2 in comparison to Scmb C, as previously seen in Fig. 2E, that down-regulation of CLIC1 or CLIC4 expression in skin cells increases their susceptibility to oxidative damage. Interestingly, CLIC1-KD cells treated with exogenous rCLIC1 (C1-KD + rCLIC1) or rCLIC4 (C1-KD + rCLIC4), showed greater percentage cell viability in comparison to the non-treated CLIC1-KD cells, both in the absence and presence of oxidative damage. Treatment of CLIC1-KD cells treated with exogenously added rCLICs, showed restored cell viability, with no significant difference seen when compared to the Control cells lines expressing endogenous levels of CLIC proteins. These findings provide further evidence that addition of exogenous rCLIC1 or rCLIC4, protect cells resulting in increased cell viability. On the other hand, CLIC4-KD cells in the absence or presence of oxidative damage, showed significant increase in cell viability only when treated with rCLIC1 (C4-KD + rCLIC1), restoring them to that of the Control cells; while treatment with rCLIC4, although a slight increase in cell viability was seen, did not reach level of significance ($p < 0.05$). This result points to a potential difference in the intracellular actions of the two proteins CLIC1 and CLIC4, including their specific roles in the antioxidant processes, interactions in signalling cascades and intracellular localisation.

Imaging of CLIC1, CLIC3, and CLIC4 in human skin tissue sections and in varying aged murine skin sections

Recent studies have shown that CLIC4 plays a key role in skin fibroblast and keratinocyte differentiation [30,50–52]. *In vivo* experiments have localised CLIC4 to the epidermis of both human and mouse skin and is primarily nuclear in dormant cells, suggesting that CLIC4 participates in epidermal homeostasis through fluctuations in both expression level and subcellular localisation [30]. Suh et al. (2007) proposed that CLIC4 contributes to both cell cycle arrest and specific gene expression that is associated with the terminal differentiation of keratinocytes through altering of Cl- ion concentration and pH of the nucleus [30]. CLIC4 has also been seen to act on the transforming growth factor beta (TGF-β) pathway, resulting in nuclear translocation and conversion of fibroblasts to myofibroblasts along with the expression of matrix proteins [50]. In a publication by Joost et al. (2016), the CLIC1 gene was found to cluster with a group of pseudotime-dependent genes that were differentially expressed in the hair follicle and interfollicular epidermis and were found to be involved in cornified envelope formation, ceramide synthesis, and proteolysis [53]. Although, there is some evidence regarding the role of CLIC4 in skin, little is known about the role of CLIC1 in skin cells. To shed some light, immunofluorescence staining was used to determine the expression pattern of CLIC1, CLIC3, and CLIC4 within human and murine paraffin skin sections.

As seen in Fig. 10, immunofluorescence (IF) staining of CLIC 1, 3 and 4 within commercially purchased human skin sections showed staining of CLIC1 and CLIC4 throughout the skin tissue sections, with the strongest expression seen within the upper most layers of the skin; mainly the epidermal skin layer (see Supplementary Figure S5 for Haematoxylin and Eosin staining of the skin sections). A similar trend was also observed in the murine skin sections (data not shown). Human

