Identification and characterization of the localization and expression of CLIC4 under redox conditions in mammalian cells

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Abstract

Chloride intracellular ion channel-4 (CLIC4) is a member of the CLIC family of proteins which were originally identified as channels of intracellular membranes permeable to ion chloride ions. Its expression and localization to intracellular membrane is sensitive to oxidative stress. This sensitivity of CLIC4 is indicated with its physiological redox regulatory function either as an oxidoreductase enzyme or an ion channel in response to decrease the cellular glutathione level, ROS accumulation, and consequently non-native disulfide formation in the cells. The pathways for disulfide formation are well characterized. However, the understanding of redox state of CLIC4 and possibility to participate in reductive pathway to removing the non-native disulfide bond is still limited and whether CLIC4 as a membrane-binding protein with oxidoreductase activity might be needed in the reduction pathway either in the cytosol or ER, are the questions we need to address.

In this project the oxidative stress induced by TNF-α and CLIC4 in response to TNF-α can be re-localized to the ER from the cytosol. The ER is a host for disulfide formation within folding proteins entering the mammalian secretory pathway. The consequence of oxidative stress in the ER is the accumulation of misfolded and unfolded proteins. The mammalian cells have a family of oxidoreductase that is thought to be isomerised non-native disulfide bonds. This reductive catalytic activity of oxidoreductases is maintained via a reductive pathway. For CLIC4 to act as an oxidoreductase for performing isomerisation or reduction reactions, it must be preserved in a reduced position. Here, by mass spectrometry, using purified proteins and ER microsomal membrane following TNF-α induced oxidative stress, we illustrate CLIC4 is predominantly placed in a reduced state in the intact cells, demonstrating a reductive pathway is prepared in mammalian cells and CLIC4 can be involved with this pathway as an oxidoreductase through its either enzyme catalytic activity or ion channel activity. In this project, the glutathione has identified to be responsible for the reduction of CLIC4 during oxidative stress. Furthermore, when inhibitors of glutathione synthesis or reductase are added to the cells, CLIC4 is not reduced. The results demonstrate that glutathione plays a direct role in the isomerisation of disulfide bonds by maintaining CLIC4 in a reduced state.

To confirm the reduction effect of GSH on CLIC4 and overall microsomal membrane protein in response to TNF-α, we have applied a cysteine-reactive tandem mass tag (Iodo-TMT) to differentially label cysteine residues and analyse the overall protein expression level and

redox state into one-step analysis. The individually labeled samples have been pooled in differential combinations to create multiple six-plex samples to determine the effect of GSH on cysteine oxidation and overall protein expression in the microsomal membrane. The result highlights the redox status of CLIC4 under reduction of GSH was confirmed with MS/LC, and the TMT-labeling detected the redox state of the overall microsomal membrane proteins with respect to cysteine oxidation and protein expression. This study is important because CLIC4 as either a membrane binding protein or an ion channel can be considered as a mis-component in reductive pathway.

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Acknowledgment

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work has not been submitted for any other degree at the University of Glasgow or any other institution. "

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Definitions/Abbreviations

aa: amino acids

Amp: Ampicillin

Ala(A) Alanine

ATP Adenosine 5‟-triphosphate

AMS: 4-acetamido-4’-maleimidylstilbene-2, 2’-disulfonic acid

ATP: Adenosine triphosphate

BCUN: Carmustine

BioGEE: Biotinylated glutathione ethyl ester

BSA: Bovine serum albumin

BSO: Buthionine sulphoximine

CaCl2: Calcium Chloride

CNX: Calnexin

Cys: Cysteine

Da: Dalton

Dia: Diamide

DHA: Dehydroascorbate

DMEM: Dulbecco's Modified Eagle Medium

DTT: Dithiothreitol DR: Disulfide bond rearrangement

ddH_2O: Double Distilled Water

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleotide Triphosphate

E. coli Escherichia coli

ECM: extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EF: Elongation Factor

ER: Endoplasmic reticulum

ERQC: ER quality control

READ: ER-associated protein degradation

Ero1: ER oxidoreductase 1

ERp: Endoplasmic reticulum-resident protein

GFC buffer: Gel filtration chromatography

GSH: Reduced glutathione

GSSG: Oxidised glutathione

Grx2: Glutaredoxin2

Grx3: Glutaredoxin 3

GR: Glutathione Reductase

GS: Glutathione Synthetase

IAA: Iodoacetamide

IAA-94: Indanyloxyacetic acid

IAP(c-IAP1): inhibitor of apoptosis

IMS: Mitochondrial intermembrane space

IP: Immunoprecipitation

IPTG: Isopropyl β-D-1-thiogalactopyranoside

LB: Luria Broth

LC-MS-MS: Liquid Chromatography with tandem mass spectrometry

MALS: Multiangle Light Scattering

Met: Methionine

MP: Unknown membrane protein

mRNA: Massager RNA

MS: Mass Spectrometry

MTP: Microsomal triglyceride transfer protein

NADP+: Nicotinamide adenine dinucleotide phosphate

NADPH: Adenine dinucleotide phosphate (reduced form of NADP+)

NEM: N-ethylmaleimide

NF-kB: nuclear factor-kB

ORF: Open reading frame

Ox: Oxidised

OCR: Oxidoreductase

PAS: Recombinant Protein A Sepharose FF Resin

PBS buffer: Dulbecco's Phosphate Buffered Saline

PCR: Polymerase chain reaction

PDI: Protein disulfide isomerize

PM: Plasma membrane

PMSF: Phenylmethanesulfonylfluoride

Re: Reduced

RRL: Rabbit reticulocyte lysate

RT: Room temperature

ROS: Reactive oxygen Species

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis:

SEC: Size Exclusion Chromatography

SP cell: Semi-permeabilised cell

TCEP: Tris(2-carboxyethyl) phosphine

TMX2: Thioredoxin-related transmembrane protein 2

TNF-α: Tumour Necrosis Factor-alpha

TNFR: Tumour Necrosis Factor-alpha Receptor

TNFBR: Tumour Necrosis Factor-alpha Binding Receptor

TMT: Tandem Mass Tag

tRNA: Transfer RNA

Trx: Thioredoxin

TrxR1: Thioredoxin reductase 1

UPR: Unfolded protein response

UT: Untreated

UTP: Uridine triphosphate

UTR: Untranslated region

4VP: 4-Vinilpyridin

Chapter 1 Introduction

1.1 Chloride Ion Channel

All living cells are surrounded by phospholipid bilayer membranes that separate the cell cytoplasm and its intracellular components from the extracellular environment. Eukaryotic cells have additional enclosed compartments demarcated by intracellular membranes. These biological intracellular membranes are composed of amphiphilic phospholipids, which have a hydrophilic phosphate head group and a hydrophobic tail comprised of two fatty acid chains. Due to this chemical structure, phospholipids spontaneously arrange into lipid bilayers when exposed to aqueous environments. Their hydrophilic head groups remain in the outer bilayer exposed to the aqueous environment, while the fatty acid tails pack together to form a hydrophobic core (Nicolson GL, et al., 2014) (Figure 1-1).

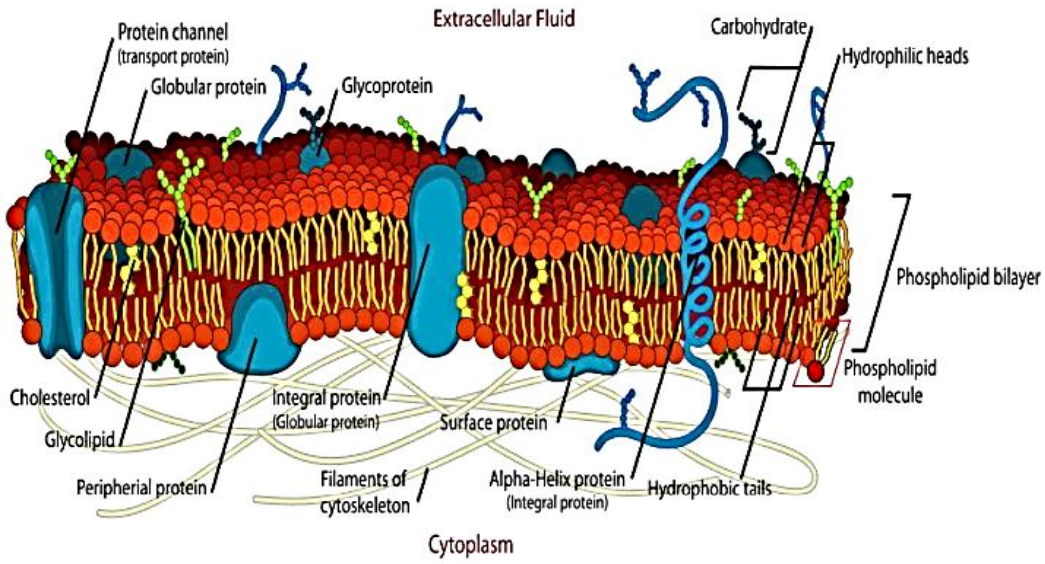


Figure 1-1 a model of the cell plasma membrane. Integral membrane proteins (blue) span the phospholipid bilayer (red). The "hydrophilic" & the "hydrophobic" sections of the phospholipid bilayer are organized as two panes of the membrane (Nicolson GL, et al., 2014).

Chloride ions are generally physiologically ample and chiefly steered negatively charged ionic species and are essential for the normal functioning of many cells. (Suzuki M, et al., 2006). Chloride ion channel proteins are found in all cell types ranging from prokaryotes to mammalian cells. They are located within the plasma membrane of cells, as well as on the membranes of intracellular vesicles, where they enable the transportation of Cl^- ions across the lipid bilayer. These membrane transport proteins are also amphiphilic and span

the entire lipid bilayer. The direction of the CLIC4 is conducted from the C-terminal that located in the cytosol toward to N-terminal into other intracellular compartments for example ER (Duncan RR, et al., 1997). They have a water-attractive surface that is in connection with the water-diluted solution or with the polar head groups of the membrane phospholipids, and a water-repellent strap that intermingles with the alkyl side chains of the phospholipid fatty acid chains in the hydrophobic core of the membrane (Golcza k, et al., 2010). It is via this structural arrangement that these integral membrane proteins form channels in the membrane, allowing the channel of ions to move inside and outside of the cells and which can open and close or "gate" in response to certain stimuli or cellular conditions (Jentsch TJ, et al., 2012). Ion channel gating is classified into three categories: "voltage-gated channels" which open and shut due to fluctuations in the membrane potential, "ligand-gated channels" that are initiated by the binding of definite molecules to their active sites, and a third group of "ion channels" that are gated via mechanical stimuli, e.g., cell swelling or other mechanical membrane perturbations (Lodish H, et al., 2000). Chloride channels participate in and regulate different cellular processes including pH regulation, trans-epithelial transport, and regulation of cellular electrical fieriness and redox regulation (Golcza K, et al., 2010).

1.2 Chloride Intracellular Ion Channel (CLIC) Family

The chloride intracellular channel proteins (CLICs) are unusual positive charged molecules and selective channel proteins (Duncan RR, et al., 1997). Ion channel proteins are intracellular protein that located in the plasma membrane and intracellular membrane. CLIC proteins are predominantly localized in the membranes of several intracellular organelles inside the cell including the “nucleus (Tilley SJ, et al., 2006), mitochondria (Fernandez-Salas E, et al., 1999), secretary vesicles (Redhead C, et al., 1997), Golgi vesicles (Edwards JC, et al., 1999), and endoplasmic reticulum (ER)” (Duncan RR, et al., 1997). Six CLIC members exist in humans including CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, and CLIC6(Cromer BA, et al., 2002) (Figure 1-2). All members contain an approximately 240 amino acid residue cassettes (Littler DR, et al., 2010), while CLIC5 and CLIC6 are two larger variants, with an extended N (amino) terminal domain (Golczak M, et al., 2002). CLIC1 and CLIC4, both of which were characterized with oxidoreductase activity and ion channel

function (Alkhameci H, et al .2015). CLIC2 and CLIC3 also showed some evidence of oxidoreductase activity.

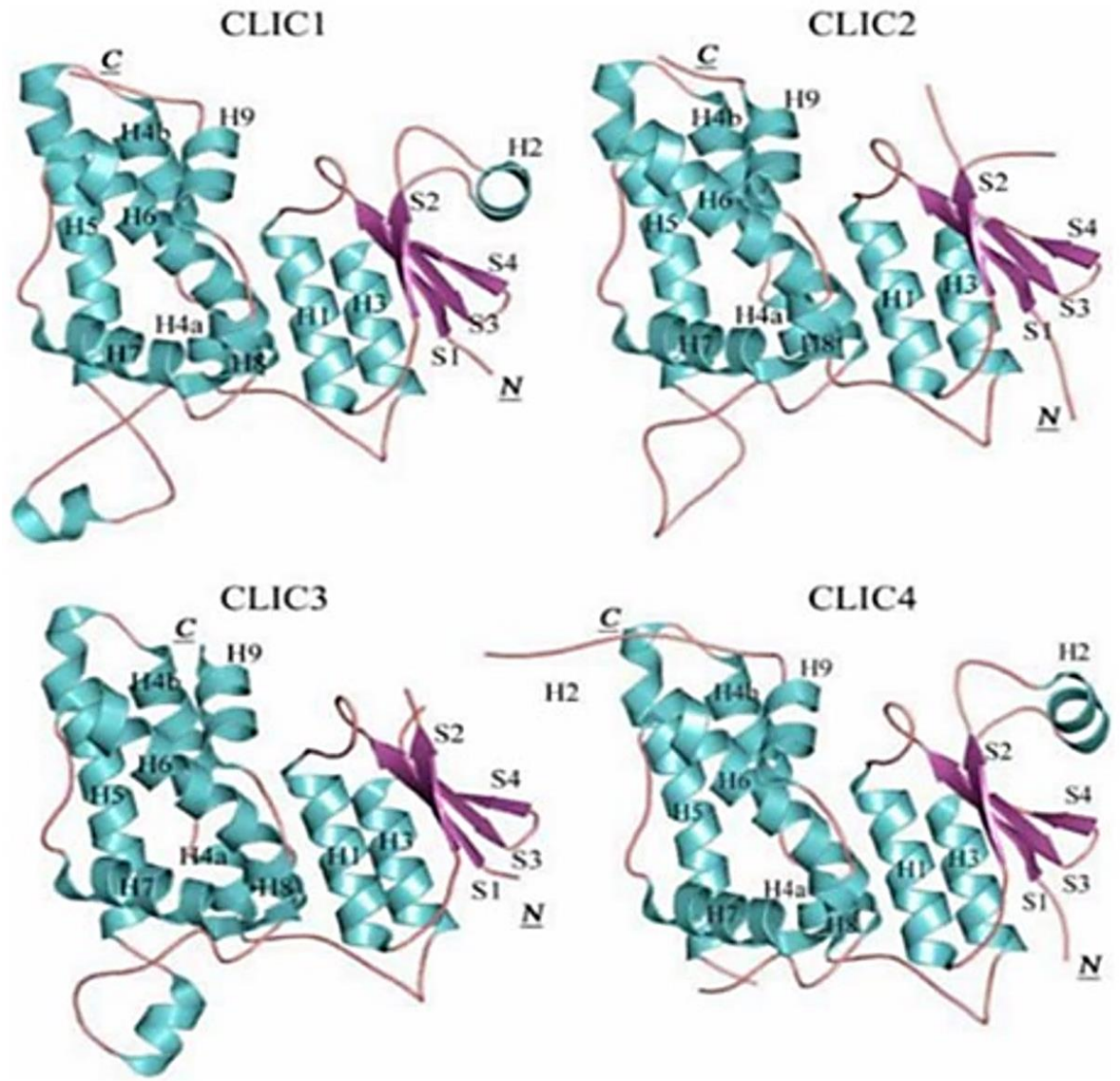


Figure 1-2 "Structures of human CLIC family members that recognized for oxidoreductase activity determined by X-ray crystallography. Secondary structural elements are colored and labelled according to type: alpha-helix (cyan, H1-H9), beta-strand (purple, S1-S4) and random coil (pink). The N-terminus and C-terminus are also labelled. Structures shown include CLIC1 (PDB: 1K0M), CLIC2 (PDB: 2R4V), CLIC3 (PDB: 3FY7) and CLIC4 (PDB: 2AHE)". Taken from (Littler DR, et al., 2006).

Six members of the CLIC family have now been shown in vertebrates (CLIC 1-6). Members of the CLIC family display high sequence similarity between them, ranging from 47% to 76% (Littler DR, et al., 2010) (Figure 1-3).

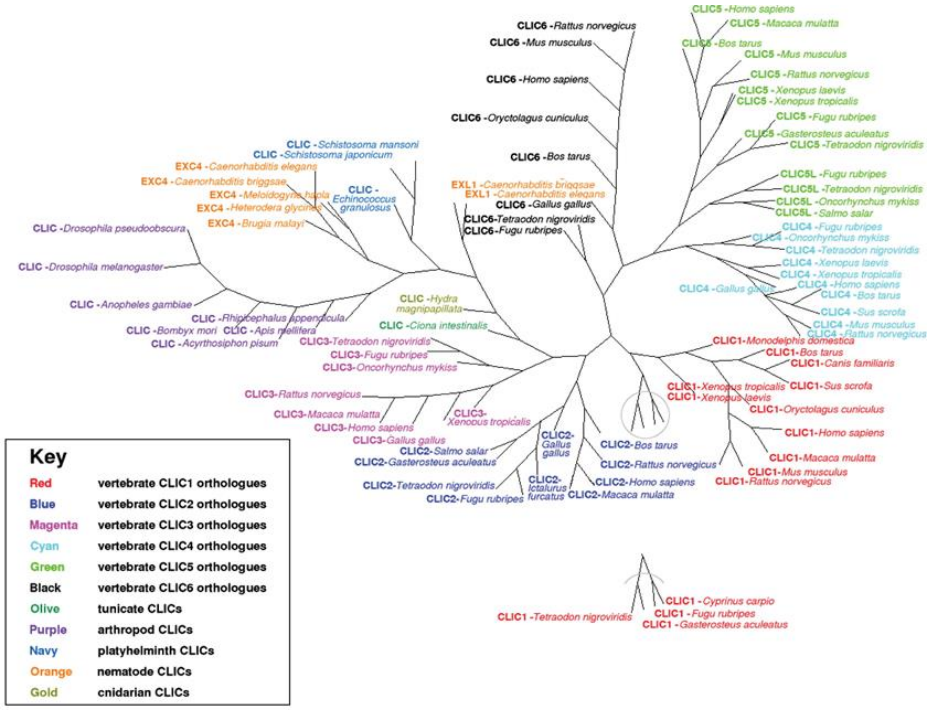


Figure 1-3 Phylogenetic analyses of CLIC proteins. The phylogenetic tree of vertebrate CLIC and invertebrate CLIC-like proteins; branches are colored according to the key on the left. Adapted from (Littler DR, et al., 2004).

1.3 Structural Characteristics of the CLIC Proteins

1.3.1 The N- and C-terminal Domains of the Protein CLIC1

CLIC1 consists of 241 amino acids with a molecular weight of 26.9 kDa (Littler DR, et al., 2004). CLIC1 is a monomeric protein and contains ten α-helices and four β-strands, resulting in 47.7% α-helices and 8.3% β-strands in its secondary structure according to X-ray crystallography studies (Harrop SJ, et al., 2001). The X-ray crystallographic structure of the soluble form of CLIC1, determined at a resolution of 1.4Å at pH 5.0, was demonstrated to have the dimensions of approximately 5.5 nm, 5.2 nm, 23 nm (Figure 1.4) (Litter RD, et al., 2010). CLIC1 consists of two domains: an all α-helical C-domain and a thioredoxin N-domain. The N-domain consists of all four strands of mixed β-sheets (strands β1, β2, β3 and β4) and three α-helices (α1, α2, and α3). The C-domain contains α-helices α (4-10) as well as an anionic “foot loop”, which is a characteristic feature of the CLIC proteins. For CLIC1, the active site is placed in the N terminal domain and contains a central catalytic active cysteine residue (Cys24 in CLIC1) (Littler RD, et al., 2004). It was demonstrated that the existence of catalytic cysteine residue (Cys 35 for CLIC4 and 24 for CLIC1) is important for

the ion channel activity and oxidoreductase activity within most of the CLIC proteins (Littler RD, et al., 2004).

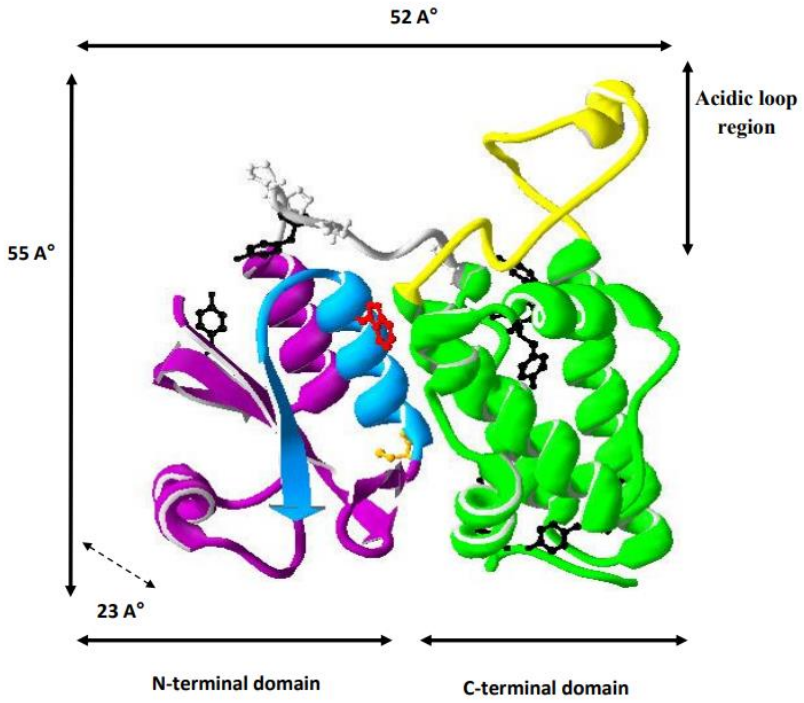


Figure 1-4 Crystal structure of reduced state of CLIC1. "The all α-helical C-terminal domain is demonstrated in green and yellow, and the N-terminal domain containing the thioredoxin fold is indicated in purple and blue. The proline-rich loop connecting the two domains is indicated in grey. The active site Cys24 is indicated in orange. The single tryptophan residue, Trp35 is in red and the eight tyrosine (two in N-terminal domain and six in the C-terminal domain) are in black. The negatively charged loop is shown in yellow and the helix that is considered likely to traverse the membrane is indicated in blue". This was modified from (Harrop SJ, et al., 2001 and Guex N, et al., 1997).

CLIC1 contains a single tryptophan, Trp35, is located at the putative N-terminal transmembrane domain. Furthermore, eight tyrosine and six cysteine residues are distributed throughout the CLIC1 protein molecule. There is also a proline-rich loop (Cys89-Asn100) connecting the N- and C- domains. This proline-rich loop is the origin of the domain interface plasticity in the protein's structure (Harrop SJ, et al., 2001). It was proposed that the N-domain would need to move away from the C-domain to initiate membrane insertion of CLIC1. As such, the loop which leads to flexibility between the two domains is likely to be involved in the transition between the soluble and the transmembrane forms of CLIC1. The putative transmembrane domain in CLICs protein makes a diffrencial among other channel proteins and enables them to act as a transmembrane binding protein. The

transmembrane binding sequencing included per (Table 1-1) desicribed and identified with sequencing in (Figure 1-5).

Table 1-1 CLICs putative transmembrane domain (PTMD)

CLIC1	CPSFQRLFMVLWLKGVTFNVTTV
CLIC2	CPFCQRLFMILWLKGVKFNVTTV
CLIC3	CPSCQRLFMVLLLKGVPFTLTTV
CLIC4	CPFSQRLFMILWLKGVVFSVTTV
CLIC5	CPFSQRLFMILWLKGVVFNVTTV
CLIC6	CPFSQRLFMILWLKGVIFNVTTV

The CLICs putative transmembrane domain (PTMD) at the N-terminal domain is conserved among all the human CLIC proteins (Harrop SJ, et al., 2001). The PTMD arises from the hydrophobicity and the α-helical propensity in the N-domain (Lecroix E, et al., 1998). The PTMD has a crucial role in the biophysical properties of the CLIC proteins. Mutations in these regions can affect different features of ion channels like their conductance, gating, or ion selectivity (Carattino MD, et al., 2105). Mutations in the transmembrane domain caused by replacing the two charged residues (Arg 29 Ala and Lys 37 Ala) where created in CLIC1. These mutations were evaluated by using different methods including artificial lipid membranes and changing the only charged residues in the putative transmembrane domain of CLIC1 with neutral alanine, leading to alteration in its electrophysiological ion channel properties (Averaimo S, et al.,2013), and all CLICs family for insertion supported with those of putative sequences and ion channel activity can be detected for this putative transmembrane domain.