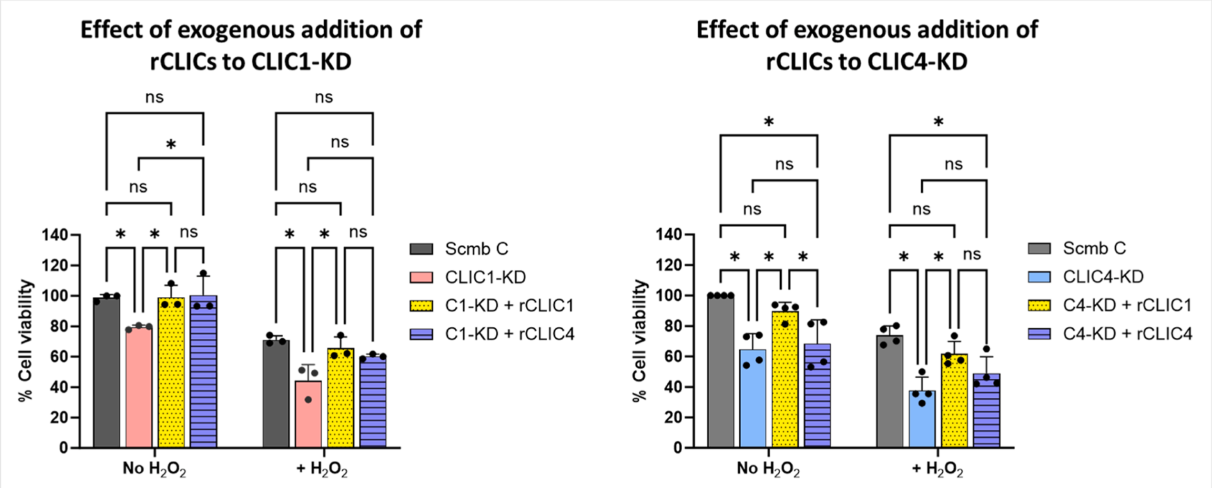


Fig. 9. Percentage Cellular Viability of siRNA knockdown HDF cells treated with different rCLICs prior to them being subjected to oxidative damage. Percentage Cell Viability of either CLIC1 knockdown (CLIC1-KD, Left) or CLIC4 knockdown (CLIC4-KD, Right) treated with rCLIC1 (yellow, dotted), or rCLIC4 (purple, stripped) following no treatment (No H_2O_2) or treated with 0.2 mM H_2O_2 for 1 hour. Cells not treated with siRNA and rCLIC proteins were used as Control (black) while cells treated with siRNA but not exogenous proteins, CLIC1-KD and CLIC4-KD shown in red and blue respectively. Two-way ANOVA with Tukey's multiple comparisons test was done. Data represented as Mean ± SD. Data was collected from 3 different passages with each passage run in triplicate. $^*p < 0.05$, ns= non-significant.