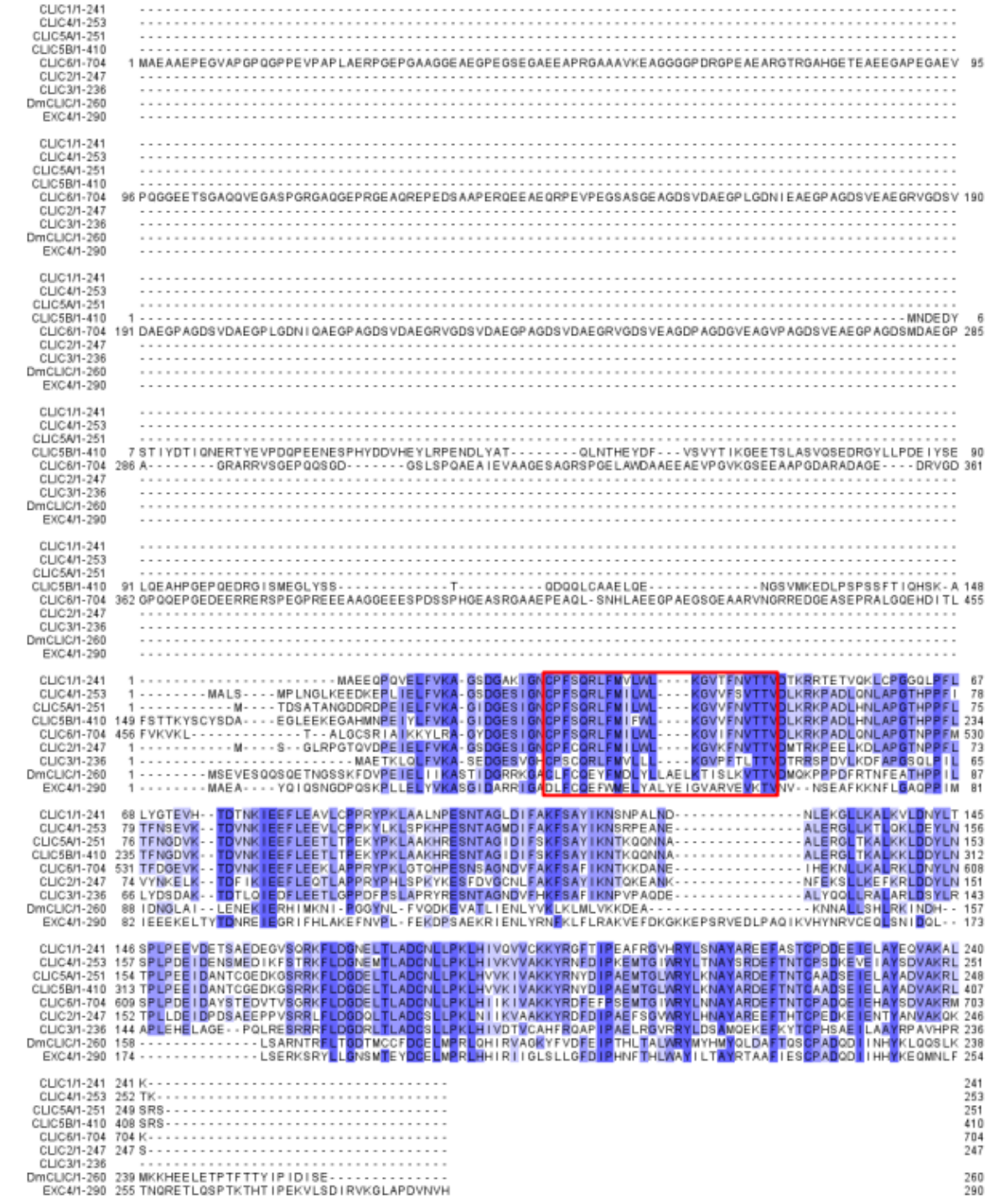


Figure 1-5 Structure-based sequence alignment of the putative transmembrane domain (PTMD) of the CLIC proteins. The PTMD at the N-terminal domain is conserved amongst all the human CLIC proteins that indicated with the red box (Harrop SJ, et al., 2001).

1.3.2 The N-and C-Domains of the Protein CLIC4

Structural CLIC4 has been determined at 1.8 A˚ resolution by X-ray crystallography. CLIC4 is monomeric, and structurally, it is like CLIC1, belonging to the GST fold class. Differences between the structures of CLIC1 and CLIC4 are localized to helix 2 in the glutaredoxin like N-terminal domain, which has previously been shown to undergo a dramatic structural change in CLIC1 upon oxidation (Littler RD, et al., 2010).

CLIC1 sequence appears to be atypical of the family, where the structural differences in this region correlate with the sequence differences. The structure of CLIC4 belongs to the GST superfamily and closely resembles that of the soluble form of CLIC1 (Figure 1-6). CLIC4 is monomeric in the crystal and has approximate dimensions of 50 × 40 × 20 $Å^3$. CLIC4 consist of two domains; an N-terminal domain (residues 16–105) with a like-thioredoxin fold, closely resembling glutaredoxin; and an all α-helical C-terminal domain. The observed structure is unique to the CLICs family proteins, and it is not seen in the GSTs, which consists of residues 16–163 and 173–257 with the break in density corresponding to the flexible foot loop between helix 5 and helix 6, (bottom left). The N-terminal side of the foot loop is anchored via interactions with residues near the reactive Cys35 of a neighbouring molecule. The clear electron density for Glu162, whose side chain forms hydrogen bonds with the backbone and side chain of Asn34 and the side chain of Lys24, was observed. This crystal contact stabilizes the structure of the leading side of the foot loop.

C-terminus of helix 6 is host of the putative internal nuclear localization sequence (NLS) of CLIC4 (residues 199–206: *K*VVA*KK*Y*R*). The structure of the NLS is almost identical to that seen in CLIC1, with the exception that the residue equivalent to Lys199 is Gln188 in CLIC1. Residues Pro252 to Lys257, which localized in the C-terminal extension of CLIC4 was important for crystallization. These residues make a crystal contact with one face of a neighbouring molecule which comprises β-strands 3 and 4 and helix 3 (Litter RD, et al., 2004 and 2005).

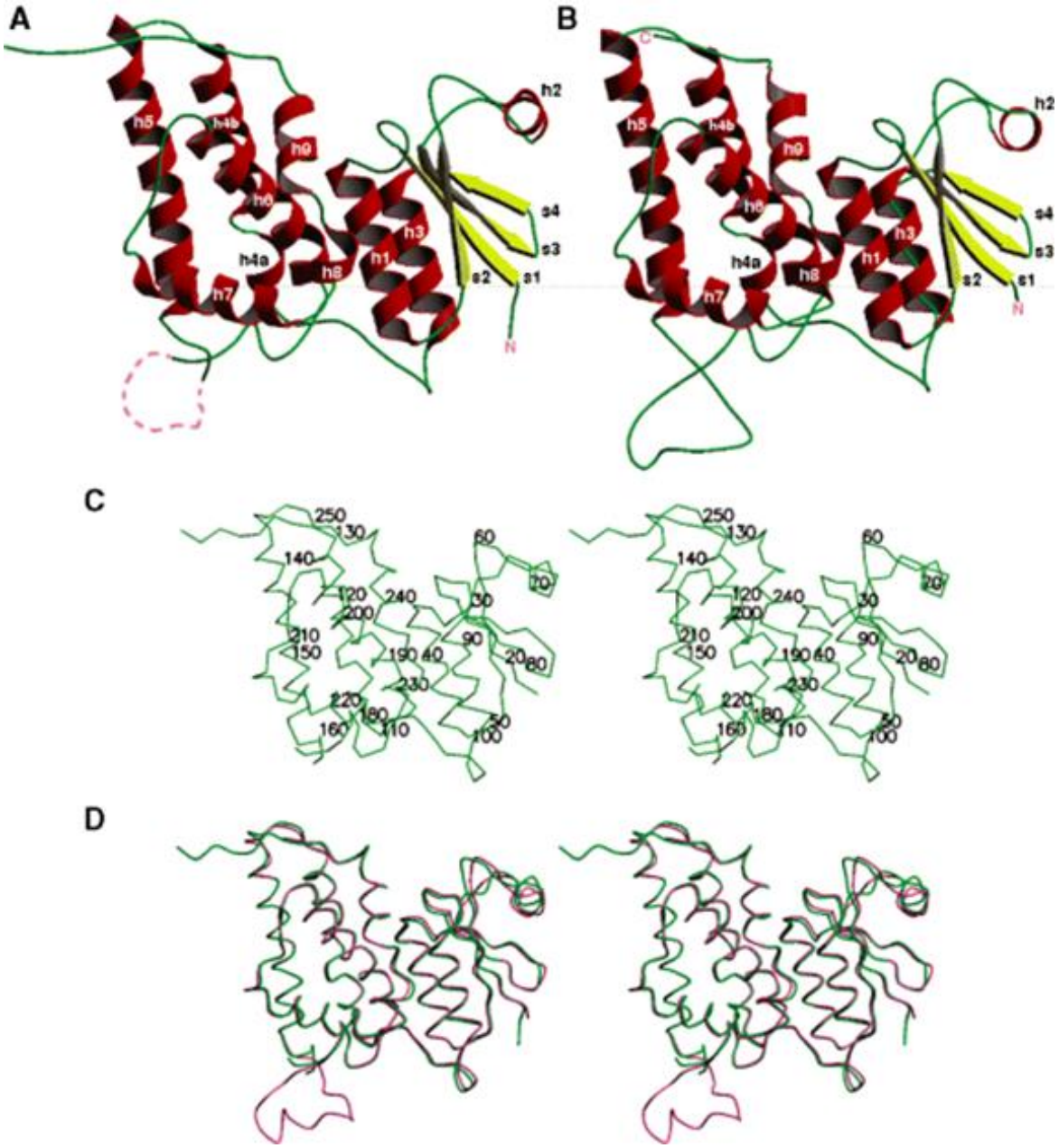


Figure 1-6 Overall crystal structure of CLIC4. "(A) the crystal structure of CLIC4 is presented with Ribbon diagram, where the last two residues of the wild-type CLIC4 sequence have been replaced by a 16-residue peptide (top left-hand corner). (B) The structure of CLIC1 in placed in at the same orientation as CLIC4 in (A). (C) the C_α trace of CLIC4 with every 10th residue numbered is indicating with a stereogram (D) A stereogram illustrating a superposition of the backbone traces of CLIC4 (green) and CLIC1 (mauve)" (the figure is adapted from Litter RD, et al., 2005).

1.4 CLICs as membrane proteins

Several proteins have now been found to exist in both soluble and membrane-bound forms. These proteins have a property that distinguishes them from typical membrane proteins, as they are soluble proteins without a leader sequence for membrane targeting but can insert into or associate with membranes. CLIC exists in soluble form and as an integral membrane protein, but it also possesses the unusual ability, to shift between two stable tertiary protein conformations, an ability described as “metamorphic” (Murzin AG, et al., 2008). These two soluble forms have been resolved by X-ray crystallography, as the reduced monomeric form and in its oxidized state (Goodchild SC, et al., 2009). To understand the ion channel activity of CLICs family as soluble and integrate forms to be capable of transferring cytosolic components between intracellular compartments, we need to consider some details of ion channel activity and ability for the membrane binding of two main family members under redox conditions.

1.4.1 CLIC1 as a membrane protein

CLICs family consist of two forms of soluble and integrate forms of structure. For CLIC1, the crystal structure of soluble form was explained in reducing and oxidizing conditions by Littler and colleagues (Littler DR, et al., 2008). This indicated that under reducing conditions, CLIC1 adopts a monomeric GST–like structure, containing a glutaredoxin-like active site. However, in an oxidative condition, CLIC1 adopts a non-covalent dimeric state. The dimer form arises via the formation of an intramolecular disulphide bond between Cys 24 and Cys59 within each monomer. The assumption of the dimeric state involves dramatic structural changes, where most of the N-terminal domain adopts a different secondary and tertiary structure. It was shown that CLIC1 undergoes a conformational change to insert into artificial bilayers and the fluorescence resonance energy transfer (FRET) spectroscopy shows a distance between the tryptophan in position 35 in the N-terminal domain and the three conserved cysteines (Cysteine 89, Cysteine 178, and cysteine 223) in the C-terminus. This indicated that in the presence of a lipid bilayer, conformational unfolding occurs between the N- and C-terminus (Goodchild SC, et al., 2010). Hence, the insertion of CLIC1 into the membrane can be regulated by redox conditions (Sing H, et al., 2007). Furthermore, the putative trans-membrane domain plays an important role in insertion of

CLIC1 due to residues Cys24 – Val46, which can form a putative trans-membrane domain (Golczak M, et al.,2002) (Figure 1-7).

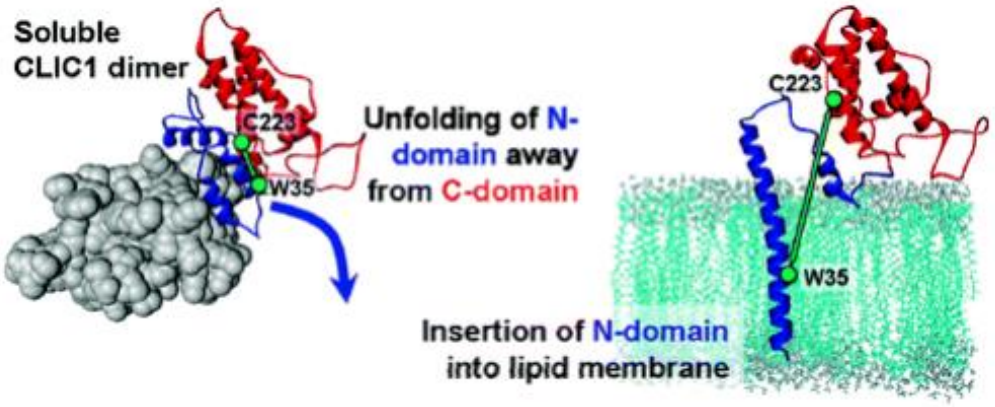


Figure 1-7 Membrane insertion model of CLIC1 protein. "The N-terminal domain inserts into the lipid bilayer (highlighted in blue), while the C-terminal domain remains cytoplasmic (highlighted in red)" (Landry D, et al., 1993).

Due to glutathione (GSH) binding site and a catalytical active cysteine (Cys24) is located at the beginning of helix α2 in the N-domain in response to oxidizing conditions can enhance its channel activity and its insertion into artificial membranes (Harrop SJ, et al., 2001). Hence, the GSH-binding site might be involved in recognizing the mixed disulfide bond of some GSH-modified proteins and thus target the chloride channel to a specific subcellular location (Harrop SJ, et al., 2001). However, this assumption was not supported by trapping mutation studies and CLIC1 (C24A) mutant is still able to insert into membranes, even though the mutant Cys24 was replaced with alanine (Sing H, et al., 2007) and only Cys59 is critical for insertion and ion channel activity. CLIC1 can make an intermediate structure under oxidizing conditions, in which CLIC1 has been shown to form a reversible non-covalent dimer with an intramolecular disulfide bond between Cys24 and Cys59 (Figure 1-7 A) (Littler DR, et al., 2008). The dimer structure is reversible, demonstrated by conversion of dimeric CLIC1 protein back to its monomeric state when exposed to 5 mM of the reducing agent DTT (Harrop SJ, et al., 2001).

The dimer seems to be preferentially formed only in the absence of lipids, while in the presence of a lipid membrane, the newly exposed hydrophobic surface of CLIC1 monomers under oxidizing conditions prefers to interact with the membrane (Littler DR, et al., 2008). The dimeric CLIC1 has been demonstrated to undergo a dramatic structural rearrangement from its monomeric state. Instead of the four β-strands, the N-terminal domain becomes

all α-helical and loops to form a hydrophobic interface. Two soluble CLIC1 monomers can thus interact via their hydrophobic interface, to form a CLIC1 dimer (Figure 1-7 B). Thus, it has been suggested that CLIC1 adopts a structure like this dimer structure, exposing its hydrophobic interface to then insert into the membrane (Littler RD, et al., 2010). This is supported by evidence showing that both monomeric and dimeric CLIC1 could insert into the membrane and form functional ion channels in artificial membranes (Zhang, Y, and Woolhead A.C, et al., 2020).

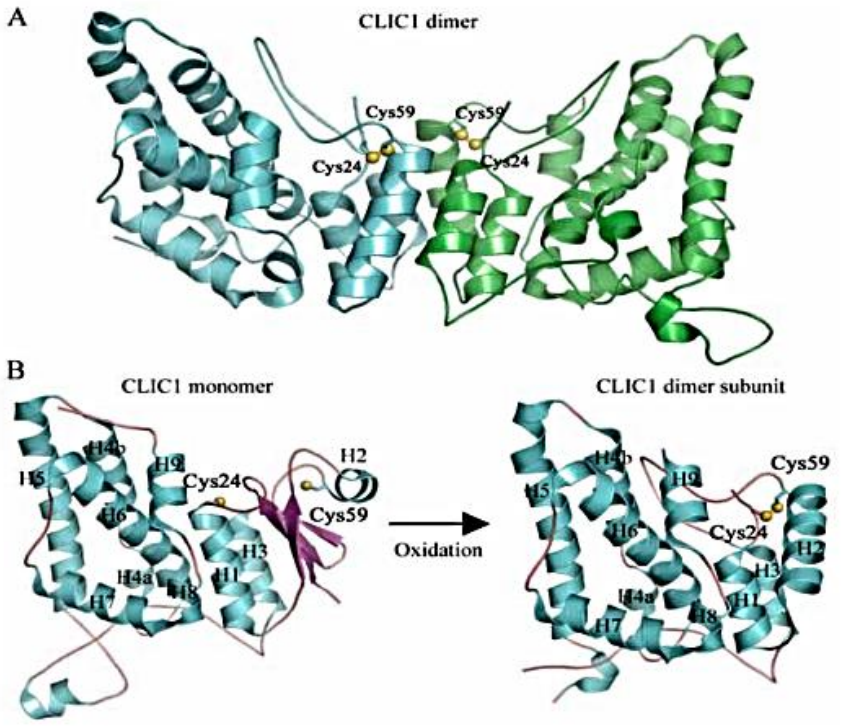


Figure 1-8 The oxidized CLIC1 dimer. A) The all alpha helical CLIC1 dimer showing the location of catalytical cysteine residues (Cys24 and Cys59) that form the intermediate structure is transiently between monomer and dimer positions, which is unique to CLIC1. The two subunits are indicated in cyan and green. **B)** The CLIC1 monomer is demonstrated via secondary structure elements, with respect to Cys24 and Cys59 labeled (Littler RD, et al., 2010).

The redox can control structural rearrangement in CLIC1 and facilitate the formation of a disulfide bond between Cys24 and Cys59 to show a dimeric position in the CLIC1. Interestingly, the monomer oxidation can increase the membrane binding of CLIC1 compared with the dimeric oxidation, concluding the dimerization process is not essential for membrane insertion and it could be considered as an explanation for other CLICs

membrane proteins such as CLIC4 that, as a monomeric protein, can function as an ion channel but in response to

oxidative conditions (Goodchild SC, et al., 2009). Interestingly, the redox state in the case of CLIC1 insertion is not involved with the redox catalytic activity of Cys24, and Cys24 does not show any central regulatory mechanism for membrane binding of CLIC1; hence, the ability of CLIC1 for insertion comes from Cys59 residues, which is essential for stabilizing the dimer structure. We consider this information to better understand the ion channel activity of CLIC4 as a monomeric protein with sensitivity to oxidative stress, which has previously shown to be regulated by redox due to belonging to the Glutathione S transferase superfamily (Alkhameci H, et al., 2015).

1.4.2 CLIC4 as a membrane protein

CLIC4 Like other CLIC proteins, can appear in both forms either as a soluble form, like GST similarity, or an integral membrane form, which is resistant to alkali treatment. Furthermore, the Proteinase K treatment of microsomes containing CLIC4 results in a 27 kDa reduction in the size of the protein, leaving a 6 kDa fragment. Thus, those result can support the hypothesis, in the integral membrane form of CLIC4 has a single transmembrane site located near the N-terminus and running from approximately Cys-35 to Val-57 (Duncan RR, et al., 1997).

The patch-clamp experiments have shown the channel properties of CLIC4 associated with plasma membrane with channel activity in transiently transfected HEK293 cells, demonstrating an anion channel activity of around 1 pS conductance, while in the artificial vesicles containing the purified CLIC4 into lipid bilayers resulted in anion channel activity with a conductance of 10–50 pS (Littler R.R, et al., 2010). The reason for the difference between these two results is not clear but both results confirm the ion channel activity of CLIC4. The inhibition of the CLIC4 conduction through several antibodies in the HEK293 cells indicated that the C-terminal portion of CLIC4 (residues 60–253) exists in the cytoplasm (Figure 1-9). Thus, like CLIC1, recombinant CLIC4 appears to be capable of forming ion channels in synthetic bilayers under non-reducing conditions in the absence of any partner proteins and those of information can provide the ion channel activity of CLIC4 could be involved with redox response in the oxidative stress conditions.

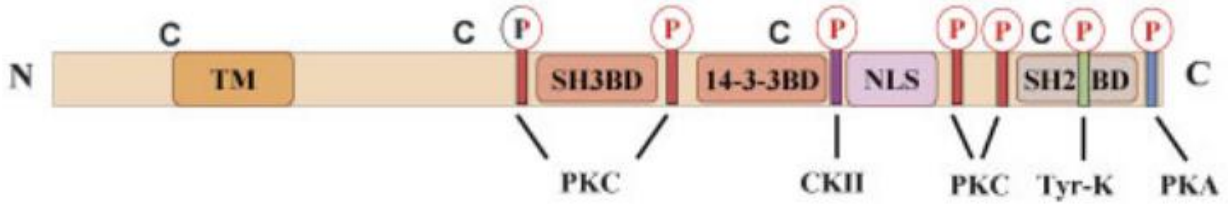


Figure 1-9 A linear schematic representation of CLIC4 protein shows the particular domains for membrane insertion (TM), nuclear localization signal (NLS), protein-protein interaction (SH3BD, 14-3BD, SH2BD), phosphorylation sites (PKC, CKII, PKA, Tyr Kinase) and critical cysteine sites (C); the figure is taken from (Shu KS, et al., 2007).

Moreover, like CLIC1, recombinant CLIC4 appears to be capable of forming ion channels in synthetic bilayers under nonreducing conditions in the absence of any partner proteins and those of information can provide the ion channel activity of CLIC4 could be involved with redox response in oxidative stress. Given that CLIC1 channels are redox regulated, under oxidation and reduction conditions that are provided via 2mM H_2O_2 for 2 h at 18 °C, CLIC4 continued to run as a monomer on size exclusion chromatography column. This differs from the behaviour of CLIC1, which forms a non-covalent dimer concomitant with the formation of an intramolecular disulfide bond between Cys24 and Cys59. This difference between CLIC1 and CLIC4 is not unexpected since Cys59 in CLIC1 is not conserved in other CLIC proteins and corresponds to Ala70 in CLIC4. That means CLIC1 has a potential to make an intermediate structure between cysteine 24 and 59 and CLIC4 is not able to make an intermediate structure with another non-catalytic Cysteine (Cys100, Cys189 and Cys236), but it can form an intermediate structure with GSH (Alkhameci H, et al., 2015).

However, the oxidation of CLIC4 via incubation with 0.4mM H_2O_2 at room temperature for one hour dramatically increased its affinity for liposomes compared with reduction condition that provided with 5mM dithiothreitol, no CLIC4 channel activity was observed in the tip dip bilayer electrophysiology system. Thus, the channel formed by purified recombinant CLIC4 in artificial lipid bilayers (liposomes) appears to be under redox control (Woolhead C A, et al., 2019). The conclusion of this study paves the way to understand that the redox condition can regulate the ion channel activity for CLIC4 compared with CLIC1. The redox regulation of ion channel activity of CLIC4 structurally is related to the glutathione S transferase family, where CLICs proteins are involved with this family.

1.5 Structural Similarities between the GST subfamily and CLIC Protein.

The CLIC family has been classified as belonging to the glutathione S-transferase (GST) protein superfamily. The GST superfamily is a group of soluble homodimer proteins with a thioredoxin fold in their N-domain and an all-alpha-helical in the C-domain. Based on structural predictions using its primary sequence (Dulhunty A, et al., 2001), CLIC1 and CLIC4 can be considered as a member of the GST superfamily. This was then confirmed by Harrop SJ, et al (2001) who determined the crystal structure of CLIC1, showing the characteristic structural fold of the GSTs was also adopted by the CLIC proteins. The GSTs are grouped into classes including alpha, beta, delta, epsilon, theta, zeta, mu, pi, tau and omega (Figure 1-10).

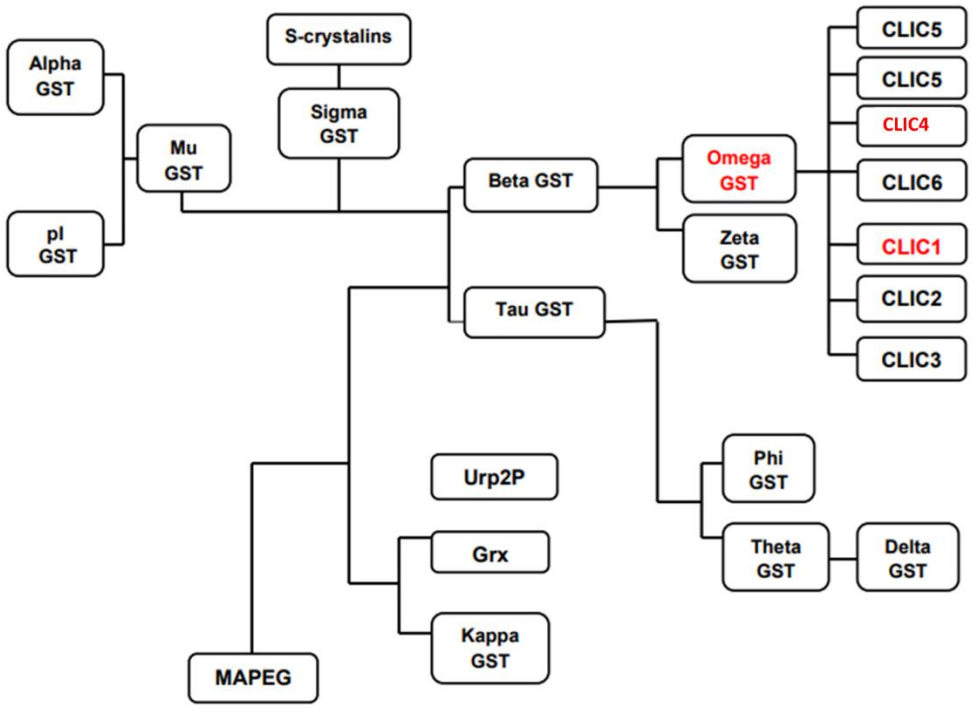


Figure 1-10 Dendrogram of the GST super family. Sequence relatedness between the members of the CLIC family adapted from (Berryman M, et al., 2000) and the members of the GST family adapted from (Sheehan D, et al., 2001).

Members of this super family are mostly found to exist as dimeric proteins in the cellular cytosol, with a molecular weight of around 24-25 kDa for each subunit (Atkinson HJ, et al., 2009). Most of it closely resembles the human Omega-GST-1 (GST Ω1-1); they share a number of amino acids, approximately 241 (Littler RD, et al., 2010). Furthermore, these

two proteins contain a single cysteine residue (Cys24 in CLIC1 and Cys32 in GST Ω1-1) in their active sites and can form a mixed disulfide bond with glutathione (Rossjohn J, et al., 1998). Furthermore, CLIC4 with one single Cys 35 in its active site may be able to form a disulfide with glutathione as well (Figure 1-11). In addition, the inhibitor indanyloxyacetic acid-94 (IAA94), which inhibits the ion channel activity of the CLICs family, can be considered an inhibitor for the GSTs. The GSTs-like activity of CLICs protein family leads them into two main reactions of their functional role; either oxidoreductase activity or ion channel activity (Alkhameci H, et al., 2015).

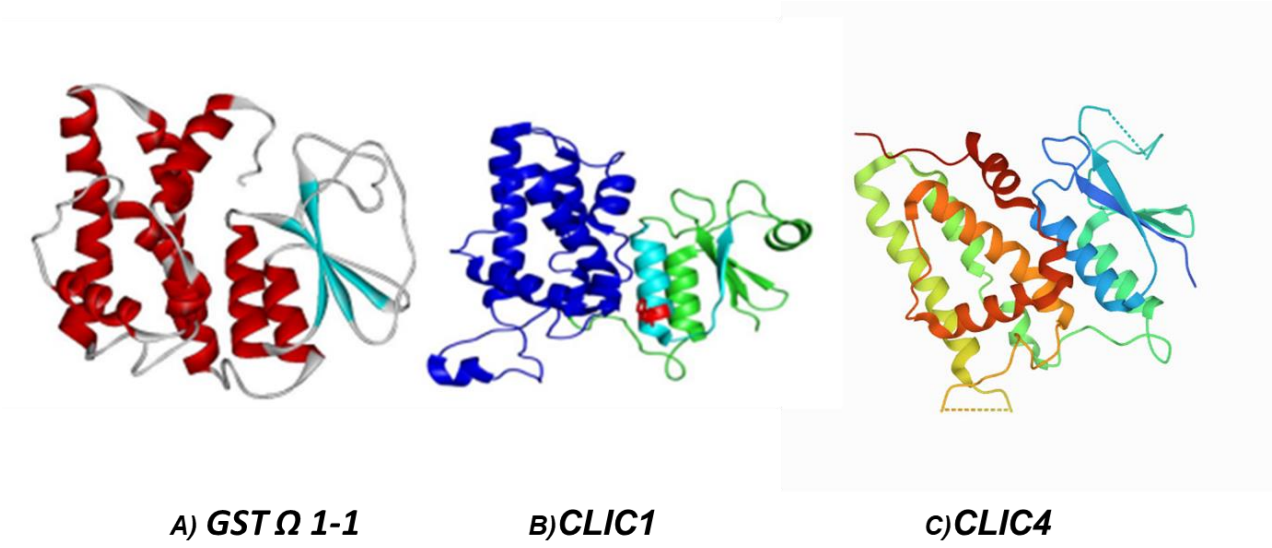


Figure 1-11 The structural similarity of A) GST Ω 1-1, B) CLIC1 structure C) CLIC4 structure. CLIC1 and GST Ω 1-1 consist of N-terminal domain with the thioredoxin indicated in gray in GST Ω 1-1 and in green in CLIC1. The all α-helical C- terminal is indicated in red in GST Ω 1-1 structure and in blue in CLIC1, C) CLIC4 like GST Ω 1-1 consists of N-terminal like thioredoxin domain, which is indicated with light blue and an all α-helical C-terminal is recognized with mixed colored (Ponsioen B, et al., 2009).

1.6 Antioxidant and deglutathionylation enzymes

Glutathione is a three-peptide component and exists in the millimolar concentration range in the liver (Mieyal JJ, et al, 2008). It is the major non-protein thiol compound that acts as an inherent antioxidant and works with oxidized glutathione (GSSG) as an intracellular redox buffer (Dalle-Donne I, et al., 2008). GSH, containing a cysteine, glutamate and glycine held together through unusual peptide bonds, in which the carboxyl group of the glutamate sidechain is linked to the amine group of cysteine. While the carboxyl group of cysteine is

linked to the amine group of glycine in a conventional manner. The concentration of GSH in the mammalian cells can be considered in a range from 1~5 mM depending on the cell type. The majority exist in the form of GSH with only a small portion existing as GSSG. ROS tends to accept electrons so that GSH acts as an antioxidant by serving as an electron donor. In this process, glutathione is turned into GSSG. Under normal physiological conditions, the GSH: GSSG ratio is around 50:1 to 100:1 but the ratio can be changed in pathological conditions e.g., oxidative stress. Glutathione may be able to be added or removed from target proteins through a specific process known as de/glutathionylation which is catalyzed by specific enzymes (Meyer Y, et al., 2009).

Glutaredoxin (Grx) is suggested to be the major deglutathionylating enzyme in mammalian cells (Mieyal JJ, et al., 2008). Grx1 is the cytosolic form of Grx in mammalian cells and is exclusively selective for protein-SSG compared with other forms of disulfides (e.g., S-S disulfide bond, S-nitrosylation, etc.), and it is extremely essential in reducing protein-SSG; thus, it is considered as a specific deglutathionylating enzyme. Moreover, in Grx1 knockout mice, no deglutathionylating activity was detected (Meyer Y, et al., 2009), and can be considered as a support for Grx1 function. Besides Grx1, other enzymes have been implicated in deglutathionylation or serving in antioxidant defense system, including thioredoxin (Trx2), peroxiredoxins (Prx3/5), thioredoxin reductase (Trxr2), and glutathione reductase (GR) (Mieyal JJ, et al., 2008).

However, there is a misunderstanding of the substrate specificity of the substrate, and the specific substrates of these enzymes still exist and not well understood (Linmta B, et al., 2006). Thioredoxin and Glutaredoxin proteins share several structural similarities. Their active site residues are located within their N-terminal domain (Holmgren A, 1976). Upon previous study, the thioredoxin system (Holmgren A, 1983) and the glutathione system (Wells WW, et al., 1993) play a crucial role in redox control for cellular function (Holmgren A, 1995).

The NMR spectroscopy and the X-ray crystallography have been utilized to investigate the structures of dithiol and monothiol Glutaredoxin (Holmgren A, 1979). Structurally, the Glutaredoxins belong to the Thioredoxin fold family of proteins. This motif is composed of a four stranded ß-sheet surrounded by three α- helices (Figure 1-12), which is also conserved by the GSTs and the CLICs proteins. The active enzyme site is placed in this fold motif and is known as a G-site (Meyer Y, et al., 2009) and it usually utilizes the N-terminal

cysteine as its active residue. Both Thioredoxin and Glutaredoxin families share similar active site motifs either the dithiol (Cys-X-X-Cys) or monothiol (CysX-X-Ser) motif (Lillig CH, et al., 2008).

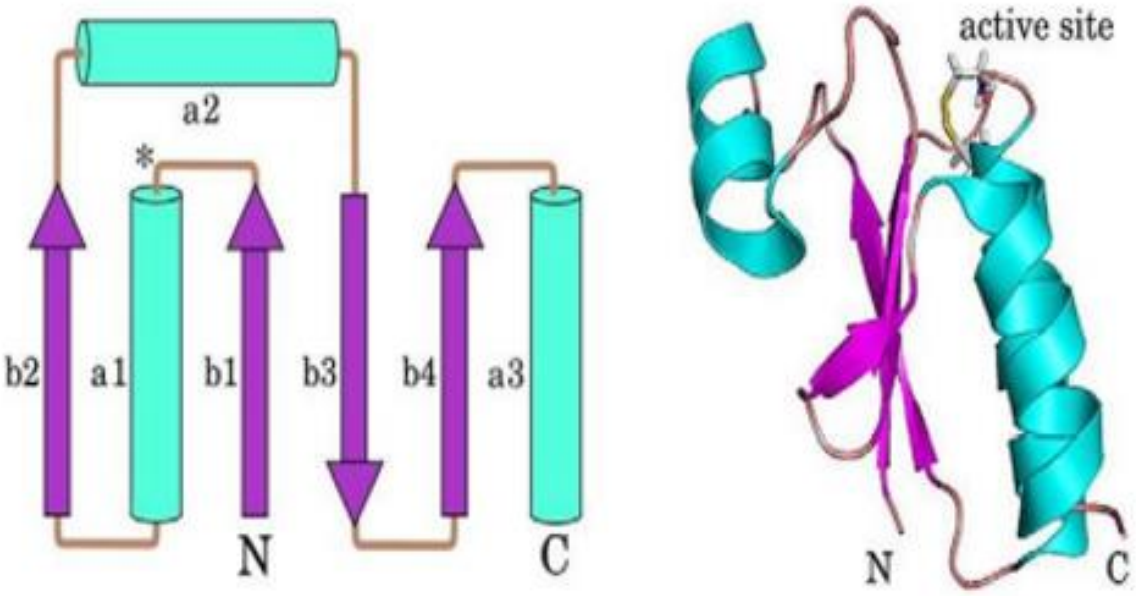


Figure 1-12 Glutaredoxin structure. The structure of oxidized E. coli Grx1 is shown on the right, and the bacterial glutaredoxins present the most principal representations of the thioredoxin fold (on the left) (Lillig CH, et al., 2008).

This glutaredoxin-like motif with a redox-active cysteine residue is present in the CLIC proteins. It can form a disulphide bond with GSH, like the GSTO Ω1-1 class of proteins (Littler RD, et al., 2008). This active cysteine frequently consists of two CXXS or CXXC motif, which is placed in the N-terminal domain. Most of the CLIC proteins contain the CXXS motif; however, CLIC1, CLIC2 and CLIC3 are exceptions, as they contain the thioredoxin-like motif with the double cysteine (CXXC), these two cysteines can form an intramolecular disulphide bond (Littler RD, et al., 2008) (Figure 1-13).

Figure 1-13 Conserved G-site motifs in members of the CLIC family. Multiple sequence alignment of human CLICs protein: CLIC 1-6, GST-omega, and Grx1-3 are highlighted in grey and are the glutaredoxin/thioredoxin active-site motif in the (G-site). The Accession numbers of proteins: CLIC1 (CAG46868), CLIC2 (CAG03948), CLIC3 (CAG46863.1), CLIC4 (CAG38532), CLIC5 (AAF66928), CLIC6 (NP_444507), GST-omega (AAF73376), Grx-1 (BAAO4769), Grx2 (AAK83089) and Grx-3 (AAH0528289) (Alkhameci H, et al., 2015).

Moreover, a structural study of the interaction between CLIC1/GSH complex demonstrated that the GSH group is poorly subjected to interaction with CLIC1, and this interaction can occur under a low-affinity binding (Harrop SJ, et al., 2001), and this property, in turn, can lead to the observation of the existence of an opening loop or an “empty slot” that is in place in the neighborhood of the GSH binding site of CLIC1 protein (Littler RD, et al., 2005); again this empty site, in turn, could be considered as a binding site for an unknown substrate and might be involved with transferring some components between intracellular organelle; hence ion channel activity of CLICs proteins can be involved with GSH binding site. This GSH binding site of CLICs family related to GST-O-1 structure with ability to modulate ion channel function in CLICs family, and ion channel activity of CLICs protein can be owing to this structure.

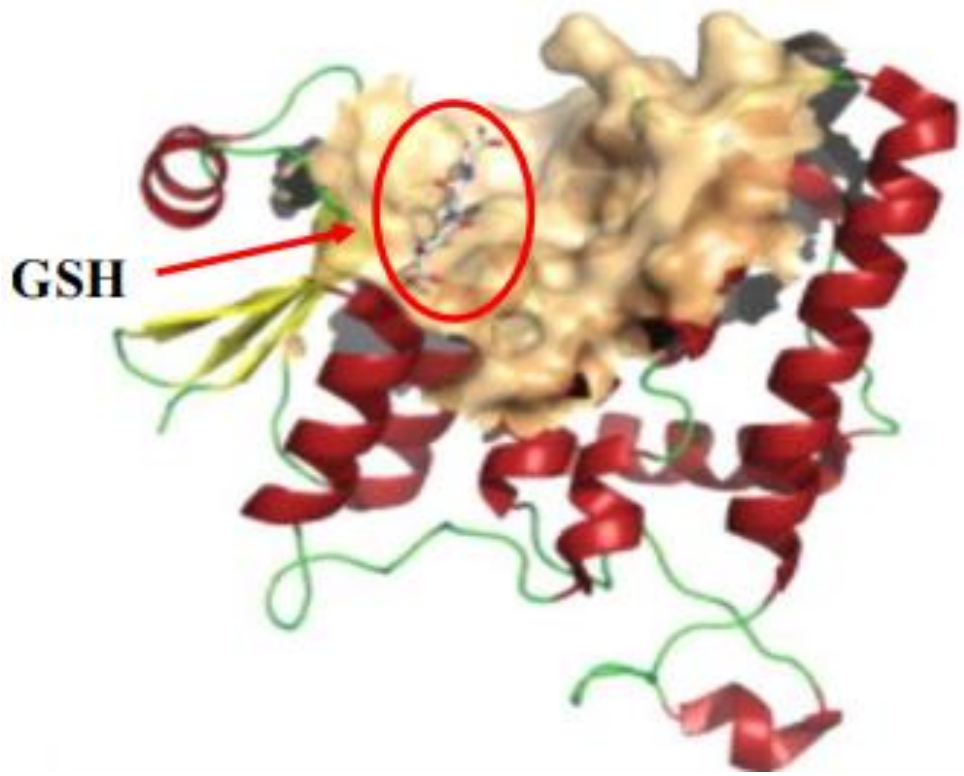


Figure 1-14 Glutathione (stick model) in CLIC1 protein as shown with the red arrow. In addition to the existence of an opening or an empty site near the GSH binding site, this can be a binding site for macromolecules, polypeptides, and unknown substrate (Littler RD, et al., 2010).

1.7 Structural of ion Channel

1.7.1 The Structural comparison of omega-GST (GSTO1) and CLIC1

Glutathione S-transferase omega-1 (GTSO1-1) is an enzyme that, in humans, is encoded by the GSTO1 gene. This protein has either a dehydroascorbate reductase activity with oxidoreductase function in the glutathione-ascorbate cycle as a part of antioxidant metabolism or a glutathione-dependent isomerase to isomerase or reduction of disulfide bonds (Alkhameci et al., 2015). The GTSO1-1 structure, due to having G-site near the active site, can function as an ion channel and/or an oxidoreductase module. X-ray crystallography revealed that the soluble form of CLIC1 adopts a three-dimensional fold, the same as the GST superfamily, and in particular the GST omega class (Harrop SJ, et al., 2001). The CLIC1 structure consists of an all alpha-helical C-terminal domain and an N-terminal thioredoxin domain comprised of four beta strands sandwiched between three alpha-helices that contain the glutaredoxin like monothiol motif [Cys-Pro-Phe-Ser]. The active cysteine residue, Cys24 in CLIC1, was found to covalently bind GSH in a manner the

same as the GST-omega proteins that possess a monothiol G-site [Cys-Pro-Phe-Ala] (Board PG, et al., 2000) (Figure 1-15).

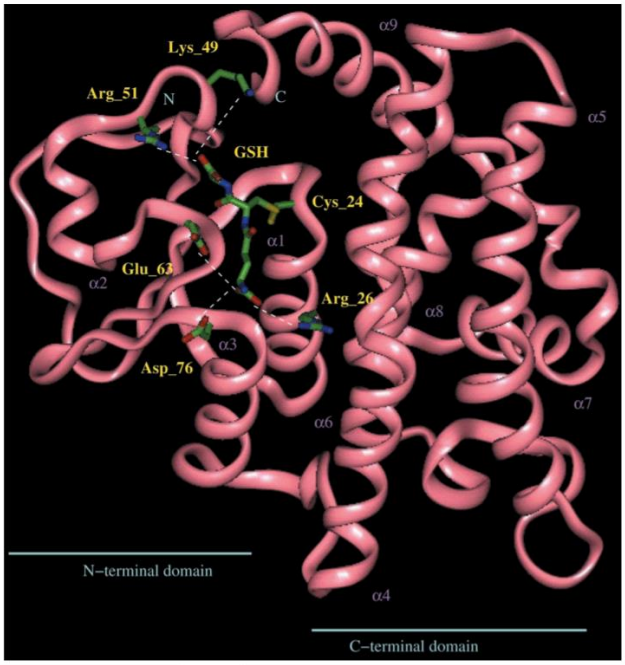


Figure 1-15-1 "A homology model of CLIC protein NCC27 and the atomic coordinates of GSTO1–1 the figure shows the CLIC sequence is compatible with the GSTO1–1 fold. The C-terminal extension is modelled to form a helical structure covering the top of the active site, a feature so far only seen in the Alpha and Omega GSTs. Placing GSH into the putative active site of the CLIC protein reveals that it can form interactions analogous to those observed in the Omega class GST."

Mutagenesis experiments identify cysteine 24 as the catalytic cysteine residue in CLIC1, which is characterized as a catalytic cysteine residue related to its oxidoreductase activity and ion channel activity of CLIC1, although the membrane insertion is totally different and cysteine 59 is responsible for insertion process to make the stability form of CLIC1 in the membrane-binding process.

1.7.2 The Structural comparison of GST omega class 1 (GSTO1) and CLIC4

CLIC4 as one of the CLICs protein structurally related to glutathione S transferase can be compared with the GST omega class upon cysteine residues that mediate the binding of glutathione and secondary substrates in GST structure (Pensione B, et al., 2009). CLIC4 consists of 4-cysteine residues that are involved with two functions catalytic and non-catalytic. The catalytic function could be involved with translocation, and the non-catalytic involved with the stability of folding protein in tertiary structure. The binding site for catalytic activity of cysteine 35 is located in the same place with GSTO-1 and can be involved with ion channel activity of CLIC4 along with its oxidoreductase. Through mutational analysis, recent studies identified 4 conserved cysteine residues, including the reactive Cys35, which are essential for CLIC4 to respond to oxidative stress, and are critical for substrate processing in GSTs. Although CLIC4 suffers from a lack of classical GST activity, CLIC4 can be mostly characterized by a single active-site cysteine residue (CXXS), which shows various unique thioltransferase and reductase activities as well as stress response properties that are still incompletely characterized (Whitbread et al., 2005). Hence, the mutant form of catalytic cysteine residue could be influenced by its both functional role either through enzyme catalytic activity or ion channel activity.

1.8 The oxidoreductase activity of CLICs protein related to omega-GST (GSTO1)

In 2015 Alkhameci demonstrated that the soluble form of the CLIC4 protein has an enzymatic activity that is distinct from the ion channel activity of their integral membrane form. This CLIC4 enzymatic activity may be important for protecting the intracellular environment against oxidation (Chalothorn D, et al., 2009). It is also likely that this enzymatic activity regulates the CLIC4 ion channel function (Alkhameci H, et al., 2015).

In addition, they demonstrated that CLIC proteins have glutaredoxin-like glutathione-dependent oxidoreductase enzymatic activity. CLICs 1, 2 and 4 demonstrate typical glutaredoxin-like activity using 2-hydroxyethyl disulfide as a substrate. The ability of CLICs protein to be involved with an oxidoreductase activity is related to Glutathione S transferase and presenting the G-site for binding to GSH. The glutaredoxin G-site is either monothiol, containing a single cysteine residue (Cys-Gly-Phe-Ser) or dithiol (Cys-Pro-Tyr-Cys) and generally act as thiol-disulfide oxidoreductase, while the monothiol members by

forming mixed disulfides between GSH, target proteins, or low-molecular weight thiols can act as a redox sensor in signaling pathway (Meyer Y, et al., 2009), while CLICs 1, 4, 5 and 6 contain the monothiol acive site motif (CXXS), and CLICs 2 and 3 (Harrop SJ, et al., 2007) contain the dithiol motif (CXXC), It has been speculated that the soluble form of the CLICs would also function as oxidoreductase enzymes (Littler RD, et al., 2017), rather than ion channel function (Board PG, et al., 2000).

The enzymatic activity of the GST-omega proteins is structurally related to the thioredoxins which are resembles of the glutaredoxins (Board PG, et al., 2000), can reduce of intracellular disulfides by catalyzing reactions through GSH, NADPH, and glutathione reductase (GR), (Holmog ren A, et al., 1995).

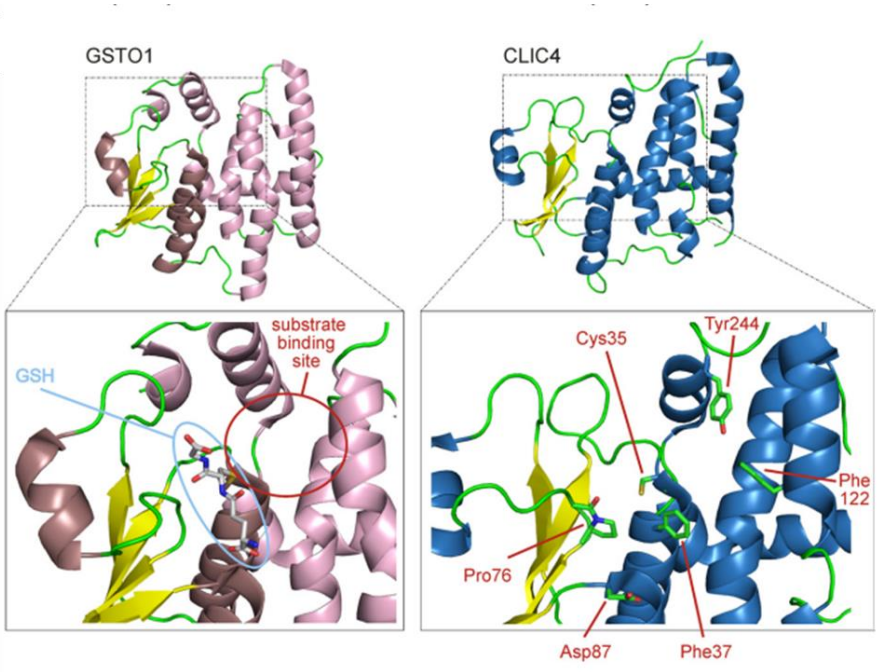


Figure 1-15-2 A Structural comparison of omega-GST (GSTO1) and CLIC4 A structural comparison of omega-GST (GSTO1) and CLIC4. The indicated residues were mutated, based on their equivalence to the residues that mediate the binding of glutathione and secondary substrates in GSTs (Ponsioen B, et al., 2009).

1.9 The Predicted Cellular Protective Effects of the CLIC proteins against Oxidative Stress

Oxidative stress means an imbalance between reactive oxygen species (ROS) and the availability and activity of antioxidants. Generally, oxidative stress is characterized by overproduction of reactive oxygen species (ROS), which include hydrogen peroxide, superoxide radicals and hydroxyl radicals (Cao Z, et al 2011). ROS can induce various reversible oxidative modifications to target cysteine residues of susceptible proteins. These excess levels of ROS, in turn result in modification of proteins with non-disulfide formation or accumulation of unfolded protein in the cells notably ER (Ryter SW, et al., 2007). Reversible modifications under moderate oxidative stress may protect proteins against permanent modification or incorrect folding and modulate their functions (Biswas S, et al., 2006). Modification of cysteine residues results in their conversion to formation of intra- and inter- molecular disulfide bonds in proteins, as well as formation of disulfide bonds between protein thiols and low molecular weight thiols such as cysteine and glutathione (GSH) in a post-translational protein modification process called glutathionylation (Biswas S, et al., 2006). Thioredoxins and Glutaredoxins have been proposed to play a major role in the response to oxidative stress (Gluta Cotgreave IA, et al., 1998). glutaredoxin proteins catalyse both the formation and the reduction of mixed disulfides between protein thiols and GSH. This indicates that glutaredoxins also play a crucial role in the reversible glutathionylation of proteins.

Given structural studies have shown CLIC4 binds GSH via a glutathione binding site (Littler RD, et al., 2010) and the recent discovery that the CLICs also have GSH-dependent oxidoreductase activity, it has been speculated that glutathionylation is a mechanism by which the CLIC proteins may regulate their ion channel activity and/or act as a substrate for isomerizing or reducing of non-native disulfide bonds formation. Due to high levels of GSH inside the cell's cytosol, the intracellular environment is, in general, highly reduced. Significantly, oxidative stress can alter this environment allowing exposed cellular thiols to become oxidized (Hernandez-Fernaud JR, et al., 2017). The formation of mixed disulfides bond between glutathione (GSH) and a cysteine residue of protein causes to generation of ROS leads to cellular apoptosis. Therefore, Gluathionylation, which is It is a post-translational modification which controls protein function and serves as a reversible protective mechanism of protein thiols that can occur under oxidative stress (Valenzuela SM, 2014). In this project, oxidative stress is induced by Tumour Necrosis Factor alpha

(TNF-α). TNF-α induces a reduction of cellular Glutathione (GSH) levels, and probably induces disulfide formation, which might be responsible for accumulation of ROS in mammalian cells; hence, TNF-α induces oxidative stress in a reactive oxygen species (ROS) dependent fashion (Xue X, et al., 2005).

1.10 Tumour Necrosis Factor alpha (TNF-α)

1.10.1 TNF-α and receptor

TNF-alpha is an inflammatory cytokine and is responsible for a diverse range of signalling events within cells, leading to necrosis and apoptosis in response to oxidative stress (Atzeni et al., 2013). TNF-α exerts many effects by binding, as a trimer, to either a 55 or 75 kDa cell membrane TNF receptor termed TNFR-1 and TNFR-2, respectively. Both these receptors belong to TNF receptor superfamily (TNFRSF). The defining trait of these receptors is an extracellular domain comprised of two to six repeats of cysteine-rich motifs and thus only with correct folding conformation as a trimer-station of TNF receptor superfamily can take place when TNF-α binds to TNFR1 in the extracellular domain of TNFR1 (Figure 1-14) (Shu HB, et al., 1996), to activate the survival pathway through NF-kB.