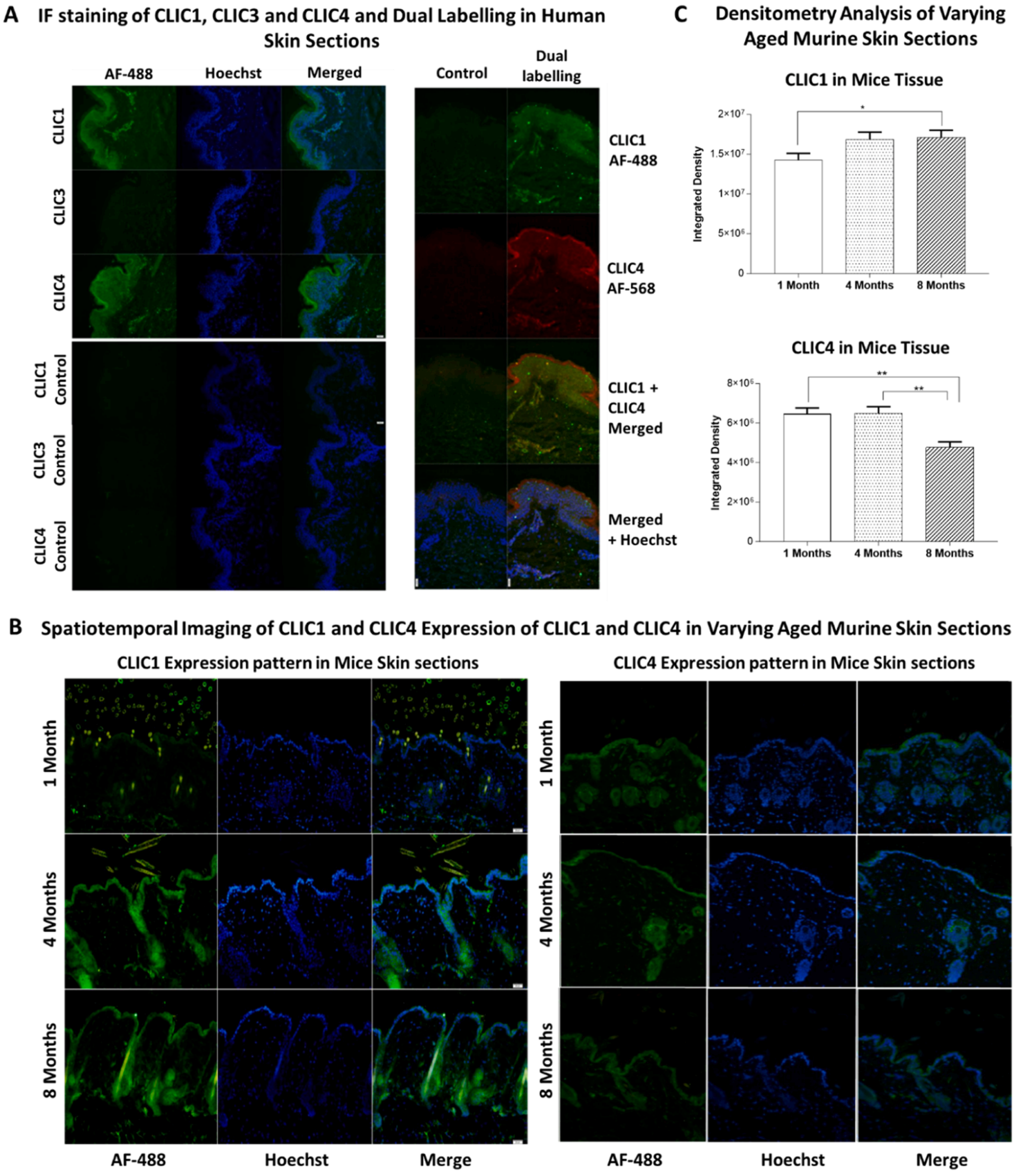


Fig. 10. Imaging of CLIC1, CLIC3, and CLIC4 in Human Skin Tissue Sections and in Varying Aged Murine Skin Sections. A) Visualisation of CLIC1, CLIC3, CLIC4 and Dual labelling of CLIC1 and CLIC4 in Human Skin Paraffin Sections with Control. **B)** Spatiotemporal Imaging of CLIC1 and CLIC4 Expression in Varying aged Murine Skin Tissue Sections. The Olympus BX51 Upright Fluorescent Microscope was used with a 20x magnification. Primary antibodies were omitted from the negative controls. Scale bars indicate 20 μm and apply to each row of panels. **C)** FIJI/ImageJ Analysis of Varying Murine Skin Sections. CLIC1 and CLIC4 stained murine skin samples were analysed for integrated density of fluorescent particles and are expressed as mean ± SEM. One-way ANOVA with Tukey's multiple comparisons test was done. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$ for Murine Samples with data collected from multiple different regions of interest within each sample.

skin sections probed for CLIC1 (green fluorescent staining) and CLIC4 (red fluorescent staining) simultaneously, as shown in Fig. 10A, showed a strong co-localisation signal for CLIC1 and CLIC4 within the upper layers of the skin. CLIC4 had stronger epidermal layer staining than CLIC1, clearly indicated by the red outline along the upper most layers of the skin sections. However, due to the lack of specific differential staining of the epidermis and the dermis layers, it was not possible to fully distinguish between these layers. On the other hand, CLIC3 was not visible and did not show any describable expression pattern unlike CLIC1 or CLIC4. Hence it was concluded that there was no CLIC3 expression or is expressed at below detection threshold within the skin sections. This was a very interesting observation and might account for the lack of protection that we saw earlier when fibroblast or keratinocyte cells were treated with recombinant rCLIC3 proteins. Whereas, both CLIC1 and CLIC4 proteins showed clear and visible expression throughout the skin sections and were able to protect cells from oxidative damage. This strongly suggests crucial roles of CLIC proteins, particularly CLIC1 and CLIC4, in maintaining skin redox homeostasis that is most likely dependent on the expression and localisation of these proteins within the fibroblast and keratinocyte cells.

The skin acts as the body's barrier to the external environment and is constantly being bombarded by pollutants and exposed to oxidative damage [54–57]. It is estimated that approximately 90 % of skin damage occurs from oxidative stress resulting in a plethora of negative effects from apoptosis, cell ageing and even malignant cancer transformation [54–57]. In order to combat oxidative damage, the human skin system employs gradients of antioxidants which with excessive and chronic exposure to oxidising agents and with aging becomes redundant [58]. To see if there are any changes in expression pattern of CLIC1 or CLIC4 within the skin system with aging, whole murine skin sections consisted of TLR4/Rage strains were probed for CLIC1 or CLIC4 across the following age groups: 1.5 months (male), 4 months (female), and 8 months (female) (multiple skin sections from three different mice were analysed per age group). The IF images and corresponding densitometry analysis of the CLIC1/CLIC4 fluorescent detected in the aging skin sections are shown in Fig. 10B CLIC1 and CLIC4 staining is clearly visible throughout the murine skin sections. As the age of the skin donors increased from 1 to 8 months, noticeable changes were seen in the staining pattern of these proteins, which likely also reflects known changes in the skin architecture with age (See Supporting Materials, Supplementary Figure S5). As seen in Fig. 10B, the distribution of CLIC4 is mostly diffuse throughout the skin tissue section in the 1-month sample, whereas in the older aged samples, CLIC4 localisation is seen to be concentrated towards the outer edge of the skin. Visible changes were also observed for the distribution and localisation of CLIC1 across the different age groups. Densitometry analysis of these sections (Fig. 10C) shows a 1.5-fold decrease in the expression level of CLIC4 while there is a significant increase in CLIC1 expression in mouse skin sections at 8 months in comparison to their respective younger skin section counterparts. These results therefore show that with aging there are changes in distribution and localisation of both CLIC1 and CLIC4 in murine skin.

Immunofluorescent staining of varying aged Human Skin Paraffin sections, commercially purchased from donor tissues collected from the abdominal region and covering ages 17 years (male), 55 years (female), and 96 years (female), also show similar patterns in CLIC1 and CLIC4 expression with age, to those seen in the murine skin sections (data shown in Supplementary Figure S6). These results however must be considered preliminary, given that each age group is represented by tissue from only one individual. Furthermore, possible variations in the human skin sections based on precise biopsy location may also arise. Therefore, more detailed investigations are required to further elucidate CLIC protein spatio-temporal tissue distribution and expression.

Upon consideration of the widespread cellular expression and subcellular localisation of the CLIC proteins, together with their high degree of evolutionary conservation [5–7], along with the co-expression of multiple CLICs, it seems clear that CLIC proteins play critical cellular roles that are vital for cell survival. A study by Padmakumar, V. et al. (2012) showed CLIC1 to modestly increase in spleen, lungs, and skin in a CLIC4(NULL) mouse model and was speculated as a compensatory mechanism for the loss of CLIC4 as CLIC1 is the closest homologue to CLIC4 in sequence compared to the four other CLIC family members [59]. Our current preliminary tissue study, also suggests a link between CLIC members, with IF data demonstrating that as CLIC4 expression decreased in the aging skin tissues, CLIC1 displayed a modest increase in expression with considerable changes in its distribution pattern, going from a broad epithelial distribution in the younger skin samples compared to a more restricted distribution within the upper skin layers in the older skin samples. Also, both CLIC1 and CLIC4 showed cellular protective antioxidant capabilities against H_2O_2-induced oxidative damage in primary human skin cells but at varying degrees. One possibility might be that there is a difference in cellular localisation of CLIC1 and CLIC4 and this may result in CLIC1 and CLIC4 being involved in different cellular processes to combat oxidative damage, although this was beyond the scope of this study, it warrants further investigation. In addition, further investigation is also required to deduce their mechanism of action in order to better understand their role as antioxidants and cellular protective agents. A study by Choi et al. (2006) showed that the ribosomal protein S3 (rpS3) is a key protein by which cells counteract deleterious effects of oxidative stress, thus playing a crucial role in DNA damage repair [60]. Hence, they set out to fuse the rpS3 with PEP-1 peptide and transduced it into human skin fibroblast cells. It was shown that the fusion protein increased cell viability and significantly reduced DNA lesion in the UV exposed cells. Further, IHC showed that the fusion protein was able to efficiently penetrate both the epidermis and dermis, suggesting that the PEP-1-rpS3 fusion protein could be used in protein therapy for UV related disorders [60]. Given that the CLICs, in particular CLIC1 and CLIC4, also impart increased cell viability and protection against oxidative damage in both human and murine fibroblast and keratinocyte cells, it would be interesting to further investigate the potential roles and/or the therapeutic benefits of this class of proteins as the next generation of natural antioxidants.

Conclusion

In this investigation, our results clearly indicate that the oxidoreductase activity of CLIC1 and CLIC4 proteins play a crucial role in protecting skin derived cells against H_2O_2-induced oxidative damage, thereby highlighting the cellular antioxidant capabilities of the CLIC proteins. Overexpression of CLIC1 or CLIC4 in murine fibroblast cells demonstrated their ability to protect the cells from oxidative damage resulting in reduced ROS generation, thereby indicating their involvement in regulation of skin cells' redox environment. While knockdown of these proteins in both human and murine skin cells resulted in decreased cell viability, increased ROS levels, reduced oxidoreductase activity and down-regulation of CLIC1 and CLIC4 expression made the cells more susceptible to oxidative damage. This study also highlighted for the first time the role of CLIC proteins as protective antioxidants when added exogenously at optimal dosage concentrations to fibroblast and keratinocyte cells in culture. We have demonstrated that exogenous addition of CLIC1 or CLIC4 provide similar protection against H_2O_2-induced oxidative damage to that of Grx and GST-Ω proteins, while exogenous addition of CLIC3 at varying concentrations was found to have no significant effect on the cell viability of skin cells both in the absence or presence of $H_2O_{2.}$ Furthermore, we confirmed that the protective activity is dependent on the oxidoreductase activity, since mutation of either one of two different glutathione S-transferase activity–related residues in CLIC1 (C24A and K37A) diminished the protein's ability to protect cells from H_2O_2-induced oxidative damage. Our findings strongly support a role for CLIC1 and CLIC4 as important contributors to regulation of the cellular ROS levels in skin cells, and thus recognition of their inclusion as members of the cellular antioxidant defence armoury, ensuring the cell's overall health and viability.