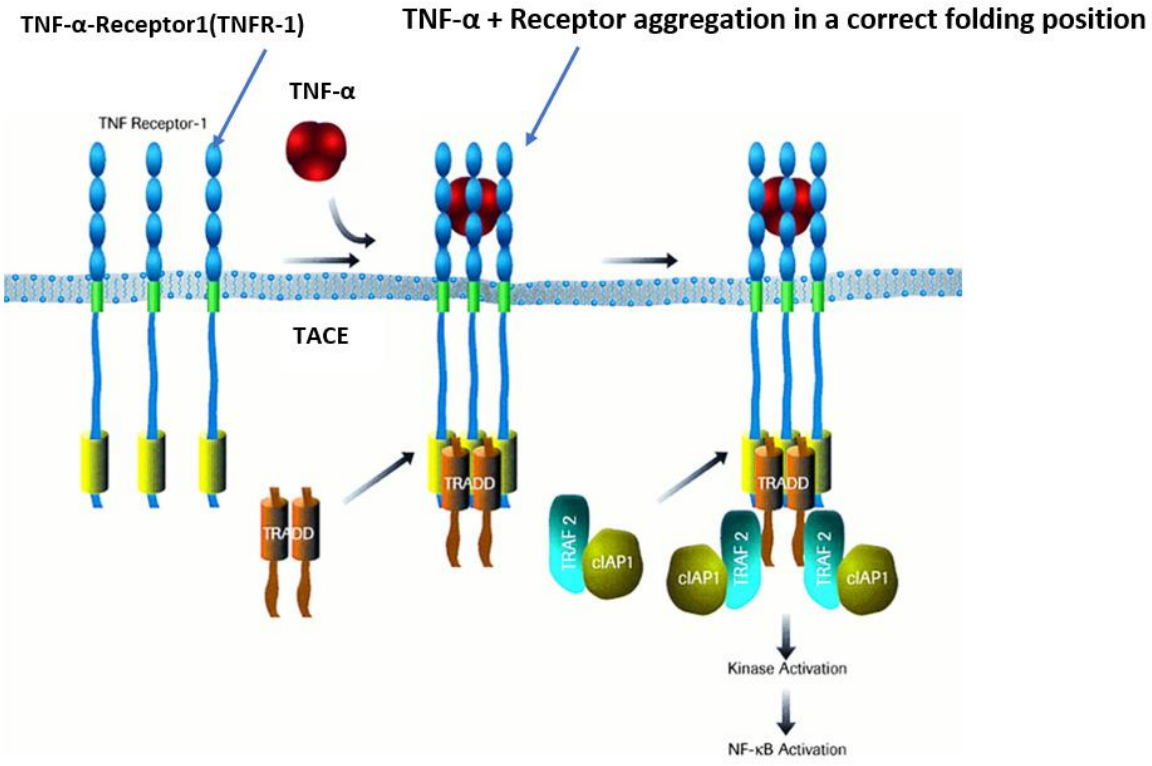


Figure 1-16 Activation of TNF-α signalling survival pathway. To start survival signalling pathway of TNF-α, a valid aggregation state of TNF- α and TNFR-1 proteins is critical to its bioactivity and function. Shedding of TNFR1 by TNF*α*-converting enzyme (TACE) is a primary mechanism for the regulation of TNF*α*-mediated events (Cook et al., 2009). The second initial factor to cascade the signalling event is TRADD with 34KD and over-expressing of TRADD causes the activation of both apoptotic pathway and survival pathway. The intermediate factor for survival pathway is NF-KB, which be activated by phosphorylation and kinase activation. The intracellular domain of TNF-R2 interacts with a TRAF2 and TRAF1 heterocomplex, which can then recruit c-IAP1 (anti apoptosis factor) and make initial complex for activation of NF-KB through phosphorylation and kinase signaling pathway. The figure is adapted from (Shu HB, et al., 1996).

1.10.2 TNF-α and NF-kb

The binding of TNF-α to its two receptors, TNFR1 and TNFR2, results in recruitment of signal transducers the activation at least three distinct effectors via complex signalling cascades to activate caspases and two transcription factors including activation Protein-1 and NF-kB (Nuclear Factor-KappaB) (Cerretani et al., 2011). NF-KB appears as a dimer (homo or hetero) composed of members of the NF-κB subfamily (*i.e.,* p50, p52), which is an important transcription factor in the stress response of survival genes (Han, D et al., 2009). In most cells, the most activate form of NF-κB is the p50/p65 heterodimer, which binds to the κB sites in the DNA. When TNF binds to TNF receptor, complex I forms, and will activate IκB kinase (IKK). NF-κB is normally found anchored in the cytoplasm with IκB-α. (Ghosh S, et al., 2002). IKK will phosphorylate IκB-α, which triggers ubiquitination and degradation of IκB-α. The degradation of IκB-α releases NF-κB, allowing NF-κB to translocate to the nucleus and to promote the transcription of survival genes; one those survival genes could be CLIC4, which was confirmed by Guoan, who indicated after treatment the cells with TNF-alpha CLIC4 was expressed in a high level in Keratinocyte of mouse (Figure 1-18).

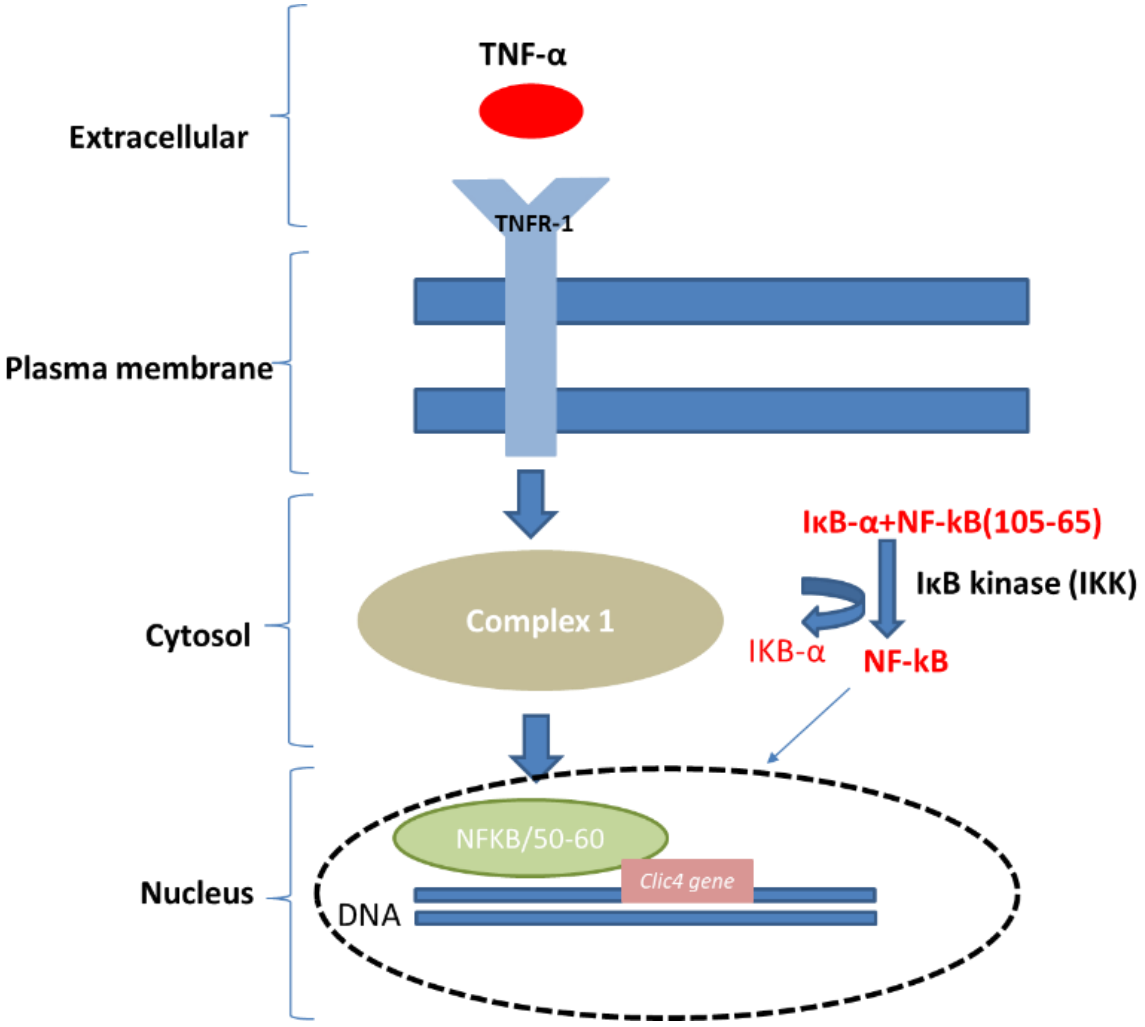


Figure 1-17 Schematic representation of the molecular mechanism of activation of NF-kB following correct interaction of TNF-α and receptor in the cytosol. Extracellular signals such as TNF-α can activate NF-κB-inducing kinase (NIK), which, in turn, phosphorylates via the IκB kinase (IKK) complex. Association of the p105-p65 heterodimer with the IKK complex could result in the phosphorylation of p105 and subsequently its ubiquitination and processing by the proteasome. The p50-p65 complex then can associate with IκBα. Association of the p50-p65-IκBα complex with the IKK complex results in the phosphorylation and subjection to a subsequent ubiquitination and degradation by the proteasome. The released p50-p65 heterodimer translocate to the nucleus, where it activates the transcription of survival gene.

1.10.3 TNF-α and CLIC4

In keratinocytes, CLIC4 expression is induced by subjecting to cytokines, such as TNF and TGF-β (Fernandez-Salas E, et al., 2002). The expression of CLIC4 upregulated either by NF-Kb as a transcription factor of TNF-α induced survival pathway or by redox regulation through reduction of Cellular GSH level via P38 MAP kinas through TNF-α induced apoptosis pathway (Hashimoto et al., 2008). The promoter of CLIC4 gene contains 4 putative binding

sites for NF-κB (Guoan H, et al., 2011), the activation of NF-kB can induce transcription of CLIC4, and it is predicted that innate cytokine responses could induce CLIC4 expression (Shu KS, et al 200 7). Indeed, CLIC4 expression was also upregulated after exposure to TNF-α and in response to cytokine and overexpression of CLIC4 mRNA in keratinocyte (Shu KS, et al., 2005).

Since CLIC4 can be considered as a survival response, it can be involved with two TNF-α pathways, survival pathway with intermediate NF-kB and apoptosis pathway with intermediated JNK and p38 MAP kinase. Both of those pathways are induced by TNF-α binding in a correct aggregation manner with TNF-α receptors. In the survival pathway, NF-KB as a transcription factor with binding to NF-kB binding site that located on CLIC4 promoter can induce transcription of CLIC4 gene, increase CLIC4 mRNA, and finally upregulate CLIC4 expression in the cytosol of (keratinocyte) mammalian cells.

Overexpression of CLIC4 following TNF-α tratment can induce intracellular translocation of CLIC4 in the mammalian cells, as CLIC4 can translocate to the nucleus in response to apoptotice signal-induced by TNF-α, in which those signals have been generated by phosphorylation of JNK, p38 and TGF-β response (Shu KS et al 2007). The apoptotic pathway induced by TNF-α can be activated through the activation of JNK as a key regulator of apoptosis pathway and caspase8, TNFα induced NFκB activation by inhibiting PPM1a/PPM1b mediated IKKβ dephosphorylation. It is intriguing that TNFα causes CLIC4 upregulation and nuclear translocation, which may have some bearing on a potential role of CLIC4 in augmenting TNFα responses in a positive feedback loop. Similarly, CLIC4 could influence alteration of cellular stress response by increased phosphorylation of JNK (Figure 1-18).

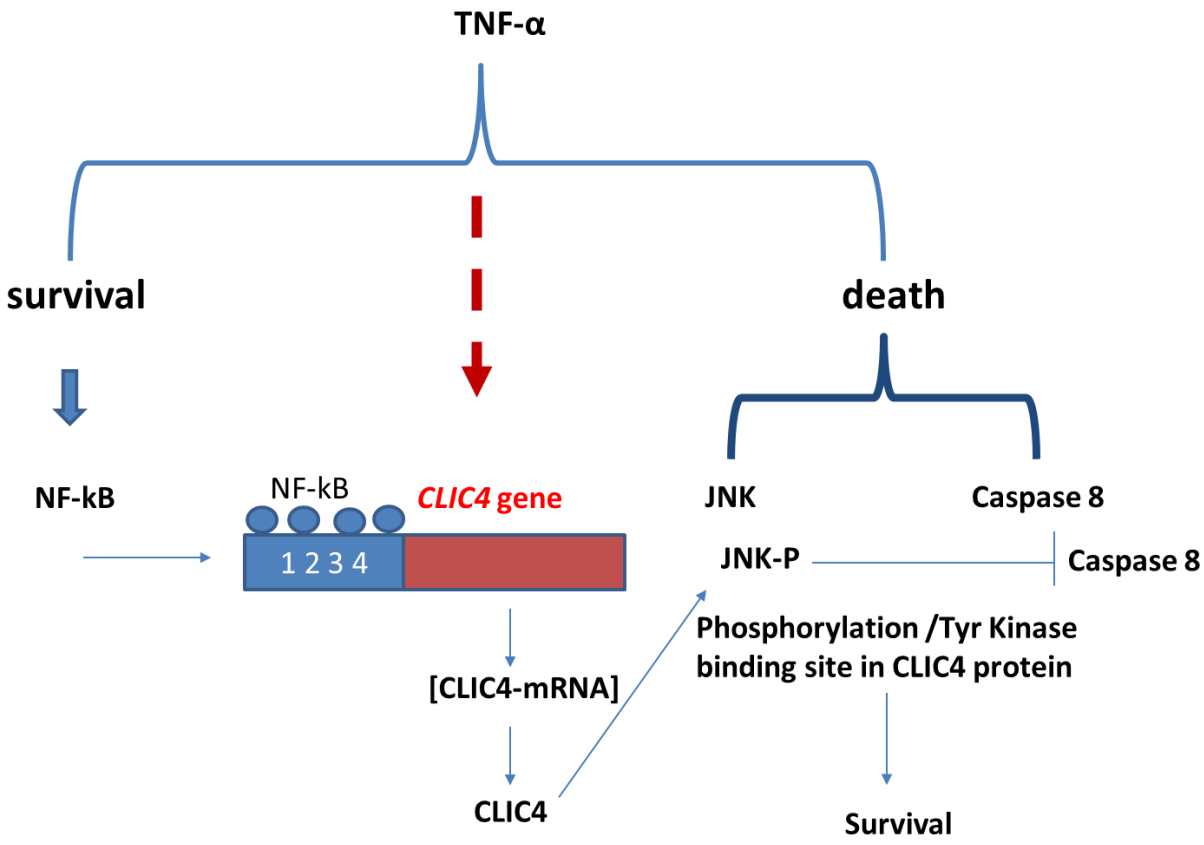


Figure 1-18 CLIC4 can work as an intermediate between both survival and apoptosis pathways of TNF-α induction and alter cellular stress response by increased phosphorylation of JNK and inhibition apoptosis (Shukla A, et al., 2010).

The p38 mitogen-activated protein (MAP) kinase regulates TNF-α-induced RANTES production in the endothelial cells in response to redox alteration, and inversely can be regulated by intracellular GSH levels (Hashimoto et al., 2008). In fact, expression CLIC4 under TNF-α induction can be up-regulated by alteration of redox balance in the cells, which could be represented via an imbalance in reduced and oxidized glutathione within the cells. The oxidative ratio can be indicated by a decrease in the GSH level and increase in GSSG. Hence, p38 MAP kinase activation is regulated by cellular redox balance that can indirectly promote CLIC4 expression and nuclear translocation (Shukla A, et al., 2010). However, CLIC4 nuclear translocation under TNF-α induction was induced via redox imbalance in the cells that are promoted by p38 MAP kinas; in turn, p38 MAP kinase activation is regulated by cellular reduction/oxidation (redox) state (Hashimoto A, 2008). However, the role of cellular redox as regulated by intracellular glutathione (GSH) in TNF-α-induced p38 MAP kinase activation and p38 MAP kinase-mediated RANTES production in

the endothelial cells, which acts as a precursor of GSH synthesis, thus scavenging ROS, can be induced ROS accumulation and reduction of cellular GSH; hence, TNF-α can be responsible for the reduction of the cellular level of GSH and might be responsible for ROS accumulation in the cells ROS (Xue et al., 2005), and in turn, ROS can inhibit NF-κB transcription of CLIC4 genes to promote apoptosis after TNF stimulation.

Reduction of GSH and ROS accumulation can be considered for unfolded protein in the ER or formation of non-native disulphide in protein entry to secretory pathway. Since CLIC4 promoter has been identified for multiple binding sites for MYC, NF-kB, and TGF-beta, CLIC4 could be implicated with this detoxicate signalling pathway through overexpression and consequently intracellular organelle translocation, notably translocation to the Endoplasmic Reticulum (ER) from the cytosol and mostly in the lumen due to a higher concentration of oxidized glutathione (GSSG).

1.10.4 TNF-α and Endoplasmic reticulum (ER)

Oxidative stress-induced by TNF-α might be responsible for the accumulation of unfolded proteins and misfolded in the ER, respectively. The accumulation of unfolded protein in the ER causes ER overload and result in ER stress, while the accumulation of misfolded protein in the ER causes oxidative stress. Mammalian cells trigger a specific response known as the unfolded protein response (UPR) to remove oxidative stress, start the reduction or isomerization of a non-native disulfide bond with ER oxidoreductase by mediating GSH, NADPH, and other cytosolic components via reductive pathway (Xue X, et al., 2005 and Jossep C, et al., 2005). Although recent studies have indicated crosstalk between ER stress and oxidative stress, the mechanistic link is not fully understood. Moreover, TNF-α induces a reduction of cellular GSH levels, which might be responsible for the accumulation of ROS and consequently cell death under oxidative stress. Given that TNF-α-induced NF-κB activation in response to oxidative stress is responsible for transcription of some survival proteins that have a binding site on their promoter to increase overexpression of CLIC4 in the cells, which in turn, caused to increase the accumulation of CLIC4 in the cytosol in response to TNF -α for transferring CLIC4 from the cytosol to the ER (Figure 1-19)

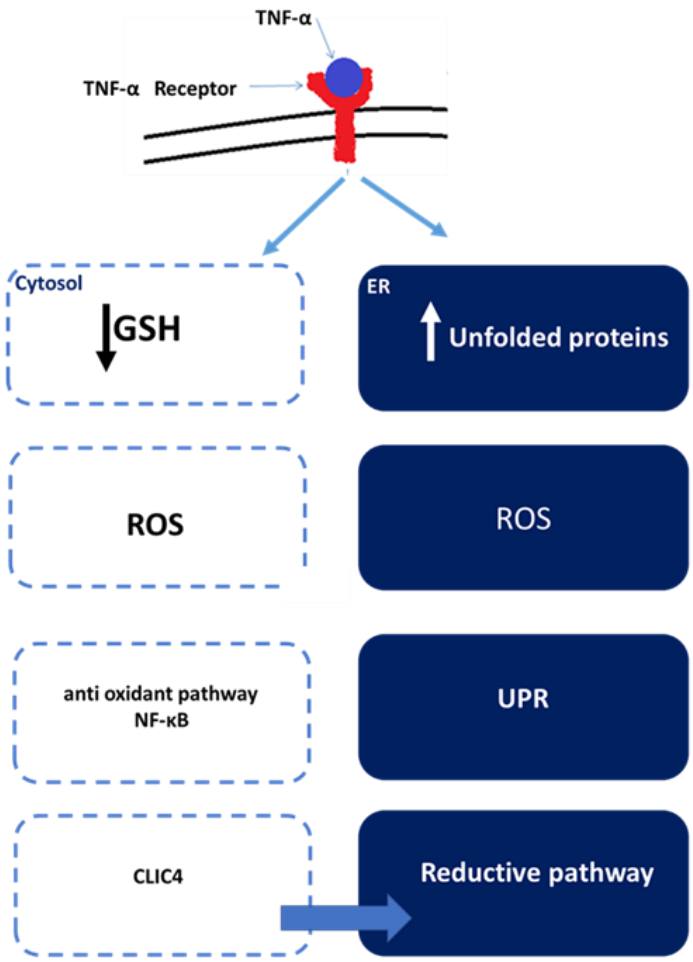


Figure 1-19 Schematic representation of TNF-α induction of re-localization of CLIC4 from the cytosol into ER. TNF-α can induce re-localization of CLIC4 from the ER into the cytosol with reduction of cellular level of GSH and Ros accumulation, in turn, be presented with unfolded and misfolded protein in the ER, and consequently activations of UPR response and reductive pathway (Xue x et al., 2005).

1.11 Endoplasmic reticulum (ER)

The ER is a large compartment with a membrane enveloped that can found in eukaryotic cells (Voeltz, 2013). ER is the entry site of the secretion pathway and can be considered as a host for disulfide formation in protein entering in secretory pathway in mammalian cells. Protein biogenesis can take place in ER, folding in granular part and lipid and steroid synthesis in soft part in this compartment (Lin et al., 2008, and Blower, 2016), so ER plays a vital role in protein synthesis and transport. The ER can be divided into different domains such as peripheral tubular, cisternae, and nuclear envelope (Figure 1-20) (English and Voeltz, 2013). The nuclear envelope works as a barrier to control selective transporting of molecules into and out of the nucleus, which consists of a double membrane bilayer

surrounding the nucleus (West et al., 2011). The peripheral ER consists of tubular and cisternae, which are in contact with nuclear envelop to form an interconnected network. The ER cisternae is placed in a closer position than cisterna to the nuclear envelope (Puhka et al., 2012), due to the cisterna indicated for more ribosome with a big surface and a larger luminal volume. In the luminal part, disulfide bond formation is achieved by (*de-nevo*) and a disulfide exchange reaction (Oka J et al., 2010). In this project for ER study, SP cells, as a source of ER, and microsomal membrane have been applied. ER can derive from the microsome and SP cells.

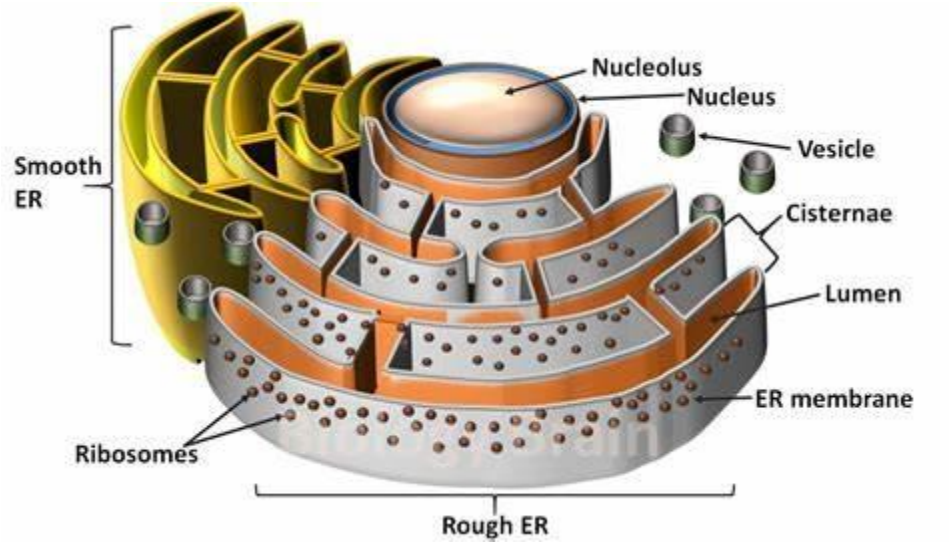


Figure 1-20 ER compartment: ER including functionally and structurally distinct domains. A nuclear envelope in contact with granular ER and, which is required for several cellular processes, mainly transporting. Figure obtained from (https://www.biologybrain.com/diagram-of-endoplasmic-reticulum-definition).

a) Microsomes

ER derived microsomal membrane is usually applied to in-vitro protein biogenesis. Microsomes are vesicles that derive from ER with a diameter of 100 – 200 nm, can be fractionated from cell cultures or other tissues (Sukhodub AL, et al., 2005) by differential centrifugation. Microsomes are included in smooth and rough ER with Enzyme activity due to several chaperon and lumen content. The microsomes, due to being RNA rich, can be involved with protein synthesis, folding, and modification that all events that can happen

in the intact cells (Sabatini et al., 2014); then, the nuclease treatment can remove protein synthesis in microsomal membrane (Robinson Ph, et al., 2017).

b) SP cells

The semi-permeabilized SP cells can be derived from ER by preparation of cell culture with treatment of a digitonin that is regularly used for nuclear import assay (Adam et al., 1990). The digitonin can replace the cholesterol by making the pore in the plasma membrane. The amount of cholesterol in mammalian cells is totally different from one membrane to other membrane as 40-90% of total cellular cholesterol is placed in the plasma membrane and the rest can be distributed to others (Liscum and Munn ,1999). In the permeabilization of cells, all intracellular organelle membrane leaves and is too much under control of digitonin concentration and incubation time (Litvinov et al., 2018). The digitonin treatment (concentration range 20-40 µg/ml) for 5 min on ice can permeabilize the plasma membrane but allow to keep ER and Golgi with cis and trans area with efficient function (Punter et al., 1992).

1.11.1 Protein biogenesis

ER is host of post-translational modification for the membrane and secretory proteins to obtain their native and functional structure to enter the secretory pathway in the mammalian cells. Then, the Golgi apparatus would be the host for corrected folded proteins and then transported and finally trafficked to their right target location. Meanwhile, ER was characterized for a quality control system to identify misfolded proteins, which will be retained and translocated to the cytosol and degraded with (ER Associated Degradation system) ERAD (Sitia and Braakman, 2003). All these steps are called Protein biogenesis; please see below (Figure 1-21).

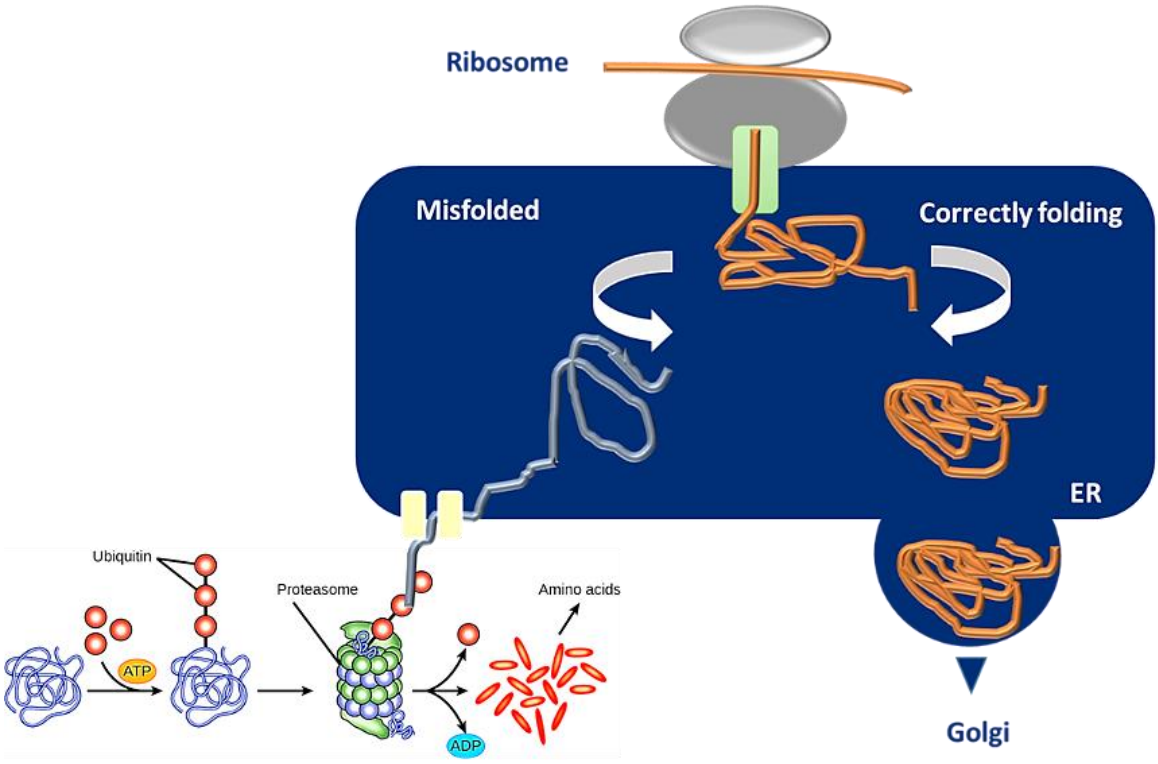


Figure 1-21 Protein biogenesis of membrane and secretory proteins: The nascent chain can transport into the ER lumen via co-translational translocation system that is provided with sec61 translocon. Following post-translational modification and corrected folded proteins transported to the Golgi apparatus to be targeted into right location and misfolded protein was identify with check point system of ER (ERQC) and be retained to the cytosol and degraded with Ubiquitatione through proteosome complex (ERDA) (Sitia and Braakman, et al., 2003).

1.11.2 Protein folding

The genomes of the third of all proteins were coded for entering ER to undergo protein folding and modification to obtain their native and functional structure (Braakman and Bulleid, 2011, Braakman and Hebert, 2013). Therefore, the protein folding process can be a curtail step of protein biogenesis and consist of two main parts: 1) co-translational folding, and 2) post-translational folding. Co-translational folding can occur during ribosome translocon complex and post-translational folding can happen after releasing the nascent polypeptide chain and be fully translocated in the ER (Ellgaard et al., 2016, Braakman and Hebert, 2013). Protein folding is a continuous process without any clear termination step (Bulleid N, et al., 2011). The main restriction for protein folding is sufficient space (Kleizen et al., 2004). Although the translocon complex can provide enough

space for α-helix formation, full folding can be done in the ER lumen (Woolhead A.C, et al., 2004). The rate of folding for limitations in space in the ER lumen, and the limitation of the existence of chaperon and enzyme folding, can happen very slowly (Braakman et al., 1999). The environment can separate nascent chains from each other to avoid any aggregation. The secondary structure formation rate could be much faster than the translational step (4-5 residues per seconds) (Braakman et al., 1999). Folding protein needs some folding factors including Enzyme and chaperon in the ER lumen. Some chaperones are multifunctional and upon folding steps, they can be classified into several groups: 1) chaperone (mainly heat shock proteins), 2) (peptidyl cis or trans isomerase, 3) oxidoreductase Enzyme 4) glycan-binding proteins (Englander and Mayne, 2014). But some chaperons are multifunctional, for example, BIP works either as a folding factor or works as a regulator of unfolded protein during ER stress (Rapoport, 2007). The folding of proteins can undergo several modifications to make sure to provide a functional protein by overlapping several duties within the cells.

1.12 Protein modifications

Modification of proteins can occur when proteins are in the stage of translocation, which can change the physical features of the polypeptide chain happening to the nascent polypeptide chain from the beginning of translation until the protein obtains its native structure and leaves the ER (Braakman and Bulleid, 2011, Braakman and Hebert, 2013). The well-known modifications include disulfide bond formation, N-linked glycosylation, and proline hydroxylation. These modifications are crucial for protein folding, and the folding of the polypeptide chain can influence the modification.

1.12.1 Disulfide formation

Disulfide formation is an essential post-transitional modification for protein folding entering the mammalian secretory pathway. Assembly of folding protein is supported with a covalent interaction between two cysteine residues, and it would occur via the oxidative pathway. The oxidative pathway for disulfide bond formation has been well characterized where PDI is known to oxidize substrate proteins and is itself maintained in an oxidized

form by Ero1 (Frand AR, et al., 1998 and 1999), in which the final acceptor of electron can be molecular oxygen (Tu B P, et al., 2002).

a) Disulfide formation in prokaryotes

The prokaryotic mechanisms of disulfide bond formation and isomerization are better characterized than eukaryotic (Berkmen, 2012). In E. coli, disulfide formation can occur in the cytoplasm and reduction and isomerization can be carried out in the Dsb system (Disulfide bond formation) (Figure 1-10). Disulfide formation is catalysed by DsbA protein in a substrate protein and can be performed via disulfide exchange with the oxidised active sites (CXXC) in DsbA (Bardwell et al., 1993). The reduced form of DsbA will be reoxidized via a DsbB as a membrane protein following completing reaction disulfide exchange (Kadokura et al., 2003). In this process, the electrons will be donated through DsbB to ubiquinone (UQ) during aerobic or anaerobic respiration because of oxidizing flanked cysteines in growing polypeptide through DsbA (Kadokura et al., 2004). DsbC may be able to edit any non-native disulfide bonds formed during the oxidation process (Shevchik et al., 1994); hence, DsbC has to maintained in a reduced state by the membrane protein DsbD and receive electrons from NADPH via Trx (Rietsch et al., 1997). Therefore, disulfide formation needs the existence of an oxidized system and reduction and isomerization of misfolded or nonnative disulfide needs a reduced system in prokaryotic cells as well (Figure 1-22).

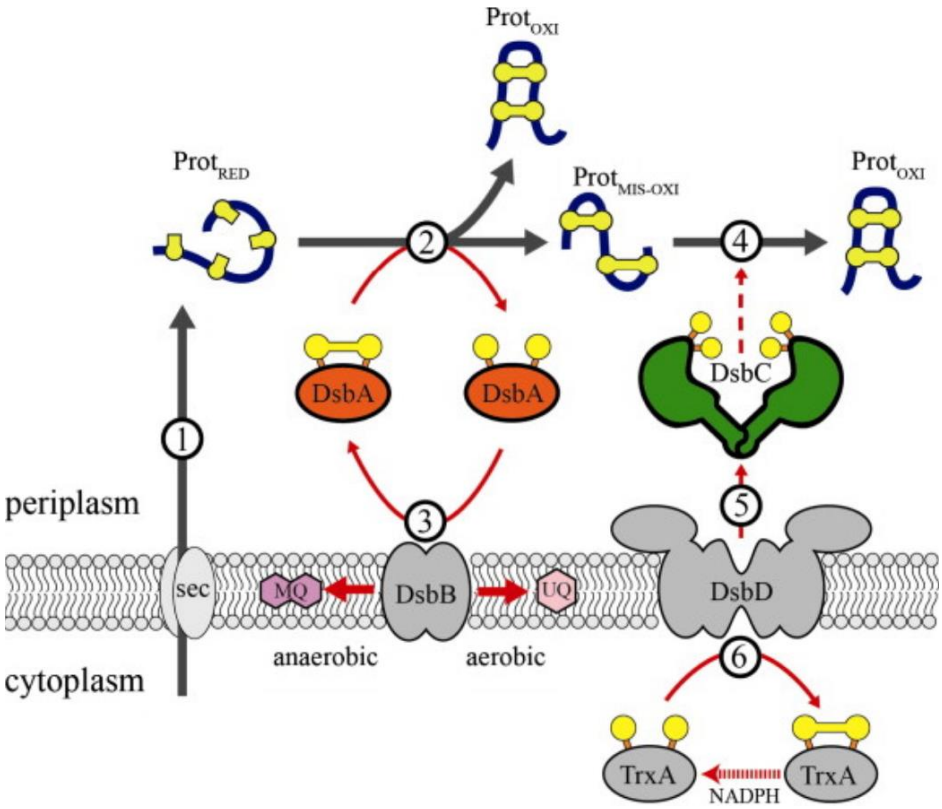


Figure 1-22 Disulfide bond formation and isomerization in E.coli. Disulfide formation process (①②③); Disulfide isomerisation process (④⑤⑥), in yellow box. ① Translation and translocation, sec61 ② Disulfide formation via DsbA, oxidized and reduced state of protein indicated with port red and oxidized ③ reoxidation of DsbA by DsbB, via MQ and UQ, ubiquinone; ④ Isomerization of the non-native disulfide bond by DsbC; ⑤ the reduced state of DsbC is maintained by DsbD; ⑥ DsbD accepts the electron from NADPH, via thioredoxin. Pro-mis ox= non-native disulfide formation.

b) Disulfide formation in eukaryotic cells

Disulfide formation in mammalian cells can be characterized by a covalent interaction between two free (SH-) thiols in ER and intermembrane space (IMS) of mitochondria via different mechanisms (Riemer et al., 2009b). The main role player in disulfide formation in the mitochondria can be considered by two important proteins and assembly protein 40 (MIA40) (Chacinska et al., 2004) and FAD-linked sulfhydryl oxidase ERV1 (ERV1) (Mesecke et al., 2005).

In the ER as a host for disulfide formation in the secretory or membrane binding proteins, two main players as PDI and Ero1 carried out disulfide formation: the redox buffer consisting of reduced and oxidized of glutathione, which might be responsible for disulfide

bond formation directly and indirectly and even isomerization process (Chakravarthi et al., 2006, Ellgaard and Ruddock, 2005).

Recently it has been shown that up to 50% of the glutathione present in the ER appeared as mixed disulfides with protein; hence, glutathione in the ER is even more oxidizing rather than reduced. ER is known as a host for disulfide formation, although the ratio of GSH:GSSG is presented at 1:1, this ration un the cytosol is found at 1:100 (Hawang C, et al., 1992), It has been demonstrated that ERo1 is responsible for oxidizing PDI, although before ER o1 discovery, GSSG was a good candidate for oxidizing the PDI (Bass R, et al., 2004). PDI is responsible for formation disulfide bond (Jansens A et al., 2002), however formation disulfide bonds are not a rate limiting step so there is not surprising that ER is indicated for oxido reductase (Jansens A, et al., 2002). The complete family of oxidoreductases enzyme for reduction or isomerization of non-native disulfide bonds, those including protein-disulfide isomerase (PDI), ERp57, ERp72, P5, and PDI-related protein (PDIR), which is essential and recognized for forming native disulfide bonds (Bulleid N, et al., 2005). Thus, for formation and reduction or isomerization of native and non-native disulfide bonds in the ER, an oxidative and reductive pathway would be required to keep hemostasis in the cells.

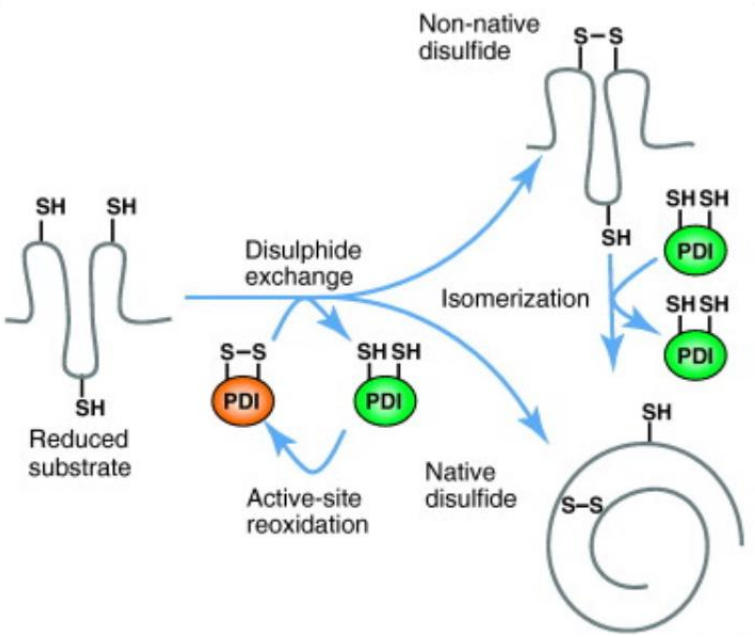


Figure 1-23 Disulfide formation and isomerization. The schematic representation shows the disulfide bonds formation through disulfide exchange reaction with intermediation of PDI, resulting in the formation of native and non-native disulfide formation. The active form of isomerase is PDI that can reduce the nonnative bonds

to native. The reduced form of PDI can isomerase the non-native to native disulfide. (Bulleid and Ellgaard, 2011)

1.13 ER and reductive pathway

The reductive pathway consists of three essential components: 1) oxidoreductase enzyme for reduction or isomerization (Bulleid N, et al., 2005) 2) cytosolic reductive equivalent (NADPH, GSH) (Robinson Ph, et al., 2020) to maintain the oxidoreductase in a reduced form 3) membrane binding protein for transferring cytosolic compartments from the cytosol into the ER (Cao X et al 2019). The main player in reductive pathway can be considered with oxidoreductase Enzyme; this oxidoreoctase enzyme must be in an active form to make reduction or isomerization. Thus, the active cysteine residues of functional CXXC motif, which is a common structural homology within redox protein thioredoxin, can exist in either an oxidized redox state or reduced redox state. The oxidized state of oxidoreductase enzyme is characterized where the cysteine residues are in the disulfide form or in a reduced redox state where the cysteine residues assume the dithiol form. Therefore, for an oxidoreductase to be capable of forming a disulfide bond, it must itself be oxidized to be capable of accepting electrons. Conversely, an oxidoreductase can only be functional as an isomerase or reductase when it is in a reduced form to be capable of donating electrons. After each cycle of reduction or oxidation, the oxidoreductase must be returned to its original, active redox state before it can fulfill further catalytic rounds. Thus, maintaining the active form of oxidoreductase is essential for reduction non-native disulfide. Reduction of these disulfides is crucial for correct folding and for degradation of misfolded proteins. The importance of a reductive pathway to remove non-native disulfides is exemplified by cells that produce large amounts of disulfide-bonded proteins, such as insulin in pancreatic β cells.

1.14 CLIC4 as an oxidoreductase

Members of the CLIC family are soluble proteins capable of spontaneously inserting into lipid membranes - in particular intracellular membranes - to form chloride selective ion channels (Harrop SJ, et al., 2001). Before 2015, characterization of CLIC protein has focused on their ion channel activity and membrane insertion, with no functional work for their

soluble form. In 2015 Alkhameci and her colleagues found that CLIC proteins can act as a glutathione dependent oxidoreductase in the 2-hydroxyethyl disulphide HEDS enzyme assay. HEDS is a low molecular weight compound found to act as a specific and sensitive substrate, suitable for use in assaying the glutaredoxin CLIC proteins as enzymatic activity (Holmgren A, 1995 and Vlamis-Gardikas A, 2002).

The enzymatic activity of CLICs 1, 2, and 4 was evaluated by measuring the consumption of NADPH. The HEDS assay demonstrated an increase in consumption of NADPH that was resulted in a decreased OD at A340 nm in the presence of the positive controls HcTrx-5 and Grx-1, well-known glutathione-dependent oxidoreductases. Upon HEDS assay, the glutaredoxin proteins can act as enzymes, by deglutathionylating mixed disulphide between glutathione (GSH) and the beta-mercaptoethanol region of the HEDS reagent (Herrero E, et al., 2007). The dimer form of CLIC1 was therefore tested for oxidoreductase enzymatic activity in the HEDS assay system. The CLIC1 dimer was found to reduce the HEDS substrate and demonstrated a similar rate of oxidized NADPH production of 0.02 uM/min, compared to monomeric CLIC1, with a rate of 0.03 uM/min.

Therefore, Alkhameci for the first time assigns a functional activity to the soluble form of the CLIC4, rather than the activity of their integral membrane form. This CLIC4 enzymatic activity may be important for reduction of non-native disulfide proteins in the ER lumen and likely regulates the CLIC4 ion channel function by binding to GSH in the G-site to catalytic cysteine within the redox motif. In addition, CLIC4, which makes an intermediate structure with GSH can demonstrate oxidoreductase activity with the reduction of disulfide bonds in insulin. Furthermore, functional activity for the soluble form of CLIC4, which appears to be autonomous to their well characterized membrane ion channel activity, potentially classifies it as moonlighting protein (Jeffery CJ, et al., 1999). Finally, the enzymatic function of the CLIC proteins may regulate their ion channel activity due to inhibition of ion channel activity of CLIC1 via IAA-94, which can inhibit its enzymatic activity (Alkhameci 2015).

For CLIC4 to act as an oxidoreductase for performing isomerisation or reduction reactions, it must be preserved in a reduced redox state to remain active. Reduced and oxidized redox states of a protein are indicated with cysteine redox state that is markedly identified with post-translational modifications of protein (Molteni et al 2004). Members of the oxidoreductase family are characterized by a functional CXXC motif that shares structural

homology with the small redox protein thioredoxin (Edman, 1997 and Freedman, 2004). CLIC4, due to lack of a standard functional motif, is indicated with (CXXS) rather than (CXXC) and needs to couple with GSH to be activated as an oxidoreductase. The catalytic cysteine residues in this motif can exist in either an oxidized state or in a reduced state and allow CLIC4 to catalyze three types of essential redox reactions depending on the initial redox state. 1) a disulfide bond may be formed through the oxidation of a substrate protein; hence, requiring CLIC4 to gain two electrons, 2) non-native disulfide bonds may be shuffled or isomerized without any exchange of electrons, 3) incorrect parings may be broken or reduced, requiring the CLIC4 to donate two electrons to the substrate; all those reactions for CLIC4 could be dependent on GSH. Therefore, for CLIC4 to be capable of forming a disulfide bond, it must itself be oxidized to be capable of accepting electrons. On the other hand, CLIC4 can only be functional as an isomerase or reductase when it is in a reduced form with formed form to be capable of donating electrons. After each cycle of reduction or oxidation, the oxidoreductase must be returned to its original, active redox state before it can fulfill further catalytic rounds. Thus, there is a requirement for oxidative and reductive pathways within the cell. Consequently, there is a requirement for a specific labelling followed by mass spectrometry (MS) to identify the abundance of cysteine residues and the mass specterometry with liquid chromatography MS/LC along with a TMT labelling to detect a relative comparison of protein expression levels in redox states (Chen X et al., 2021).

1.15 GSH

GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is synthesized in the cytosol from the precursor amino acids glutamate, cysteine, and glycine (Figure 1-23). Reduced glutathione is a good candidate to maintain the oxidoreductase in a reduced form. A candidate for the reduction of the CLIC4 is reduced Glutathione (GSH).

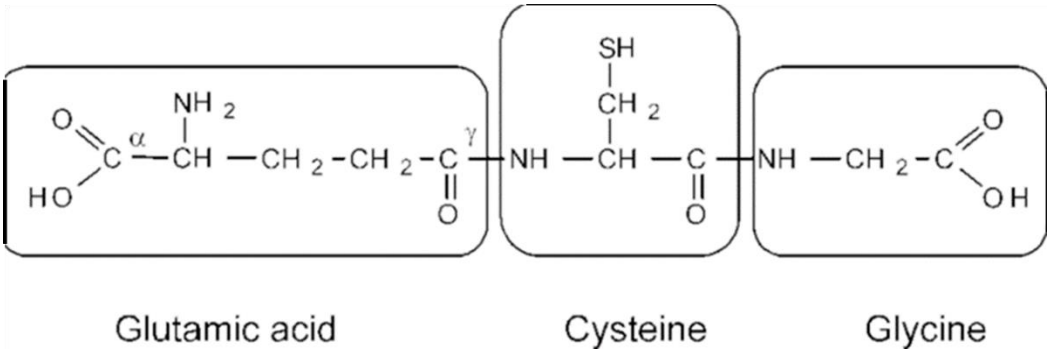


Figure 1-24 Glutathione structure consists of three GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is synthesized in the cytosol from the precursor amino acids glutamate, cysteine, and glycine (Littler RD, et al., 2010).

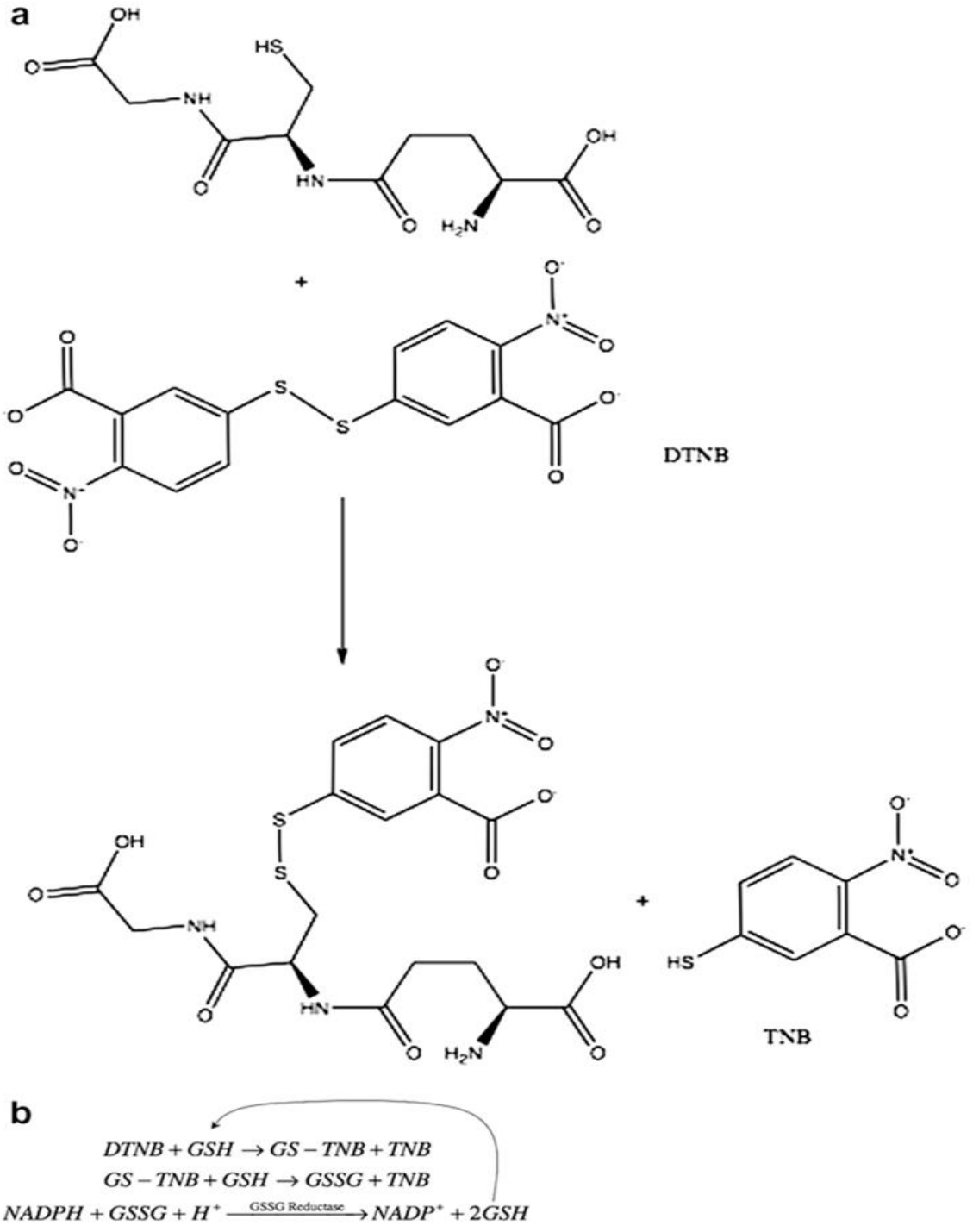
1.15.1 Measurement of GSH levels

One of the important issues in determining the mechanisms of both oxidative stress and redox signalling is the measurement of the different forms of thiols in cells. The predominant forms are the reduced form of GSH and oxidized form of GSSG. Cysteine is a precursor amino acid of GSH. Cysteine residues of protein thiols can exist as a mixed of disulfide between cysteine and GSH. It is important to recognize that an increase in the oxidized forms of these thiols in the cytosol will be transient even during oxidative stress. Therefore, it can be very difficult to measure thiol oxidation, particularly that occurring in signal transduction. GSH reacts with dithionitrobenzoic acid (DTNB) (Akerboom and Sies, 1981), and by reducing GSSG total GSH (GSH + GSSG) can be measured. DTNB reacts with GSH to produce a conjugate and TNB anion that can be detected by fluorescence or absorbance (Figure 1-26).

To measure total GSH, a recycling assay is used in which GSH reacts with the conjugate producing GSSG and another molecule of TNB, which can increase fluorescence or absorbance. The enzyme glutathione reductase then reduces the GSSG releasing the GSH that can react with another molecule of DTNB. Therefore, instead of a single determination of how much DTNB reacts with GSH, the rate of TNB production is measured, as that is proportional to the initial amount of GSH. To measure GSSG, however, one must first modify the GSH present at the beginning, so it is removed from the recycling assay. Modification of GSH is done with N-ethylmaleimide (NEM) or vinyl pyridine. To measure

protein mixed disulfide, the GSH can be released from the protein mixed disulfide with sodium borohydride ($NaBH_4$), and the GSH is then measured in the recycling assay.