CRediT authorship contribution statement

KR Hossain: Writing – original draft, Investigation, Formal analysis, Data curation. **A Alghalayini:** Writing – review & editing, Investigation, Formal analysis. **DR Turkewitz:** Writing – review & editing, Investigation. **C D'Amario:** Investigation. **Catherine A Gorrie:** Writing – review & editing, Methodology, Formal analysis. **M Wallach:** Supervision, Resources. **SM Valenzuela:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Stella Valenzuela reports financial support was provided by Bod Science Pty Ltd and reports a relationship that includes: Patent pending. We also declare that Optimus Salvus Ltd have acquired CLIC intellectual property from Bod Science, and have approved publication of these results.

Acknowledgments

This research was funded by Bod Science Ltd. (ACN 601225441) and supported by The School of Life Sciences at University of Technology Sydney (UTS).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.arres.2025.100132.

Data availability

Data will be made available on request.

References

[1] E. Argenzio, W. Moolenaar, Emerging biological roles of Cl- intracellular channel proteins, J. Cell Sci. 129 (2016) 4165–4174.

[2] B. Cromer, C. Morton, P. Board, M. Parker, From glutathione transferase to pore in a CLIC, Eur. Biophys. J. 31 (2002) 356–364.

[3] R.S. Gururaja, D. Ponnalagu, N. Patel, H. Singh, Three decades of chloride intracellular channel proteins: from organelle to organ physiology, Curr. Protoc. Pharmacol. 80 (2018) 11.21.11–11.21.17.

[4] D. Littler, S. Harrop, S. Goodchild, J. Phang, A. Mynott, L. Jiang, S. Valenzuela, M. Mazzanti, L. Brown, S. Breit, P. Curmi, The enigma of the CLIC proteins: ion channels, redox proteins, enzymes, scaffolding proteins? FEBS Lett. 584 (2010) 2093–2101.

[5] D. Littler, S. Harrop, L. Brown, G. Pankhurst, A. Mynott, P. Luciani, R. Mandyam, M. Mazzanti, S. Tanda, M. Berryman, S. Breit, P. Curmi, Comparison of vertebrate and invertebrate CLIC proteins: the crystal structures of Caenorhabditis elegans EXC-4 and Drosophila melanogaster DmCLIC, Proteins 71 (2008) 364–378.

[6] A. Elter, A. Hartel, C. Sieben, B. Hertel, E. Fischer-Schliebs, U. Lüttge, A. Moroni, G. Thiel, A plant homolog of animal chloride intracellular channels (CLICs) generates an ion conductance in heterologous systems, J. Biol. Chem. 282 (2007) 8786–8792.

[7] R.S. Gururaja, D. Ponnalagu, S. Sukur, H. Singh, S. Sanghvi, Y. Mei, D. Jin, H. Singh, Identification and characterization of a bacterial homolog of chloride intracellular channel (CLIC) protein, Sci. Rep. 7 (2017).

[8] B. Cromer, M. Gorman, G. Hansen, J. Adams, M. Coggan, D. Littler, L. Brown, M. Mazzanti, S. Breit, P. Curmi, A. Dulhunty, P. Board, M. Parker, Structure of the Janus protein human CLIC2, J. Mol. Biol. 374 (2007) 719–731.

[9] S. Harrop, M. DeMaere, W. Fairlie, T. Reztsova, S. Valenzuela, M. Mazzanti, R. Tonini, M. Qiu, L. Jankova, K. Warton, A. Bauskin, W. Wu, S. Pankhurst, T. Campbell, S. Breit, P. Curmi, Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-A resolution, J. Biol. Chem. 276 (2001) 44993–45000.

[10] D. Littler, N. Assaad, S. Harrop, L. Brown, G. Pankhurst, P. Luciani, M. Aguilar, M. Mazzanti, M. Berryman, S. Breit, P. Curmi, Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4, FEBS J. 272 (2005) 4996–5007.