On method that has been developed to measure nitrosoglutathione involves the production of GSH from it followed by reaction with orthophthaldehyde (OPT) to produce a fluorescent compound (Figure 1-25, d) (Tsikas et al., 1999) while another method uses a biotinylated fluorescent label in a method called the biotin-switch (Gladwin et al., 2006). First, however, as with the measurement of GSSG above, it is necessary to remove any GSH in the original sample with methyl methanethiosulfonate before reducing GSNO to release GSH. Various reagents have been proposed as best for differentially reducing GSNO as well as PSNO especially as the presence of GSSG or protein mixed disulfides can also yield GSH upon reduction (Gladwin et al., 2006). After reaction with OPT, the products are separated by HPLC with a fluorescence detector.


a
DTNB
TNB
b
DTNB + GSH → GS − TNB + TNB
GS − TNB + GSH → GSSG + TNB
NADPH + GSSG + H+ → NADP+ + 2GSH
GSSG Reductase

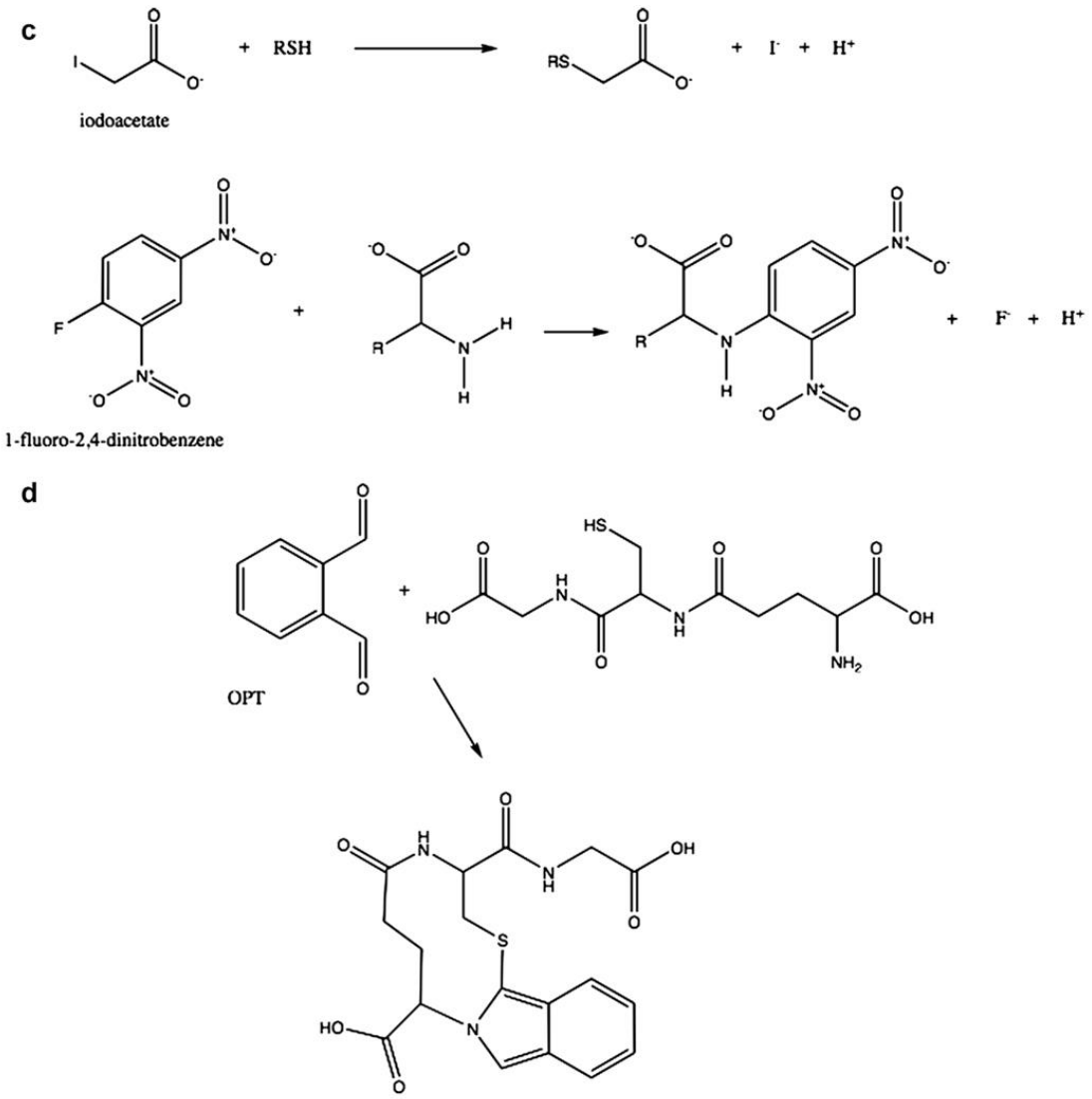


Figure 1-25 Measurements of thiols. (a) Reaction of GSH with DTNB produces a TNB, which is measured Spectro-fluorometrically or spectro-phometrically; (b) total glutathione can be determined by recycling of GSSG produced in the reaction in (a) and measuring the rate of TNB; (c) Glutathione and related compounds are first derivatized with iodoacetate followed by a second derivatization with 1-fluoro-2,4-dinitrophenol. The second products are then separated by HPLC and measured Spectro-fluorometrically; (d) Reaction of glutathione with ortho-Phthaldehyde (OPT) yields a product that can be measured Spectro-fluorometrically.

1.15. 2 GSH and disulfide formation

The cell consists of millimolar concentrations of GSH (up to 10 mM) that are preserved in a reduced form via a cytosolic NADPH-dependent reaction and can be catalysed by glutathione reductase (GR). Protein disulphide bonds do not usually form in the cytosol owning to the existence of a high level of the concentrations of GSH (Bulleid N, et al., 2007). By contract, the ER lumen consists of a comparatively higher concentration of the oxidized

glutathione GSSG (Hwang et al, 1992). This allows the formation of native disulphide bonds in the ER via a series of enzymatic pathways, which involve not only in disulphide-bond formation, but also in the isomerization of non-native disulphide bonds (ChaKravarthi ST, et al., 2007). Both oxidation and reduction can be catalysed by protein disulphide isomerase (PDI) and probably other oxidoreductases, which can share a common motif as (CGHC) with PDI (Ellgaard & Ruddock, 2005). The motif contained two cysteines with catalytic activity and can be involved with either formation of a disulphide to become active as an oxidase, or two free cysteines as a dithiol to act as an isomerase to participate in disulfide exchange reaction. The oxidized form of glutathione (GSSG) is commonly considered to be involved with oxidizing of PDI, but it has been indicated that the oxidation of PDI is related to protein Ero1 either in vivo or in vitro (Tu et al, 2000; Tu & Weissman, 2002). These results indicate that the disulphide-bond formation can occur independently of GSSG, although the ratio of reduced to oxidized glutathione ([GSH]:[GSSG]) is an optimal for disulphide-bond formation and is important for disulfide formation in the ER (Lyles & Gilbert, 1991). There is still no answer to the question as why an oxidizing balance of glutathione is required in the ER and how this balance can be preserved in the ER. However, GSH might have an important role in this process.

1.15.3 GSH and disulfide reduction

GSH can be considered to have an essential role as a reductant, rather than as an oxidant because through studying the mutant gene for coding γ-GCS in mice, the embryonic lethal was found (Shi et al, 2000). Similarly, a yeast strain lacking a functional copy of the GSH1 gene was unable to grow and by adding mM GSH with DTT, the growth was restored (Cuozzo & Kaiser, 1999), suggesting the function role of glutathione can be complemented by the addition of reducing agents and indicating that the main role for glutathione is in reduction rather than oxidation (Chakravarthi S, et al., 2007). The potential ability for GSH in the ER compartment can be determined by reducing non-native disulphide bonds. The formation of non-native disulfide bond can result from oxidative stress or enzymatic oxidation (Jansens et al, 2002). The formation of disulphide bonds increased when the levels of total glutathione were decreased; hence, GSH can have a crucial role in the isomerization of non-native disulfide bonds (Molteni et al, 2004). However, an increase in GSSG can have a significant effect in the formation of non-native disulfide bonds

(Chakravarthi & Bulleid, 2004). Furthermore, the depletion or decrease in levels of cytosolic GSH can increase the oxidation of PDI and can affect its ability for isomerisation of disulfide bond. Thus, GSH can directly or indirectly be involved with isomerization of non-native disulfide bonds.

1.15.4 GSH and oxidoreductases

In the mammalian cells, the ER oxidoreductases enzyme is usually located in a reduced form in the normal steady state condition (Jessop K, et al., 2004). Recovery of one of oxidoreductase enzyme was required the cytosolic component and mostly GSH. In addition, GSH was found to reduce oxidoreductase in vivo and in vitro conditions after removal of oxidizing agent and GSH can form a transient structure with two catalytic cysteines in the active stie of this oxidoreductase that was confirmed with biotinylating (Fratelli et al, 2002), and it was indicated that GSH ion of the main reductant to reduction of ER oxidoreductase.

A large amount of GSH was reported that exist in the ER as mixed disulfide with proteins in the ER (Bass et al, 2004). The mixed disulfide resulting from these mixed disulphides could be formed either during the oxidation of proteins by GSSG or by the reduction of proteins by GSH. Many substrate proteins are able to fold spontaneously in a glutathione buffer in the absence of oxidoreductases. However, the rate of folding is increased dramatically in the presence of enzymes such as PDI (Weissman & Kim, 1993) or ERp57 (Zapun et al, 1998). For GSH to reduce a non-native disulphide bond, it must first glutathionylate one of the cysteine residues. A second glutathione molecule must then attack the glutathionylated protein, thereby releasing GSSG and a reduced substrate protein. By contrast, the C-terminal cysteine of the CXXC motif of PDI is positioned perfectly to resolve the mixed disulphide. Thus, the CXXC motif present in the ER oxidoreductases ensures efficient reduction or isomerization. Polypeptide-binding domains in PDI also increase the efficiency of disulphide-bond isomerization (Winter et al, 2002). Therefore, although GSH is potentially able to reduce non-native disulphide bonds in newly synthesized proteins, there is a kinetic advantage for this reduction to be catalysed by the ER oxidoreductases.

1.15.5 [GSH]: [GSSG] ratio in the ER

A model that seeks to explain the role of glutathione within the ER must take into account how the redox balance becomes distinct from the cytosol. In an attempt to understand how this balance is maintained, it is important to consider some of the sources of reducing or oxidizing equivalents that occur from disulphide-bond formation within the ER lumen (Fig 1). Reducing equivalents are continuously introduced during protein translocation as cysteine residues in nascent chains. These residues can undergo oxidation to form disulphide bonds and thus reduce PDI or an equivalent ER oxidoreductase. PDI oxidation by Ero1 leads to the production of ROS (Gross et al, 2006). The fate of any ROS produced is unclear; they could diffuse into the cytosol, or they could react with GSH in the ER, leading to an increase in GSSG. In addition, the reduction of non-native disulphide bonds would lead to an increase in the level of GSSG, particularly if the resulting cysteine residues remain reduced or reform native disulphide bonds through an Ero1-catalysed oxidative pathway. If Ero1 has a significant role in disulphide-bond formation, then the net consequence would be an increase in the concentration of GSSG relative to GSH. Conversely, if GSSG oxidizes PDI, then the net result would be an increase in the concentration of GSH relative to GSSG. Therefore, balancing Ero1 and GSSG oxidation of PDI could regulate the [GSH]:[GSSG] ratio. The cell contains millimolar concentrations of GSH (up to 10 mM) that is maintained in a reduced form via a cytosolic NADPH-dependent reaction catalysed by glutathione reductase (GR) (Chakavarthi S, et al., 2007). Protein disulphide bonds rarely form in the cytosol because of the high concentrations of GSH (Joosep C et al., 2005). In addition to a role in disulphide-bond formation, it has been suggested that glutathione provides a redox buffer against ER-generated oxidative stress. However, cell lines isolated from the mutants indefinitely could grow by incubation in a medium contained with N- acetyl- cysteine NAC) (Shi ZZ, et al, 2000).

The ratio of GSH:GSSG in the secretory pathway is very low suggesting increasing consumption of glutathione to maintain the oxidoreductase enzyme in an active form for reduction, correction, and editing unfolded or mi-folded proteins entering in secretory pathway, but the overall GSH buffer system still maintained in a reduced form (Cuozzo and Kaiser, 1999). Furthermore, the rate of activity of ERO1 in the mammalian cells is directly dependent on the amount of GSH, while a high level of GSSG can be considered because of ERO1 activation (Cuozzo and Kaiser, 1999).

How the GSH:GSSG ratio is regulated inside the ER, is an important question corresponding to GSH in the ER, which It was considered that the microsome that derived from ER can import GSH, but not for GSSG, and GSSG is not able to transport to the cytosol at substantial levels (Banhegyi et al., 1999). The vesicular transport can contain GSSG, but the mechanism is not clear (Appenzeller-Herzog et al., 2010). But transporting GSH from the cytosol and removing GSSG from the ER still maintain unclear.

Trx1/TrxR1 or the GSH/GR/Grx pathway are two major pathways that might prevent disulfide formation in proteins synthesized in the cytosol, NADPH is the main player for both of them for the ultimate electron donor, so lack of NADPH can aberrant disulfide formation in the reticulocyte lysate (Bulleid N, et al., 2017). Thus, NADPH and GSH are two main cytosolic factors that are necessary for reductive pathway to eliminate the oxidative stress effect in the ER by a reduction of disulfide formation but delivery of these components into the ER is still not understood. Therefore, there is a requirement to identify the membrane protein involved with transfer electron and cytosolic components from the cytosol into the ER. CLIC4, due to either its ion channel activity or oxidoreductase activity, could be a good candidate in the reduction of the non-native disulfide in response to oxidative stress. In the final step of this project to make sure the GSH has the reduction effect on CLIC4 or even overl proteins, I apply the mass specterometry analysis and TMT labelling.

1.16 Mass specterometry and TMT labelling aid to redox analysis

Changes in the redox state mediated by cysteine modifications to provide a link between oxidative stress and physiological condition which in turn could be a consequence of a change in disulfide bond formation (Blatnik, M et al., 2008 and Bulleid N, et al., 2009). Reversible cysteine modifications can act as a biological switch for activating or stabilizing protein tertiary and quaternary conformations, which in turn can influence the stability and function of the protein (Go, Y et al., 2013). Hence, Cysteine modifications play an important role in the structure and function of proteins because they are sensitive to redox conditions. Free cysteines or reduced form (SH) are highly reactive and can state reversible or irreversible modifications, for instance, cysteine nitrosylation (SNO), cysteine

sulfenylations (SOH), cysteine sulfonylations (SO3H) and disulfide bonds (S−S) (Giron, P et al., 2011 and Wojtyla, K et al., 2015).

Proteomic analyses allow the redox state to be linked to protein expression changes, which are insensitive to reversible oxidative changes and typically performed under reducing conditions (Sun M, et al., 2016). The fundamental of proteomic approaches is labelling reduced cysteine or modified cysteine residue to provide a link between oxidation and expression of protein (Chouchani E, et al., 2011). Several proteomics methods have been developed to evaluate modifications in cysteine oxidation under several redox conditions (Murray C and Van Eky, et al., 2012). These methods involve with selective labelling of unmodified and modified cysteine residues to trap the cysteine redox state under several physiological conditions (Wojtyla and Rogowska-Wrzesinska et al., 2015). The Isotopic labelling are identified with co-eluting of several labelled peptides in liquid chromatography (LC) along with mass spectrometry (MS) to resolve labels with distinct masses (Aebersold R, et al., 2003) and detecting relative quantitation through comparing the ratio between heavy-labeled and light-labeled peptides (Gygi S.P, et al., 1999 and Reichert L.I, et al., 2008). There is a restriction for isotope labelling, which is not completely co-eluted the peptides that chemically are distinct in mass, resulting (MS) mass spectrum totally become very more mixed due to several or multiple peaks which are in fact belonging to same peptide (Thompson MS, et al., 2004). In addition, these labels are deigned to break at a specific location to create an ion reporter that totally would be differed in mass in each tag and allow a comparison between several multiple samples in a single run at once (Chouchani E, et al., 2011), those advantages are provided with (MS/MS) with different reactive groups depending on the labelling interested (Chen X et al., 2021).

TMTs are isobaric reagents that are available for labelling redox cysteine (Giron P, et al 2011). Amine-reactive TMTs are typically used to label tryptic peptides, reacting with lysins and peptide N-terminal amines to give a relative quantitation for the overall protein concentration changes between samples. Hence, in this study it is a requirement to apply the TMT-labelling (Figur1-27).

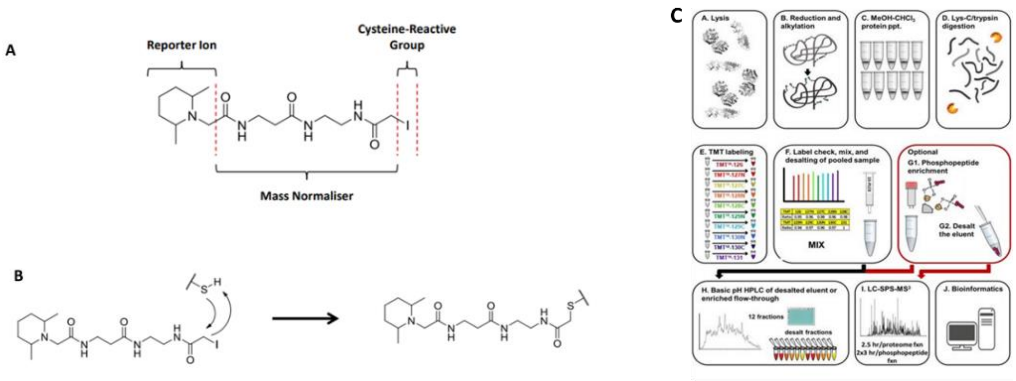


Figure 1-26 The basic structure of the Iodo-TMT reagent and proteomics process (A) The isobaric six-plex reagent set includes 5 heavy atoms distributed along the molecule, with the reporter ion section in each reagent having a different number of heavy atoms (0-5). (B) The reaction mechanism between the iodo-TMT reagent and a reduced cysteine residue. The image comes from (c) work fellow of TMT-labelling process the image comes from (Prakash As, et al., 2018).

1.18 Aims and Hypotheses of this Research Project:

Based on the previous study, CLIC4 demonstrated as an oxidoreductase enzyme and an ion channel which is sensitive to oxidative stress due to its multiple binding site promoter for cytokines and ability to participate in signaling pathways. In this project after identification of the localization and expression of CLIC4 in several subcellular compartments, we are looking for the reason for re-localization of CLIC4 into ER in response to oxidative stress that induced by TNF-alpha. As ER is a host of disulfide formation in membrane binding protein and folding within proteins entering the mammalian secretory pathway. However, the formation of disulfide bonds is not always result in the correct pairing of cysteine residues, therefore the existence of a reductive pathway in the ER to edit and remove non-native disulfide bonds, is essential. Understanding of this pathway still is poor although initial and final steps have identified. In this project we assume that CLIC4 due to ion channel activity and oxidoreductase activity can participate in this pathway either through transferring the cytosolic equivalents from the cytosol into the ER or via reduction of non-native disulfide bonds via its oxidoreductase activity (Figure 1-27). Based on this assumption, the knowledge gap is divided into three questions:

1. What is the main reason for re-localization of CLIC4 from the cytosol into the ER?

2. What is the redox status of CLIC4 in the ER that made it a good candidate to participate in the reductive pathway?

3. What is a good candidate for the reduction of CLIC4?

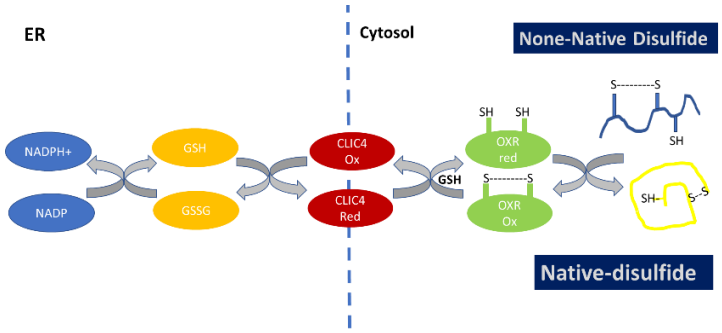


Figure 1-27 hypotetic ER reductive pathway: the shematic represntation shows a hypotical reductive pathway that implicated with reduction of non-native disulfide bonds through GSH, NADPH and an unknown binding membrane protein. CLIC4 might be considered as a good candidate for membrane binding protein because of the ability to insert to membrane owning to ion channel activity or ability for isomerization due to GSH-dependent oxidoreductase activity.

Chapter 2 Material and methods

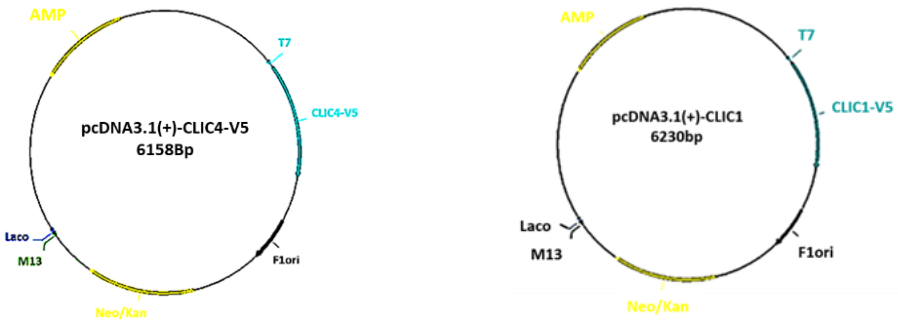
2.1 DH5α competent cells

F - Φ80lacZΔM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rk -, mk +) phoA supE44thi-1 gyrA96 relA1 λ

2.2 Plasmid Vectors

2.2.1 pcDNA3.1 (+)

PcDNA 3.1 (+), is a high-level expression vector for mammalian cell lines. Genes of interest are placed under the control of the T7 promoter. It contains ampicillin resistance which acts as a selectable marker and its multiple cloning site is useful for restriction enzyme cloning. The restriction enzymes shown are those used during cloning experiments in this plasmid vector. This vector was applied for the designed CLIC1-V5 and CLIC4-V5 construct for this project (see Appendix 2).



2.2.2 pET-28a-c (+)

pET-28-c (+) vector carries an N-terminal His-Tag-thrombin-T7-Tag configuration plus an optional C-terminal His-Tag sequence. It contains a Kanamycin resistance which acts as a selectable marker and multiple cloning site is useful for restriction enzyme cloning. In our lab, this vector was design for CLIC1+His construct for express in prokaryotic cells (see Appendix 2).

2.2.3 pET-32 a (+)

pET-32 a (+) is an AMP resistance that use as a selectable marker with several restriction sites for efficiencies cloning. In our lab, this vector was constructed for CLIC4 with 9 Cysteine protease peptide (TEV +6 His) to ability for purify CLIC4-his and CLIC4 with removal His-tag (see Appendix 2).

2.3 Stable cell line

After designing the construct, Human embryonic Kidney 293T cell line, which stably over expressed V5-tagged CLIC4 and CLIC1 in presence of antibiotic puromycin were stably transfected and cultured in 75cm² filter capped flasks and in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) at 37°C under 5% CO_2. The HEK293T wild type and HEK293T-mutant(C-A), were set up as below.

To generate the stable cell line, the HEK293T were seeded into 6cm dishes at 1:4 dilution (so should be around 60% confluent for transfections in the next day). We designed 2x transfected dishes and 2x un-transfected. Note, at the same time with transfection we put a cover glass to check the overexpression of CLIC4. The next day we check the cells to make sure to indicate a correct confluency, and then we set up the transfections via Megatran 1.0 and puromycin. This included 300ul of serum-free medium (in Eppendorf) adding 2ug DNA for each construct and adding 200 ng pPUR to ($1/10^{th}$) of pCDNA3.1(+), and add 6ul Megatran for all (1:3 ratio) and incubated for 20 minutes in the room temperature and mix and introduce to the medium in dishes at 37°C. Following test for successful transfection, we can split cells into 15cm dishes at various dilutions and add puromycin at 1ug/ml and maintain under selection for approximately 2-3 days and change the medium and puromycin every 2-3 days. We use several dilutions with plate 1 (1:2), plate2 (1:10), plate 3 (1:100) and plate 4 (1:1000). We expected the colonies will be overgrown in (1:2) and will prob get no colonies in (1:1000) dilution. The several dilutions of stably transfected cell line with puromycin selection were included 1:2, 1:10, 1:100, 1:1000. The minimum ratio and maximum ratio were considered for a overgrow and no colonies, respectively. Once the single colonies were appeared, they will transfer into 12 well plate via cloning discs. The cloning disc will pure into the trypsin in separate dish to make a wet disc. The wet disc place on top of colony and put into 1ml medium in a 12 well plate and maintain until cells are almost confluent and then test expression of colonies. Once cells be confluent in 12

well plate, then split 1/10th into (6 well plate) to keep growing, and place remainder 9/10th into another 6 well plate for testing expression via western blot analysis and will keep colonies which give the best expression.

2.3.1 Preparation of SP cells

SP-cells were prepared as described previously (Wilson, Allen et al., 1995). The sub-confluent cells were rinsed twice with 10 ml PBS (Gibco) to remove the medium which inhibits trypsin. Trypsin solution (2 ml) (prewarmed to RT) was added into the flask and incubated for at 37℃ for 3 min to detach the cells. KHM buffer (8 ml) (20mM HEPES buffer pH 7.2, including 110mM KOAc, 2mM MgOAc) was used to resuspend the cells and soybean trypsin inhibitor (final concentration 100 µg/ml) was added into the solution to inactivate the trypsin. The cell suspension was then transferred to a 15 ml tube tube which was pre-cooled on the ice. The cells were pelleted by centrifugation at 250 ×g at 4'C for 3 min. The pellet was resuspended in 6ml KHM buffer including 40 µg/ml digitonin (Calbiochem) and incubated on ice for 5 min to permeabilize the plasma membrane. The volume was made up to 14 ml and the cells were pelleted by centrifugation as above. The cells were resuspended in 14 ml ice-cold HEPES buffer (KOAc 50mM, HEPES 20mM, pH 7.2) and incubated on ice for 10 min. After centrifugation as above, the cell pellet was resuspended in 1 ml KHM buffer and 10 µl of cell suspension was transferred into an Eppendorf tube which contained 10 µl Trypan Blue (Sigma). The SP cells were counted and checked for permeabilization using a Cell Counter. SP cells were pelleted by centrifugation as above and resuspended in KHM buffer. The SP cells were pelleted by centrifugation as above and resuspended in 100 µl KHM buffer ready for determination of the redox state experiments. For CLIC4 redox state determination experiments, the nuclease treatment was not included during the preparation. In certain experiments, the SP cells were prepared from cells expressing V5-tagged CLIC4.

2.4 Reagents

2.4.1 Chemicals and reagents

If not specifically mentioned, chemicals were purchased from Sigma-Aldrich.

2.5 General Methods

2.5.1 Preparation of LB plates

LB-Agar was autoclaved to 126°C for 30 minutes and allowed to cool before ampicillin was added at a final concentration of 100 µg/mL The LB-Agar was mixed well and poured into plates under sterile conditions.

2.5.2 Preparation of bacterial competent cell stocks

Glycerol stocks of competent bacterial cells, DH5α, were streaked out onto LB-Agar plates and incubated overnight (~16 hours) at 37°C. A single colony was picked and used to inoculate 5 mL of LB media and incubated overnight at 37°C in a shaking incubator. 500µL of the overnight culture was then used to inoculate 50mL of sterile LB broth which was then incubated with moderate agitation (~220 rpm) until an Optical Density (OD) of 0.375 at 595 nm had been reached. Upon reaching the required OD, cells were chilled for 10 minutes before being transferred to sterile 50 mL falcon tubes and centrifuged at 2,500 rpm for 10 minutes at 4°C to pellet the bacteria. The supernatant was removed, and the pellet re-suspended in 10mL competent cell buffer before being centrifuged again at 2,500 rpm for 5 minutes at 4°C. The supernatant is again removed, and the pellet re-suspended in 10 mL of competent cell buffer, incubated on ice for 30 minutes and then centrifuged at 2,500 rpm for 5 minutes at 4°C. Finally, the pellet is re-suspended in 2 mL of competent cell buffer and the bacterial cells are aliquoted into sterile Eppendorf tubes, frozen with liquid nitrogen and stored at -80°C.

2.5.3 Transformation of competent bacteria cells

Transformations of well-established plasmids were carried out in DH5α cells, whilst were used for the transformation for wild type or site-directed mutagenesis plasmids. A 50 µL aliquot of competent cells were thawed on ice and 1 µL of the relevant DNA plasmid was added. The cells were then incubated on ice for a further 30 minutes before a heat shock at 42°C was carried out for 1 minute and returned to ice for 5 minutes. 500µL of LB broth was then added and samples were incubated at 37°C for 1 hour in the 73-shaking incubator. Following this, the entire transformation sample was split and spread over two LB-

ampicillin plates and incubated overnight (~16 hours) at 37°C Glycerol stocks of competent bacterial cells, DH5α, were streaked out onto LB-Agar plates and incubated overnight (~16 hours) at 37°C. A single colony was picked and used to inoculate 5 mL of LB media and incubated overnight at 37°C in a shaking incubator. 500µL of the overnight culture was then used to inoculate 50mL of sterile LB broth which was then incubated with moderate agitation (~220 rpm) until an Optical Density (OD) of 0.375 at 595 nm had been reached. Upon reaching the required OD, cells were chilled for 10 minutes before being transferred to sterile 50 mL falcon tubes and centrifuged at 2,500 rpm for 10 minutes at 4°C to pellet the bacteria. The supernatant was removed, and the pellet was re-suspended in 10 mL competent cell buffer before being centrifuged again at 2,500 rpm for 5 minutes at 4°C. The supernatant is again removed, and the pellet re-suspended in 10 mL of competent cell buffer, incubated on ice for 30 minutes, and then centrifuged at 2,500 rpm for 5 minutes at 4°C. Finally, the pellet is re-suspended in 2 mL of competent cell buffer and the bacterial cells are aliquoted into sterile Eppendorf tubes, frozen with liquid nitrogen, and stored at -80°C.

2.5.4 Small-scale preparation of plasmid DNA (MINI-PREP)

A single colony is selected from a bacterial transformation and used for the inoculation of 5 ml LB media containing 100 µg/mL of ampicillin which was then incubated overnight at 37°C in the shaking incubator. The overnight culture was centrifuged at 6000 x g for 10 minutes at 4°C to pellet the cells. The plasmid DNA was then purified from the bacterial cells using the Qiagen QIAprep Spin Miniprep Kit. Firstly, the pellet was re-suspended in 250 µL of Buffer P1 and transferred to a sterile Eppendorf tube. Cell lysis was then carried on the addition of 250 µL of Buffer P2. The cell lysis was then neutralised after 5 minutes by the addition of 350 µL of Buffer N3. The sample was centrifuged at 15871 x g for 10 minutes and the supernatant was transferred to a QIAprep spin column. The column was centrifuged at 15871 x g for 1 minute and the flow-through was discarded. The column was then washed with 750 µL of Buffer PE and centrifuged at 15871 x g for 1 minute with the flow-through again discarded. To remove any residual PE buffer the column was spun again for 2 minutes. The plasmid DNA was then eluted into a sterile Eppendorf by adding 50 µL of nuclease-free water to the column and centrifuging for 2 minutes at 15871 x g. Plasmid DNA is then stored at -20°C.

2.5.5 Large-scale preparation of plasmid DNA (MAXI-PREP)

After successful small-scale preparation of plasmid DNA, larger quantities required for transfection of cells were required. This was prepared using the Qiagen Maxi Plasmid Kit, using the materials and reagents supplied, and following the manufacturer's instructions. 300 µL bacterial cultures in LB broth containing 0.01 mg/mL ampicillin, were grown overnight at 37°C in the shaking incubator. The overnight cultures were centrifuged at 6000 x g for 10 minutes at 4°C to pellet the cells. The pellet was then re-suspended in 10 mL of P1 solution, followed by the addition of P2 solution to lyse the cells, and incubated at room temperature for 5 minutes. 7.5 mL of Buffer P3 was then added to neutralize the lysate, before 20-minute incubation on ice, followed by two centrifugation steps at 20000 x g for 30 minutes at 4°C. The supernatant was then loaded onto a Qiagen Maxi column and 74 under gravity were allowed to flow through with the DNA remaining bound to the column. Three wash steps in QC buffer followed and plasmid DNA elution was carried out using buffer QF. The eluted DNA was then precipitated by adding 10 mL of isopropanol, centrifuged at 15000 x g for 30 minutes at 4°C and washed twice with 70% (v/v) ethanol. The pellet was subsequently air-dried, and the DNA was re-suspended in 300 µL of ddH_2O. The DNA concentration was determined by spectrometry and stored at -20°C.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 DNA amplification by PCR

DNA sequences of interest were amplified by specifically designed forward and reverse oligonucleotide primers. Forward and reverse primers also included any appropriate restriction sites required for restriction digests. All primers used in this study were synthesized by Integrated DNA technologies. PCR samples were set up on ice in thin-walled PCR tubes, using plasmid DNA as the template (Table 2-1).

Table 2-1 PCR reactions

10x DNA polymerase buffer	10 µL
dNTP mix (2.5 mM each: dATP, dCTP, dGTP, Dttp)	8 µL
Forward primer (100 pmol)	1 µL
Reverse primer (100 pmol)	1 µL
Template DNA (~100 ng/µL)	1 µL
Ex Taq DNA polymerase (5 units/µL)	0.5 µL
ddH2O Final volume	100 µL

PCR was carried out in the Applied Biosciences 2720 thermal cycler using the following standard conditions (Table 2-2).

Table 2-2 PCR reaction thermal cycler

Temp	Step	Time	#Cycles
94°C	Initial Denaturing	1 minute	1 cycle
94°C	Denaturing	30 seconds	30 cycles
56°C	Annealing	30-60 seconds	
72°C	Elongation	1 minute/kb	
72°C	Final Elongation	8 minutes	1 cycle
4°C		Hold	

The standard protocol for PCR can be altered to account for the different melting temperatures of individual primers. Optimal annealing temperature is ~5°C lower than the lowest melting temperature of the set of primers. Upon completion of PCR, the products were purified by PCR clean-up and run on an agarose gel to confirm the obtained product was of the correct base pair size.

2.6.2 Site-directed mutagenesis by PCR

2.6.2.1 Single site-directed mutagenesis

Single point mutations were generated in constructs using the Quick-Change Site-Directed Mutagenesis kit (Agilent). Forward and reverse primers were designed to incorporate the desired mutation (refer to Appendix 1). PCR samples were set up on ice, in thin-walled PCR tubes and the following protocol was followed (Table 2-3).

Table 2-3 PCR reaction for mutant sample

10x DNA polymerase buffer		5 µL
dNTP mix (10 mM each: dATP, dCTP, dGTP, dTTP)		1 µL
Forward primer (100 pmol) 1 µL Reverse primer (100 pmol)		1 µL
Template DNA (~100 ng/µL)		1 µL
Pfu Turbo DNA polymerase (2.5 units/µL)		1 µL
ddH2O	Final volume:	50 µL

PCR was carried out in the Applied Biosciences 2720 thermal cycler using the following standard conditions:

Table 2-4 PCR thermal reaction

Temp	Step	Time	#Cycles
94°C	Initial Denaturing	1 minute	1 cycle
94°C	Denaturing	30 seconds	
56°C	Annealing	30-60 seconds	30 cycles
72°C	Elongation	1 minute/kb	
72°C	Final Elongation	8 minutes	1 cycle
4°C		Hold	

Upon completion of single site-directed mutagenesis, products were treated with 1 µL of the restriction enzyme Dpn1 for 1 hour at 37°C. The Dpn1 enzyme recognizes methyl groups on the backbone of the parental DNA molecules and removes them via digestion. 1 µL of the final reaction is used to transform 50 µL of competent XL-1 blue cells. Plasmid DNA is isolated through small-scale DNA preparation and was subsequently sent for sequencing to confirm whether the mutation had been successfully incorporated.

2.7 PCR purification

Products produced by PCR were purified using the PureLink PCR Purification Kit (Invitrogen). 1 volume of the PCR sample (100 µL) was mixed with 4 volumes of Binding Buffer (B2) in an Eppendorf and added to a PureLink Spin Column provided. The sample was then centrifuged at 15871 x g for 1 minute to bind the DNA to the column. The flowthrough was discarded, and the column was washed with 650 µL of Wash Buffer and centrifuged at 15871 x g for 1 minute. The flow-through was once again discarded and the column centrifuged at 15871 x g for a further 2 minutes to remove any residual Wash Buffer. The column was then transferred to a fresh Eppendorf and the PCR product was eluted from the column by applying 50 µL of ddH2O and centrifuging at 15871 x g for 1 minute. The PCR product was analysed on an agarose gel to confirm both the PCR and purification were successful.

2.8 DNA agarose gel electrophoresis

Between 1-2% (w/v) agarose powder was dissolved in 1x TAE buffer by heating in the microwave. The molten gel was allowed to cool before adding 0.5 µg/mL ethidium bromide. The molten gel was then poured into a mold containing a loading comb and allowed to set. The gel was then placed in a gel tank, the comb removed, and immersed in 1x TAE buffer. The DNA samples were prepared by adding 6x Loading dye before being loaded into individual wells on the gel. Either a 100bp or 1kb DNA ladder (Promega) was run alongside the samples as a marker for size. Electrophoresis was carried out at 80 volts for ~ 40 minutes and the gel was visualized, with images being recorded, using the BioRad Molecular Imager ChemiDoc XRS+ System.

2.9 DNA purification from agarose gel

DNA samples were resolved by gel electrophoresis and purified using the QIAquick Gel Extraction kit (Qiagen). DNA samples resolved within a gel were visualised on a UV light box and bands corresponding the correct size were excised using a scalpel and placed into sterile Eppendorf. The gel was weighed and 3 gel volumes (1 g: 300 µL) of Buffer QG were added and incubated at 50 °C for 10 minutes until the gel was completely dissolved. 1 volume of isopropanol was added to the sample, mixed, and transferred to a QIAquick spin column and centrifuged at 15871 x g for 1 minute. The flow-through was discarded and the column was washed with 750 µL of PE Buffer and centrifuged at 15871 x g for 1 minute. The flow-through was once again discarded and the column centrifuged at 15871 x g for a further 2 minutes to remove any residual Wash Buffer. The column was the transferred to a fresh Eppendorf and the PCR product was eluted from the column by applying 50 µL of ddH2O and centrifuging at 15871 x g for 1 minute. The products were collected and stored at -20 °C.

2.10 Restriction Endonuclease Digestion

Plasmid DNA or PCR products containing specific restriction sites were digested by pairs of selectively chosen restriction enzymes (Table 2-5). Samples for restriction digestions were set up as follows:

Table 2-5 restriction endonuclease digestion method

Component	Volume
Restriction enzyme buffer	10 µL
Plasmid DNA/PCR product (100 ng/µL)	1.5 µL
Restriction enzyme 1 (20 units/µL)	0.5 µL
Restriction enzyme 2 (20 units/µL)	0.5 µL
10x BSA (as recommended by manufacturer)	0.15 µL
ddH2O	Final Volume: 15 µL

Reactions were incubated at a temperature and a time in accordance with the manufacture's guidelines. Upon completion, enzymes were heat inactivated at 65 °C for 10

minutes. Samples were resolved on an agarose gel and purified by gel extraction. Samples were stored at -20 C.

2.11 Ligation of DNA

PCR products with 5‟ and 3‟ ends sliced by using restriction enzymes to start the reaction procedure of ligation. This reaction led to the insertion of product into the vector that was formed by digestion with the forementioned enzymes.

The ligation protocol is as follows:

Table 2-6 DNA ligation method

Components	Volume
10x T4 DNA ligase buffer	2 µL
Insert DNA- PCR product (~ 100 ng/µL)	6 µL
Plasmid vector DNA (~ 100 ng/µL)	1 µL
T4 DNA Ligase (400 units/µL)	1 µL
ddH2O Final Volume:	20 µL

The reaction was gestated at normal room temperature for 1 hour. Another way of starting the reaction is at 16 ° C in a cold room overnight. reaction compound (5 µL) was used to transform potential DH5α cells. Plasmid DNA was extracted by small-scale DNA preparation and subsequently sent to sequence to ensure successful binding.

2.12 DNA Sequencing

The sequencing of Plasmid DNA was managed by a small-scale DNA preparation. Samples were diluted to the concentration of 20 ng/µL. for the sequencing of DNA, samples were sent to the “School of Health Studies, University of Dundee”. The outcomes were analyzed and related with known DNA data base of nucleotides on NCBI-BLAST. Furthermore, the Swiss Institute of Bioinformatics (SIB) ExPASy translate software online.

2.13 Preparation and purification of plasmid

pcDNA3.1 (+) is designed for high-level, constitutive expression in a variety of mammalian cell lines. It contains a Geneticin® selectable marker and Large multiple cloning sites. This plasmid has introduced into E. coli strain DH5α for transformation. A colony of bacteria

carrying the plasmid has then cultured in LB broth containing 10mM ampicillin to produce large quantities of pure plasmid DNA. This process has checked by setting up an extra plate containing bacteria transformed with no plasmid DNA (as control) and cultured in a plate containing ampicillin. Purification of plasmid DNA from bacterial culture has done according to the user manual of Qiagen QIAprep Spin Miniprep Kit, and stored to be used for agarose gel analysis, plasmid identification and restriction endonuclease digestion.

2.14 Expression of CLIC4

2.14.1 Small scale protein expression

A small scale of protein expression was carried out to investigate whether the transformation was successful. A transformed BL21-DE3 colony was picked from the agar plate with antibiotic and cultured overnight in 5 ml LB (containing 100 µg/ml Amp) at 37'C, 225 × g in a shaker incubator (Innova 4400). A volume of 50 µl overnight culture was added to 5 ml fresh LB (containing 100 µg/ml Amp) and cultured for 3-4 h until the OD600 reached 0.6. A volume of 1 ml bacterial culture was centrifuged at 18,000 × g for 10 min. The pellet was resuspended in SDS loading buffer and boiled at 105 'C for 5 min (for example, if the OD600= 0.6, 100 µl SDS-loading buffer was used to resuspend the pellet). This sample was loaded on the gel as the uninduced control of protein expression. The remaining bacterial culture was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration 1 mM) for 3 - 4 h. Another 1 ml solution was measured at 600 nm, centrifuged, and resuspended in SDS-loading buffer as above. The samples were loaded on a 15% SDS-PAGE gel (running conditions, 300 V, 25 mA, 2 h). Coomassie staining was carried out to visualize the protein.

2.14.2 Large scale protein expression

A colony was picked and cultured overnight in 10 ml LB in the presence of 100 µg/ml Amp at 37 ‘C. Bacterial culture (5 ml) was added to 500 ml fresh LB with antibiotic and cultured for 3-4 h until the OD600 reached 0.6. Before the addition of IPTG, a 1 ml of culture was collected and after centrifugation, the pellet was resuspended in SDS-loading buffer as a uninduced control. The remaining bacterial culture was incubated with 500ul IPTG (stock1M=1mMfinal). Reduce the incubator temperature to30’C and continue incubation for 4 hrs to allow protein expression in bacterial cells. The OD600 of a 1ml bacterial culture after induction was measured and the bacteria was isolated by the centrifugation at 14,800 rpm for 15 min as a control for expression. The rest of bacterial culture was split into 250 ml flat bottom screw cap centrifuge bottles and centrifuged at 4’C, 3,000 × g for 15 min (Beckman, rotor JA17). The supernatant was discarded. The bacteria were pelleted and washed three times with PBS (40 ml/tube). the bacterial pellet was stored in-20’C. Samples before and after induction were loaded on a 15% SDS-PAGE gel (running conditions, 300 V, 25 mA, 2 h) under reducing conditions.

2. 15 Purification of CLIC4/CLIC1

2. 15.1 His tag affinity chromatography

The purification process of CLIC4 is carried out in different steps. In the primary step of purification, the outcomes of the previous processes after induction of the bacteria pellet by IPTG were unfrozen on ice. After completing this melting process of the bacteria pellet, it is resuspended in 20ml total of 1XTBSbuffer. Recombine the re-suspended cells; to this add 1 EDTA-free protease inhibitor tablet and 20mg of lysozyme (stock 100mg/ml). Then incubate on ice for 20 mins before lysing cells by French press with 2 passes at 8000psi. The process of centrifuge is continued in the centrifuge machine with 17,000rpm. The temperature is set to be on After centrifuged at 17,000rpm, 39.2^{o}F. The minimum time required for this process is 30 min. further purify by ultra-centrifugation at 42,000 rpm for 50mins at 4’C. During ultra-centrifugation equilibrate a His binding nickel column with 10 bed-volume of binding buffer (40mMTris, 30mMNaCl, pH8). The supernatant is required to pass through the Ni-column and then wash the Ni-column with 10 bed-volumes of wash buffer (40mM Tris, 300 mM NaCl 20mM imidazole, PH8). Elute the protein using 3 bed-

volumes of Elution Buffer (40mMTris, 300mMNaCl, 400mMimidazole, pH8). Then using the Nano-drop to determine protein concentration.

2.15.2 Gel filtration

The eluted samples from His tag affinity chromatography were concentrated to a final volume 1 ml at 3,500 rpm, 4 oC for 30-60 min using a Vivaspin Turbo 15 centrifugal concentrator (100,000 MWCO PES) (Sartorius) which was washed with 10 ml distilled water and equilibrated by 15 ml GFC buffer (20 mM Tris pH 7.5, 100 mM NaCl). The sample was applied (flow rate at 0.4 ml/min) in the equilibrated gel filtration column (Superdex 200 10/300) (GE Healthcare). The column was washed (flow rate at 0.4 ml/min) by GFC buffer until the UV absorbance curve became flat. Purity of protein was analysed after gel filtration using SDS-PAGE (15% SDS gel) under reducing conditions with an Coomassie stained. The fractions containing target protein were collected and concentrated until the protein concentration reached 500 mM/ml. The protein concentration was measured at an absorbance of 280 nm (Cary 300 Bio UV-Vis, Varian). The absorbance of the solution was measured at 280 nm in water including 1% SDS. The concentration of proteins were calculated using the equation: $c = A / (e \times b)$ (A is the value of A280, e is extinction coefficient of the protein, and the b is the path length (cm)). The aliquots of purified protein were frozen in liquid nitrogen and then stored -80 oC.

2.16 Purification of CLIC4-V5 from mammalian cells

HEK293T-CLIC4-V5 that stably transfected with recombinant CLIC4-V5 construct were pre-cleared by incubating the sample with "protein A-Sepharose (PAS)" for 30 minutes at 4°C, formerly incubation with "anti-V5-conjugated agarose beads (Sigma)" for 16 h at 4°C. Afterward, washing of the bead three times with lysis buffer was done; accompanied with 0.5% SDS and then eluted the cross-linked complexes by incubating with 10 mM DTT for 5 minutes. Subsequently, the sample was boiled for 5 minutes to dissociate the immunocomplex from the beads. Spin at 13,000 × g for 1 min at 4°C to recover sample from beads and run on the gel for Coomassie blue staining and “western blotting” with both anti V5 and anti CLIC4 antibodies to find a right band.

2.17 Preparation of the cell extract and the microsomes

2.17.1 Cell extract preparation

The cell extract was prepared as previously described (Mikami et al., 2006). Cells were cultured using cell culture roller bottles (1700 cm2, filter screw cap) (Greniner Bio-one) in an CO_2 incubator (Galaxy S, Wolf laboratories) (5% CO_2) at 37̊C in DMEM. The confluent cells were detached from the roller bottles by incubation with 100 ml PBS containing 1 mM EDTA for 10 -15 min at 37̊C. These cells then were pelleted by centrifugation at 250 ×g for 3 min and washed three times with washing buffer (35 mM HEPES–KOH pH 7.5, 140 mM NaCl, and 11 mM glucose) to remove the remaining DMEM and EDTA thoroughly. After washed once in extraction buffer (20 mM HEPES–KOH pH 7.5, 135 mM potassium acetate, 30 mM KCl, and 1.65 mM magnesium acetate), the cells were harvested by centrifugation at 2000 × g for 3 min. An equal volume of extraction buffer (less extraction buffer could be used to obtain a more concentrated cell extract) was used to resuspend the cells. The cells were disrupted in a Mini-Bomb Cell disruption chamber (KONTES) using a nitrogen pressure of 1.0 MPa for 30 min on the ice. Cell homogenates were centrifuged at 6000 × g for 5 min at 4̊C and the post nuclear supernatant was retained. To pellet the membrane, the supernatant was centrifuged at 50,000 × g (Rotor TL100.3) (OptimaTM Max-XP Ultracentrifuge, Beckman Coulter) for 15 min at 4'C. The supernatant was removed to a new eppendorf tube. The aliquots of the supernatant were frozen in liquid nitrogen and stored at -80 °C. The membrane was used to prepare microsomes. In some experiments, a half of cell extract was treated with PD-10 desalting columns (GE healthcare) to remove small molecular weight compounds.

2.17.2 Microsomes preparation

The membrane pellet was resuspended in buffer A (50 mM Tris-HCl pH7.4, 0.25 M Sucrose, 50 mM KCl, 6 mM MgOAc, 1mM EDTA) to give an A280 value of 50 units/µl (the absorbance was determined in water in the presence of 1% SDS). If needed, a nuclease treatment of microsomes was carried out by the incubation with Staphylococcus aureus nuclease (Calbiochem) (final concentration 150 U/ml) in the presence of CaCl2 (5 mM) at RT for 12 min to remove the endogenous mRNA. The nuclease was inactivated by the addition of EGTA (final concentration 4.5 mM). Sucrose cushion buffer (50 mM HEPES-KOH pH 7.5,

100 mM KCl, 0.5 M Sucrose) was added to the thick wall polycarbonate tubes (Beckman Coulter) and nuclease treated microsomes were then layered over the sucrose cushion buffer. The microsomes were isolated by centrifugation at 60,000 × g (Rotor TL100.3) for 15 min at 4°C. The microsomes were resuspended in Buffer A (50mM Tris-HCl pH7.4, 0.25 M Sucrose, 50mM KCl, 6 mM MgOAc, 1mM EDTA) and aliquoted into eppendorf tubes (50 µl/tube). The microsomes aliquots were frozen in liquid nitrogen and stored -80 °C.

2.18 Protein concentration measurement

The Bio-Rad Protein Assay was used to measure total protein concentration of the cell extract or microsomes. The Dye Reagent concentrate (Bio-Rad) was diluted 5 times using distilled water (1 ml Dye reagent with 4 ml water). The diluted dye was filtered through a Minisart filter (pore size 0.2µm, Sartorius) to remove particulates. Five dilutions of a bovine serum albumin (BSA) standard were prepared to generate a standard curve (BSA concentration range was from 0.05 mg/ml to 0.5 mg/ml). The BSA solutions and samples were normally assayed in triplicate. BSA dilutions and samples were pipetted into Greiner-Bio Cell 96 Well black Cell Culture Microplates (10µl/well) (Greiner Bio). The samples were incubated with 200µl diluted dye reagent at RT for at least 10 min. The absorbance was measured at 595 nm (SPECTROstar Nano, BMG LABTECH).

2.19 Mitochondrial and cytosolic fractions

Confluences 4x (15cm dishes), transiently transfected HEK293T cells with CLIC4-V5 were either left untreated or treated with (500pM) TNF-a (sigma) for 15 minutes and with (2mM) for 1-hour H_2O_2. The medium was removed, and then it is washed for the first time with 4ml PBS supplemented along with the EDTA-free protease inhibitor tablet, and then 5 ml ice-cold PBS were added, and harvested cells by a rubber policeman (cell scraper). Cells were centrifuged at 1000 RPM, for 5 minutes then pellet resuspended in cold PBS (4 ml per plate) and pellet again resuspended in mitochondrial isolation buffer with protease inhibitors (2-3ml per 15-cm plate of starting cells), were incubated on ice for 10 minutes. Homogeneous (used a tight-fitting glass Teflon homogenizer) using 50 strokes., was spin down homogenized cells at 700g, for 10 minutes, at 4°C, Spin down supernatant at 700 g, for 10 minutes, at 4°C (Repeat step 7 until a pellet was no longer visible in the supernatant

at 20,000 g, 15 minutes, at 4°C. The resulting pellet was washed with mitochondrial fraction.

2.20 Preparation of Nuclear, cytosolic, and membrane fractions

Confluent 6cm dishes, transiently transfected HEK293T cells with CLIC4-V5 were either left untreated or treated with (500pM)/(10µM) TNF-α (sigma) for 15 minutes and with (2mM) for 1 hour/(5mM) for 10 minutes H_2O_2. The medium was removed and then washed firstly by 3ml PBS supplemented and EDTA-free protease inhibitor tablet. 2.9ml ice-cold PBS were added and were harvested cells by a rubber policeman (cell scraper) into a 15ml tube Spin at 3000 rpm for 5 minutes, the supernatant was removed, and cells were resuspended in 1ml ice-cold buffer A. Cells then were shifted to 1.5ml tube, put on the ice bath for the time of 10 minutes. Cells were lysed by moving in a lightweight needle close to a 1ml syringe, 30 times. The remaining sample was spun at 1000g, for 7 minutes at 39.2F. Pellet was a nuclear fraction; supernatant contains cytosol and membrane.

2.20.1 Preparation of Nuclear fraction

The pellet was washed on priority in (500ul) buffer A (supplemented with protease inhibitor tablet), then spin at 1kg, for seven minutes at 39.2°F, and discarded supernatant. Pellets are then resuspended in 100ul buffer B and incubated for 60min on a 4°C, rocker then was spun at 100,000g for 30 minutes at 4°C temperature, and the supernatant was transferred supernatant to fresh tube=nuclear extract.