[11] D. Littler, L. Brown, S. Breit, A. Perrakis, P. Curmi, Structure of human CLIC3 at 2 A resolution, Proteins 78 (2010) 1594–1600.

[12] A. Dulhunty, P. Gage, S. Curtis, G. Chelvanayagam, P. Board, The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator, J. Biol. Chem. 276 (2001) 3319–3323.

[13] J. Martin, Thioredoxin–a fold for all reasons, Structure 3 (1995) 245–250.

[14] G. Powis, W. Montfort, Properties and biological activities of thioredoxins, Annu. Rev. Biophys. Biomol. Struct. 30 (2001) 421–455.

[15] D. Sheehan, G. Meade, V. Foley, C. Dowd, Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily, Biochem. J. 360 (2001) 1–16.

[16] H. Al Khamici, L. Brown, K. Hossain, A. Hudson, A. Sinclair-Burton, J. Ng, E. Daniel, J. Hare, B. Cornell, P. Curmi, M. Davey, S. Valenzuela, Members of the chloride intracellular ion channel protein family demonstrate glutaredoxin-like enzymatic activity, PLoS One 10 (2015).

[17] J. Hernandez-Fernaud, E. Ruengeler, A. Casazza, L. Neilson, E. Pulleine, A. Santi, S. Ismail, S. Lilla, S. Dhayade, I. MacPherson, I. McNeish, D. Ennis, H. Ali, F. Kugeratski, H. Al Khamici, M. van den Biggelaar, P. van den Berghe, C. Cloix, L. McDonald, D. Millan, A. Hoyle, A. Kuchnio, P. Carmeliet, S. Valenzuela, K. Blyth, H. Yin, M. Mazzone, J. Norman, S. Zanivan, Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity, Nat. Commun. 8 (2017).

[18] A. Alghalayini, K. Hossain, S. Moghaddasi, D. Turkewitz, C. D'Amario, M. Wallach, S. Valenzuela, *In vitro* enzymatic studies reveal pH and temperature sensitive properties of the CLIC proteins, Biomolecules 13 (2023).

[19] D. Turkewitz, S. Moghaddasi, A. Alghalayini, C. D'Amario, H. Ali, M. Wallach, S. Valenzuela, Comparative study of His- and Non-His-tagged CLIC proteins, reveals changes in their enzymatic activity, Biochem. Biophys. Rep. 26 (2021).

[20] H. Al Khamici, V. Sanchez, H. Yan, C. Cataisson, A. Michalowski, H. Yang, L. Li, M. Lee, J. Huang, S. Yuspa, The oxidoreductase CLIC4 is required to maintain mitochondrial function and resistance to exogenous oxidants in breast cancer cells, J. Biol. Chem. 298 (2022) 102275.

[21] H. Do, I. Kim, B. Jeon, C. Lee, A. Park, A. Wi, S. Shin, H. Park, Y. Kim, H. Yoon, H. Kim, J. Lee, Structural understanding of the recycling of oxidized ascorbate by dehydroascorbate reductase (OsDHAR) from Oryza sativa L. japonica, Sci. Rep. 6 (2016).

[22] J. Liang, Y. Shaulov, C. Savage-Dunn, S. Boissinot, T. Hoque, Chloride intracellular channel proteins respond to heat stress in Caenorhabditis elegans, PLoS One 12 (2017).

[23] D. Ponnalagu, S. Hamilton, S. Sanghvi, D. Antelo, N. Schwieterman, I. Hansra, X. Xu, E. Gao, J. Edwards, S. Bansal, L. Wold, D. Terentyev, P. Janssen, T. Hund, M. Khan, A. Kohut, W. Koch, H. Singh, CLIC4 localizes to mitochondrial-associated membranes and mediates cardioprotection, Sci. Adv. 8 (2022).

[24] D. Ponnalagu, N. Patel, A. Chaudhury, E. Gao, W. Koch, A. Kohut, H. Singh, CLIC4 and CLIC5, mitochondrial chloride channel proteins mediate cardioprotection against ischemia reperfusion injury, Biophys. J. 114 (2018).

[25] R. Weston, R. Schmitt, M. Grotewiel, M. Miles, Transcriptome analysis of chloride intracellular channel knockdown in Drosophila identifies oxidation-reduction function as possible mechanism of altered sensitivity to ethanol sedation, PLoS One 16 (2021).

[26] Y. Zhang, W. Wang, H. Yang, Y. Li, X. Kang, X. Wang, Z. Yang, Molecular properties and functional divergence of the dehydroascorbate reductase gene family in lower and higher plants, PLoS One 10 (2015).

[27] H. Geng, C. Feng, Z. Sun, X. Fan, Y. Xie, J. Gu, L. Fan, G. Liu, C. Li, R. Thorne, X. Zhang, X. Li, X. Liu, Chloride intracellular channel 1 promotes esophageal squamous cell carcinoma proliferation via mTOR signalling, Transl. Oncol. 27 (2023).

[28] J. Lee, J. Lee, H. Kim, M. Hahn, J. Kang, H. Cho, The inhibition of chloride intracellular channel 1 enhances Ca2+ and reactive oxygen species signaling in A549 human lung cancer cells, Exp. Mol. Med. 51 (2019) 1–11.

[29] B. Singha, S. Harper, A. Goldman, B. Bitler, K. Aird, M. Borowsky, M. Cadungog, Q. Liu, R. Zhang, S. Jean, R. Drapkin, D. Speicher, CLIC1 and CLIC4 complement CA125 as a diagnostic biomarker panel for all subtypes of epithelial ovarian cancer, Sci. Rep. 8 (2018).

[30] K. Suh, M. Mutoh, T. Mutoh, L. Li, A. Ryscavage, J. Crutchley, R. Dumont, C. Cheng, S. Yuspa, CLIC4 mediates and is required for Ca2+-induced keratinocyte differentiation, J. Cell Sci. 120 (2007) 2631–2640.

[31] J. Tung, O. Hobert, M. Berryman, J. Kitajewski, Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis *in vitro*, Angiogenesis 12 (2009) 209–220.

[32] J. Tung, J. Kitajewski, Chloride intracellular channel 1 functions in endothelial cell growth and migration, J. Angiogenes. Res. 2 (2010).

[33] Z. Uretmen Kagiali, N. Saner, M. Akdag, E. Sanal, B. Degirmenci, G. Mollaoglu, O. N, CLIC4 and CLIC1 bridge plasma membrane and cortical actin network for a successful cytokinesis, Life Sci. Alliance 3 (2019).

[34] A. Holmgren, M. Björnstedt, Thioredoxin and thioredoxin reductase, Methods Enzymol. 252 (1995) 199–208.

[35] S. Valenzuela, H. Alkhamici, L. Brown, O. Almond, S. Goodchild, S. Carne, P. Curmi, S. Holt, B. Cornell, Regulation of the membrane insertion and conductance activity of the metamorphic chloride intracellular channel protein CLIC1 by cholesterol, PLoS One 8 (2013).

[36] W. Wang, M. Wan, D. Liao, G. Peng, X. Xu, W. Yin, G. Guo, F. Jiang, W. Zhong, J. He, Identification of potent chloride intracellular channel protein 1 inhibitors from traditional Chinese medicine through structure-based virtual screening and molecular dynamics analysis, Biomed. Res. Int. 2017 (2017).

[37] M. Bernier, A. Manning, D. Hearse, Reperfusion arrhythmias: dose-related protection by anti-free radical interventions, Am. J. Physiol. 256 (1989) H1344–H1352.

[38] C. Del Prete, T. Stout, S. Montagnaro, U. Pagnini, M. Uccello, P. Florio, F. Ciani, S. Tafuri, V. Palumbo, M. Pasolini, N. Cocchia, H. Henning, Combined addition of superoxide dismutase, catalase and glutathione peroxidase improves quality of cooled stored stallion semen, Anim. Reprod. Sci. 210 (2019).

[39] H. Aksoylar, N. Patsoukis, Treatment with exogenously added catalase alters CD8 T cell memory differentiation and function, Adv. Biol. 7 (2023).

[40] G.D. Mao, P.D. Thomas, G. Lopaschuk, M. Poznansky, Superoxide dismutase (SOD)-catalase conjugates. Role of hydrogen peroxide and the Fenton reaction in SOD toxicity, J. Biol. Chem. 268 (1993) 416–420.

[41] R. Bonetta, Potential therapeutic applications of MnSODs and SOD-mimetics, Chemistry 24 (2018) 5032–5041.

[42] T. Tominaga, M. Hachiya, T. Shibata, Y. Sakamoto, K. Taki, M. Akashi, Exogenously-added copper/zinc superoxide dismutase rescues damage of endothelial cells from lethal irradiation, J. Clin. Biochem. Nutr. 50 (2012) 78–83.

[43] X. Chen, E. Zhang, L. Fang, J. Zhang, J. Zhu, W. He, X. Luo, Repair effects of exogenous SOD on Bacillus subtilis against gamma radiation exposure, J. Environ. Radioact. 126 (2013).

[44] T. Decker, M. Lohmann-Matthes, A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, J. Immunol. Methods 115 (1988) 61–69.

[45] X. Liu, L. Wang, J. Cai, K. Liu, M. Liu, H. Wang, H. Zhang, N-acetylcysteine alleviates H2O2-induced damage via regulating the redox status of intracellular antioxidants in H9c2 cells, Int. J. Mol. Med. 43 (2019) 199–208.

[46] D. Ezeriņa, Y. Takano, K. Hanaoka, Y. Urano, T. Dick, N-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H2S and sulfane sulfur production, Cell Chem. Biol. 25 (2018) 447–459, e444.

[47] P. Montero, I. Roger, C. Estornut, J. Milara, J. Cortijo, Influence of dose and exposition time in the effectiveness of N-acetyl-L-cysteine treatment in A549 human epithelial cells, Heliyon 9 (2023).

[48] H. Al Khamici, K. Hossain, B. Cornell, S. Valenzuela, Investigating sterol and redox regulation of the ion channel activity of CLIC1 using tethered bilayer membranes, Membranes 6 (2016).

[49] S. Averaimo, R. Abeti, N. Savalli, L. Brown, P. Curmi, S. Breit, M. Mazzanti, Point mutations in the transmembrane region of the clic1 ion channel selectively modify its biophysical properties, PLoS One 8 (2013).

[50] A. Shukla, Y. Yang, S. Madanikia, Y. Ho, M. Li, V. Sanchez, C. Cataisson, J. Huang, S. Yuspa, Elevating CLIC4 in multiple cell types reveals a TGF- dependent induction of a dominant negative Smad7 splice variant, PLoS One 11 (2016).

[51] K. Suh, M. Malik, A. Shukla, A. Ryscavage, L. Wright, K. Jividen, J. Crutchley, R. Dumont, E. Fernandez-Salas, J. Webster, R. Simpson, S. Yuspa, CLIC4 is a tumor suppressor for cutaneous squamous cell cancer, Carcinogenesis 33 (2012) 986–995.

[52] K. Suh, M. Malik, A. Shukla, S. Yuspa, CLIC4, skin homeostasis and cutaneous cancer: surprising connections, Mol. Carcinog. 46 (2007) 599–604.

[53] S. Joost, A. Zeisel, T. Jacob, X. Sun, G. La Manno, P. Lönnerberg, S. Linnarsson, M. Kasper, Single-cell transcriptomics reveals that differentiation and spatial signatures shape epidermal and hair follicle heterogeneity, Cell Syst. 3 (2016) 221–237, e229.

[54] W. Bergfeld, The aging skin, Int. J. Fertil. Womens Med. 42 (1997) 57–66.

[55] B. Poljšak, R. Dahmane, Free radicals and extrinsic skin aging, Dermatol. Res. Pract. 2012 (2012).

[56] M. Rinnerthaler, J. Bischof, M. Streubel, A. Trost, K. Richter, Oxidative stress in aging human skin, Biomolecules 5 (2015) 545–589.

[57] M. Schäfer, S. Werner, The cornified envelope: a first line of defense against reactive oxygen species, J. Invest. Dermatol. 131 (2011) 1409–1411.

[58] A. Godic, B. Poljšak, M. Adamic, R. Dahmane, The role of antioxidants in skin cancer prevention and treatment, Oxid. Med. Cell Longev. 2014 (2014).

[59] V. Padmakumar, K. Speer, S. Pal-Ghosh, K. Masiuk, A. Ryscavage, S. Dengler, S. Hwang, J. Edwards, V. Coppola, L. Tessarollo, M. Stepp, S. Yuspa, Spontaneous skin erosions and reduced skin and corneal wound healing characterize CLIC4 (NULL) mice, Am. J. Pathol. 181 (2012) 74–84.

[60] S. Choi, S. Kim, J. An, S. Lee, D. Kim, H. Ryu, N. Lee, S. Yeo, S. Jang, M. Won, T. Kang, H. Kwon, S. Cho, J. Kim, K. Lee, J. Park, W. Eum, S. Choi, Human PEP-1-ribosomal protein S3 protects against UV-induced skin cell death, FEBS Lett. 580 (2006) 6755–6762.