2.20.2 Preparation of Cytosol and membrane fraction

Supernatant from A was spun at 100,000g for 30 minutes at 4°C. The supernatant was removed to a fresh 1.5ml tube that was the cytosol. The pellet contained a membrane. The cytosol was precipitated with ice-cold acetone (5x volume), then was spun at 15000 rpm at 4°C for 15 min. Pellet was resuspended in 100ul buffer B (were supplemented with protease inhibitor). The membrane pellet was resuspended in 100ul buffer B (were supplemented with protease inhibitor) Buffer A: 10mM HEPES pH 7.4, 250mM Sucrose, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, and 1mM EGTA. Buffer B: 10mM HEPES pH 7.6, 2.5

% Glycerol, 420mM NaCl, 1.5mM MgCl2, 1mM EDTA and 1mM EGTA. Samples were analysed on 12% reducing SDS-PAGE, followed by western blotting using mouse anti-V5, reproved using rabbit anti-Calnexin, anti-NaKATPase for the membrane fractions blot, rabbit anti-HDAC2 for the nuclear extracts, and anti-GAPDH for cytosol.

2.21 Measurement of Protein concentration

2.21.1 Bradford assay

The basic purpose of this section is to calculate the amount of protein in the given sample. In our case, samples are either cell extract and microsomes or protein concentration for TMT labelling. How can we calculate this? The answer is based on the Bradford method are known as the Bio-Rad Protein Assay. In this method first, prepare the dilute solution of the Bio-Rad Protein Assay. The concentration ratio is 1:4 i.e., 1ml dye reagent and 4ml distilled water. The filtration process is carried out and this is done through a Minisart filter which filtered the diluted dye to mitigate the particulates. For statistical result and standard curve number of dilution, samples should be more, for this purpose '5' dilutions of a "bovine serum albumin (BSA)" which is a standard that was ready to produce a standard curve in excel (BSA concentration range was from 0.05 mg/ml to 0.5 mg/ml). These dilutions and the samples were taken into "Gr-B Cell 96 Well black Cell Culture Microplates (10µl/well) (Greiner Bio)". Finally, incubated all the samples were for a time minimum of 10 minutes along 200 µl diluted dye reagent at RT. The absorbance was estimated at 595 nm "(SPECTRO star Nano, BMG LABTECH)"

2.22 SDS-PAGE

In this section, SDS/PAGE technique is used for proteins separation. The first step for this method is to prepare samples for electrophoresis. After preparing samples they were resuspended in the buffer solution of SDS/ PAGE. (4X) (Thermofisher-Scientific) and DTT (50 mM) solution were inserted in the samples to decrease samples. It is not necessary that All samples to be reduced from some samples remain un-reduce. After resuspension reduced and non-reduced samples were heated until they start boiling before 5 minutes to process electrophoresis. Before the gel process starts samples should be cooled down by placing them on an ice bath. After that reduced and un-reduced samples were loaded in

SDS/PAGE. To connect this section to previous sections first step is to move the proteins from "gel phase to nitrocellulose blotting membrane (AmershamTM ProtranTM 0.45 µm NC) (GE Heathcare) in transfer buffer (25 mM Tris pH 8.3, 0.192 M Glycine, 0.0013 M SDS, 10-20% Methanol)". This movement could only be possible if the voltage was 300 V, the current was 250 mA, and the time should be at least 1 hour. These are boundary conditions of our system. The membrane was collected at incubating with weight to vol. Ration "10 ml 3% w/vmilk (Marvel) in PBST buffer". The membrane after that was ready to incubate in conditions: "5ml PBS adding primary [Na2HPO4 (10mM), KH2PO4 (1.8mM) pH7.4 KCL (2.7mM), NaCl (137 mM), Tween-20 (0.5% v/v)] for 1 hour". The membrane was scanned by an Imager. The experiment was repeated three times for better results.

2.23 Colloidal Coomassie blue stain

Coomassie staining was carried out to visualize protein expression. After SDS-PAGE analysis, the gel was stained with Coomassie blue (10% phosphoric acid (H3PO4), 10% ammonium sulphate (NH4)2SO4, 0.12% Coomassie G250 (brilliant blue), 20% Methanol for at least 1 h. Gel was de-stained using distilled H2O overnight and scanned to obtain image in the next day.

2.24 Western blotting

In previous sections, SDS-PAGE was performed. To connect this section to previous sections first step is to move the proteins from gel to nitrocellulose blotting membrane (AmershamTM ProtranTM 0.45 µm NC) (GE Healthcare) in transfer buffer (25 mM Tris pH 8.3, 0.192 M Glycine, 0.0013 M SDS, 10-20% Methanol)".This movement could only be possible if the voltage was 300 V, the current was 250 mA and time period should be at least 1 hour, V=300 V, I=150 mA for time 2 hours, or v=300 V, I=25 mA for overnight. These are boundary conditions of our system. The membrane was collected at incubating with weight to vol. "Ration 10 ml 3% w/v milk (Marvel) in TBST buffer (TrisHCl pH 8.0, NaCl 150 mM, Tween-20 0.5%) at RT for 1-16 h (generally 1 h). The membrane was incubated in 5 ml TBST including primary antibody overnight at 4'C or RT for 1 hour". The washing process of the membrane was carried out after primary antibody incubation, 3 washing cycle is necessary for the membrane using 10 ml TBST buffer. This activity has lasted after 5 min

duration at RT. In the second wash incubation time increased up to 45 to 60 min with the same concentration of buffer in the dark. There are 3 times of washing cells with PBS to remove excess unpleasant antibodies and other material. Finally, the scanning process was carried out by the imager to get Hight resolution pictures of our experiment. These Experiments were repeated by three times. So that all statistical values such as averages, means standard deviations with error bars representation. "The western blot antibody information was as followed: for CLIC4 and CLIC1 using as a primary antibody which is raised in mouse and mouse Anti-mouse 680 secondary antibody as the secondary antibody (Licore), for CLIC4-V5/CLIC1-V5 using an anti-V5 antibody (Invitrogen) as primary antibody and goat anti-mouse IgG (H+L) (DyLight 800) (Thermo Fisher). For organelle fractionations using specific marker for detecting organelle localization, using anti-rabbit for all primary anti body, for mitochondria using anti-ATP5-A, for cytosol using anti-GAPDH, for nucleus using anti-$HDAC_{2,}$ for microsome using anti-PDI, for ER using anti-CNX and for plasma membrane using anti-NaK. All detected with an anti-rabbit secondary anti-body (DyLight 800) (Thermo Fisher).

2.25 Immunofluorescence

HT1080 and HEK293T cells were seeded on "12 mm x 12 mm coverslips" before use, fixed with methanol (-20 °C, 10 min), washed with PBS, and then blocked with "0.2 % (w/v) BSA in PBS" before the addition of antibodies. Cell membranes were permeabilized with 0.1 % triton before incubation with primary antibodies including; rabbit anti-GAPDH, anti-HDAC2, anti-CNX, anti-ATP-5A, anti-NaK-ATPase, anti-CLIC4 and mouse anti-V5antibody and stained with (AlexaFluor488nm) and (Alexafluor568nm), which "conjugated goat anti-(rabbit immunoglobulin) and goat anti-(mouse immunoglobulin), respectively" in the final step, incubated with 4',6-diamido2-phenylinimide (DAPI) for cellular DNA staining used 1ug/ml DAPI for 2-3 minute in room temperature and washed the samples 2-3 times in PBS before proceeding to medium before imaging. The imaging for fluorescence takes placed by EPI and for confocal analysis take place for confocal microscopy, most frequently confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation.

2.26 Redox State determination in bacterial cells

“E. coli strain BL21(DE3) pLysS" that contain a new form of PET32a+ articulating His-tagged CLIC4 was a form of blessing from "Donald Campbell" (Woolhead Lab University of Glasgow, Glasgow, UK). The purification and realisation of His-tagged CLIC4 were carried out against "50mM phosphate buffer, pH 7.4”. The oxidation-reduction state of CLIC4 in vitro was examined using the procedure of alkylation with AMS. The exchange of Disulfides was prohibited with 10% (w/v) trichloroacetic acid, and the proteins were precipitated by adding “25% (v/v) acetone for 1 hour on ice. Next centrifugation, the pellets were washed two times with chilled acetone and resuspended in SDS-PAGE sample buffer (0.25 mM Tris-HCl buffer, pH 6.8 containing 4% (v/v) glycerol and 1% (w/v) SDS).” Free thiols were alkylated with “30 mM AMS” for 1 hour at normal room temperature, and the samples were parted by the technique of SDS-PAGE. The gels were stained with Coomassie Blue; reduced protein displayed a decrease in mobility.

2.27 Redox State Determination in intact cells

Two conditions might be applied to the "HEK293T cells (a human embryonic kidney cell line)" present in suspension; either left unprocessed or treated with 10mM DTT or 10uM TNF-α. The oxidoreduction state of proteins was found through "AMS to alkylate free thiols". In vivo, the oxidoreduction state was imprisoned by alkylation of free thiols by "25 mM N-ethylmaleimide (NEM)", and lysis of the cells was carried out in "50mM Tris-HCl containing 150mM NaCl, 2mM EDTA, 0.5mM phenylmethylsulphonyl fluoride, and 1% (v/v) Triton X-100 (lysis buffer)". In the next step, the denaturing of the lysate was carried out by boiling the lysate for 2 minutes in the incidence of w/v SDS. After the completion of the lysis, breakage of the existing disulphide bond was carried out by the addition of Tris[2-carboxyethyl] phosphine (TCEP) (10mM). This bond was then alkylated by adding 30mM AMS (Thermofisher) for 1 hour at normal room temperature. The presence of oxidoreductases was then detected by the separation of lysates using SDS-PAGE and Western blotting techniques. The mobility of oxidized protein was less.

2.28 Determination of the reduction of CLIC4 in mammalian cell line

The reduction of CLIC4-V5 was examined following oxidation of HEK293T-CLIC4-V5 cell lines with 10uM TNF-α. Before starting the experimental procedure, the cells were subjected to treatment with 0.5mM BSO as inhibitor of GSH synthetase for the whole night or with 0.1mM Carmustin as inhibitors GSH reductase of GSH for 3 hours. Otherwise, cells can also be left without any treatment procedures. Before oxidation, samples were reserved to check the oxidoreductive state of CLIC4. Afterward, the cells were set for incubation in 10uM TNF-α at normal room temperature for about 5 minutes. The sample was then subjected to centrifugation for the removal of TNF-α. The resulting cell pellet was again mixed in a fresh buffer that contained a suitable concentration of the inhibitory. At several points, the oxidoreductive state of the cells was entombed by adding 25mM NEM.

2.29 Determination of the reduction of CLIC4 in Microsomes

The next step was the preparation of cytosol and subsequently homogenization of $3X10^8$ HEK293T cells in 4 volumes of buffer A "50mM Tris-HCl buffer pH 7.4, 0.25M sucrose, 25mM KCl, 0.5mM MgCl2, 1mMEDTA". The homogenised cells were then subjected to centrifugation at 150,000g to settle down the membranes pellet. The cytosol was then separated from the solution on a PD10 column, which was equilibrated by buffer A earlier. Microsomes prepared from HEK293T cells (as described above) were either not further treated or treated with 10uM TNF-α for 5 minutes. TNF-α was detached from the microsomes with centrifugation. Then, the microsomes in oxidised state were incubated in isolate cytosol or buffer A. oxidized microsomes were incubated in either buffer A or isolated cytosol. At several times, the oxidation-reduction state of a sample was found through an adjustment with AMS as described overhead.

2.30 Association of HEK293T with Biotinylated Glutathione

“Microsomes from HEK293T cells were treated with 10uM TNF-α for 5 min and allowed to recover for 1 h in fresh buffer A in the presence of BioGEE (2.5 mM). After 7.5 min free thiols were alkylated by the addition of 25 mM NEM and lysed in lysis buffer. The lysates were precleared for 30 min at 4 °C by incubation with 2.5% (v/v) suspension of streptavidin-agarose that had previously been blocked with 100x binding capacity of biotin (10uM) and

then washed eight times with lysis buffer. The beads were removed, and the supernatant was incubated with 2.5% (v/v) streptavidin-agarose for 1 h at 4 °C. The beads were washed with lysis buffer four times and then resuspended in 4xSDS-PAGE sample buffer containing DTT (50mM) and boiled for 2 min. The samples were separated by SDS-PAGE and western blotted with CLIC4 antibody."

2.31 Glutathione Assay

The measurement of total glutathione concentration was carried out by a glutathione assay kit (Sigma Aldrich). Briefly, the cells at least 10^8 cells were washed with PBS, then suspend to a cell density of 1×10^8 cells per ml in PBS and transfer cells to a microcentrifuge tube. The cells were centrifuged at 600 × g to obtain a packed cell pellet. The supernatant was removed. The volume of the pellet was measured and 3 volumes of the 5% SSA solution were added to the packed cell pellet. The suspension was twice frozen and thawed (used liquid nitrogen to freeze and a 37 °C bath to thaw) and left for 5 minutes at 2–8 °C. The extract was centrifuged at 10,000 × g for 10 minutes. Then the sample was added to "0.2 mM NADPH, 0.6 mM Ellman's reagent, glutathione reductase (1–2units/ml), 150 mM phosphate buffer, pH 7.4". The absorption rate was measured at 412nm in 1 min according to GSH standards. If we want to calculate the concentration of GSSG, we need to treat the cells with 4 vinyl pyridine and then use the concentrations of GSH were predicted by take away of the total of glutathione concentration.

2.32 NADPH Assay

In this section, another method known as concentration measurement of "NADPH is used in the cell extract were determined using "NADPH abcam assay ". Incubation of the sample is the first step of this method. To perform this method, we considered a 50 µl volume of the cell extract was prepared to incubate along the same amount of alkali solution (0.2N NaOH). So, we can say that the concentration of cell extract and solution of the base should be the same for this method. 50% sample of cell extraction was incubated with 25 µlHCl (0.4 N). The incubation temperature is set to be at 140°F. The sample should be incubated for at-least 20 minutes to dissolve the NADPH in the cell extract completely. At room temperature, the incubation time is reduced to 10 min and continuously made neutral by adding Trizma chapp. "To measure the concentration of NADPH, the sample should be

normalized by placing the sample on an ice bath. The sample was cooled down to RT for 10 min. By taking number of samples, we get a standard curve using multiple concentration of NADP was generated in excel to calculate theses NADP concentration in the cell extract. After this titration experiment of acid base cell extract samples were combined with 25 µl NADPH detection reagent and instantly converted into Cornin96. A well-defined white color Solid Polystyrene Microplate (Life Scienes). Is prepared".

Prepare NADPH stock solution: 200 µL of PBS buffer was added into the vial of NADPH standard to make a 1 mM (1nmol/ µL) NADPH stock solution. Note: The unused NADPH stock solution should be divided into single use aliquots and stored at -20°C, and is stable for 1-2 months. 2. Prepare NADPH reaction mixture:1 ml of NADPH Probe was added into 4 mL NADPH Assay Buffer and mix well. Only make enough reaction mix to use in the assay, as it is not stable and cannot be stored. Note: 5 mL NADPH reaction mixture is for one 96-well. The working solution is not stable, use it promptly and avoid direct exposure to light. 3. Prepare serial dilutions of NADPH standard (0-100 µM). Add 100 µL of NADPH stock solution (from Step 1) into 400 µL PBS buffer (pH 7.4) to generate 200 µM (100 pmol/µL) NADPH standard solution. Note: Diluted NADPH standard solution is unstable and should be used within 4 hours. Take 200µL of 200µM NADPH standard solution to perform 1:2 serial dilutions to get 100, 50, 25, 12.5, 6.25, 3.13 and 0µM serial dilutions of NADPH standard. Then add serial dilutions of NADPH standard and NADPH containing test samples into a white/clear bottom 96-well microplate as described in Tables 1 and 2. Note: Prepare cells or add supernatant as desired.

2.33 Sample preparation for mass spectrometry analysis

Samples were set for oxidation-reduction status analysis with some modifications. SP cells were treated with several oxidized and reduced reagents including Diamide (5mM), DTT (10mM), H_2O_2 (5mM), and (10uM) of TNF-α and incubated in 37℃ for 5 min. Sampels were washed with fresh medium to remove oxidized and reduced reagents. Then species were washed with PBS that contained 25mM NEM to modified cysteine residues. Samples of cells were precleared by incubation with protein A-Sepharose (PAS) for 30 min at 4°C, before incubation with anti-V5-conjugated agarose beads (Sigma) for 16 h at 4°C. The beads were washed three times with lysis buffer supplemented with 0.5% SDS and then incubated with 10 mM DTT for 5 min to elute cross-linked complexes. Boil for 5 min to

dissociate the immunocomplex from the beads. Spin at 13,000 × g for 1 min at 4°C to recover sample from beads and run on the gel for Coomassie blue staining and western blotting with both anti V5 and anti CLIC4 antibodies to find a right place to cut the bands and sent to St. Andrew mass spectrometry facility to analysis.

2.34 preparation samples for TMT labeling

2.34. 1 Cell Cultures

Wild-type stable HEK293T cell lines were selected in the presence and absence of GSH treatment to investigate the effect of reduction of GSH on CLIC4 or overall protein expression. Three biological replicates each of +GSH and -GSH were cultured in complete (six samples in total) with 10% HI-FBS at 25 °C and sub passages every 2−3 day. Each replicate was split into two equal aliquots of approximately 5 × 107 cells (12 samples in total) and centrifuged at 1000g for 10 min. Samples were washed twice to remove salts and other soluble macromolecules, by resuspending in cold phosphate-buffered saline (PBS), followed by centrifugation at 1000g for 10 min.

2.34.2 Cell Lysis

One sample from each culture was resuspended in 200 µL of SDT lysis buffer (4% SDS, 100 mM Tris base, and 0.1 M DTT, pH 7.6). The remaining six samples were resuspended in 200 µL of NEM lysis buffer (250 mM HEPES, 10 mM EDTA, 0.1 mM neocuproine, 2% SDS, and 100 mM NEM, pH 7.0). Samples in NEM buffer were incubated at 37 °C in the dark for 90 min with gentle mixing to allow the NEM to react irreversibly with free thiols. Then cells were lysed by probe sonication, alternating a 2 s pulse with 1 min of cooling on ice for 10 cycles. Samples were then centrifuged for 10 min at 13000 × g to remove cell debris and other insoluble material. The supernatant was removed into separate Eppendorf tubes and ice-cold acetone (4× sample volume) was added to each sample; the tubes were vortexed briefly and then stored at −20 °C overnight. Samples were centrifuged for 10 min at 13000 × g at 4 °C and the supernatant was carefully removed. The protein pellet was washed twice with 80% ice-cold acetone, vortexed briefly, and then centrifuged to remove the supernatant. Protein pellets were stored at −20 °C until required. Isobaric Labeling. Protein

pellets were resuspended in HEPES buffer (50 mM HEPES, 1 mM EDTA, and 0.1% SDS, pH 8.0) and the protein concentration was measured by a Bradford assay. Each sample was split into two 30 µg aliquots (24 samples in total) and made up of equal volumes with HEPES buffer. Reduction of reversibly modified cysteine residues was performed with TCEP-HCl (final concentration ∼5 mM), and then samples were vortexed briefly for mixing and incubated at 37 °C for 1 h. Irreversibly modified cysteines, such as sulfonylations, are unaffected by TCEP reduction and therefore cannot be quantified by this method. IodoTMT reagents were resuspended in MeOH to a concentration of 10 mg mL−1 and samples were labeled with 10 µL of the appropriate iodoTMT reagent, as described in chapter 5. Samples were incubated at 37 °C in the dark for 1 h before being quenched with DTT (final concentration ∼20 mM) and incubated for a further 15 min at 37 °C in the dark. Individual samples were combined in equal protein concentrations into four six-plex samples; a fully reduced (Red) sample with all cysteines labeled with iodoTMT, a NEM-blocked (Ox) sample with only modified cysteines labeled with iodoTMT, and +GSH and -GSH samples comparing modified cysteines with total cysteine content for each group, as described in Chapter 5. Enzymatic digestion was carried out with the FASP protein digestion kit, digesting samples overnight at 37 °C with trypsin.

2. 34.3 Affinity Purification

Peptide samples were dried in a SpeedVac before being reconstituted in 200 µL of TBS buffer (25 mM Tris and 0.15 M NaCl, pH 7.2). Affinity purification was carried out with 400 µL of anti-TMT slurry in a spin column, following the manufacturer's instructions, including an incubation step at 4 °C overnight with end-over-end mixing. Unbound peptides were washed from the column with 5 column volumes of TBS buffer followed by 3 column volumes of water. The labeled peptides were eluted from the column with 4 column volumes of TMT elution buffer. All eluted fractions (labeled and unlabeled peptides) were dried in the SpeedVac and reconstituted in 10% MeCN + 0.1% formic acid. Approximately 6 µg of each sample was pipetted into a 96-well microplate, dried in a SpeedVac, and stored at −20 °C until ready for analysis.

2.34.4 Peptide assay

To measure the peptide, we use the Thermos Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay is an easy-to-use colorimetric microplate assay designed specifically to improve the sensitivity and reproducibility of quantitation of peptide mixtures. The kit provides the BCA reagents for the reduction of Cu^{+2} to Cu^{+1} and a proprietary chelator that has been optimized for the quantitation of peptide mixtures. In this reaction, the copper is first reduced by the amide backbone of peptides under alkaline conditions (Biuret reaction). The proprietary chelator then couples with the reduced copper to form a bright red complex with an absorbance of 480 nm. The signal produced from this reaction is 3-4-fold more sensitive for peptide analysis for a high-quality peptide digest reference standard for use in generating linear standard curves and to serve as a calibration control. The working peptide concentration range of 25–1000 µg/ml (Figure 2-2). After the measurement the peptide, Peptides were reconstituted in 10 µL of loading buffer [1% MeCN + 0.05% formic acid (v/v)] and injected onto the trap column for 4 min at a loading buffer solvent flow rate of 25 µL per min. Peptides were then transferred to the analytical column under starting chromatographic conditions; 95% buffer A (water + 0.1% formic acid)/5% buffer B (80% MeCN + 0.08% formic acid) (v/v), that step took place with proteomics centre in Glasgow University.

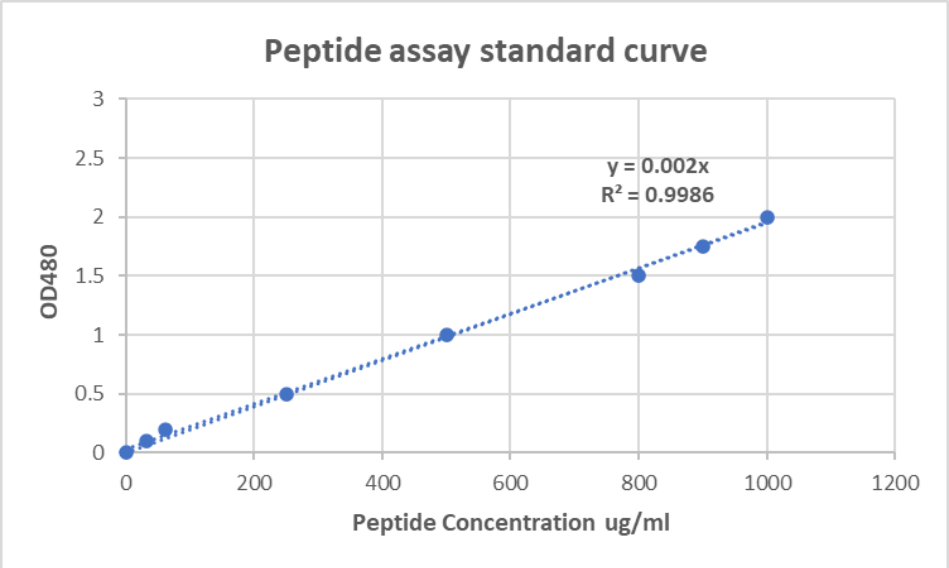


Figure 2-2 Peptide assay Standard curve for peptide assay.

2.34.5 Liquid Chromatography-Tandem Mass Spectrometry Analysis

Analysis was carried out by LC-MS/MS on an UltiMate 3000 RSLCnano liquid chromatography (nanoLC) Labeling workflow for +GSH and -GSH. Each sample was split into two equal parts and either fully reduced (red subscript) or blocked with NEM (ox subscript) before iodoTMT labeling. By using strategic iodoTMT labeling, the individually labeled samples could then be combined in different groups to create four six-plex samples, providing relative quantitation for protein expression (Red), quantitation of cysteine oxidation (Ox), percentage of cysteine oxidation in +GSH, and percentage of cysteine oxidation in -GSH (see appendix 5). The three most abundant multiply charged ions in the MS scan above the set signal thresholds were then selected for consecutive MS/MS analyses by higher-energy collisional dissociation (HCD) and collision-induced dissociation (CID). MS/LC took place at the proteomics Centre at Glasgow University.

2.34.6 Data Processing

Data processing was carried out in Proteome Discoverer software version 2.1 SP1 (Thermo Fisher Scientific) using Mascot version 2.6.1 (Matrix Science) for database searching. MS/MS spectra were searched against the CLIC4-human database (version November 2021) with the following parameters: enzyme trypsin (full), maximum missed cleavages 2, precursor mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, quantitation method IodTMT six-plex, reporter ion integration tolerance 20 ppm, and isolation interference threshold 20%. Variable modifications included oxidation (M), iodoTMT (C), N-ethylmaleimide (C), N-ethylmaleimide + water (C/K), and protein N-terminal acetylation. The protein quantity was calculated by manual formula through the abundance of oxidized protein to overall abundance between a common protein that expressed to overall protein. Those variations were set up by Proteomics analysis Centre at Glasgow University.

Chapter 3 CLIC4 in response to TNF-α re-localizes from the cytosol into the ER and maintains in a reduced state

3.1 Introduction

Oxidative stress can be regulated by many environmental and endogenous factors. In this study, oxidative stress is induced by TNF-α. TNF-α is an inflammatory cytokine and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis in response to oxidative stress (Kayedpoor P, et al., 2017). TNF-α can act through two receptors, the valid aggregation state of Tumor Necrosis Factor Receptor Superfamily (TNFSF) proteins and TNF-α receptors via extracellular cysteine-rich domain are critical to their bioactivity and functional signaling (Lang I, et al., 2016), and only with correct folding conformation can the proteins effectively ensure the smooth progress in the signaling pathway for an efficient function (Figure 3-1).

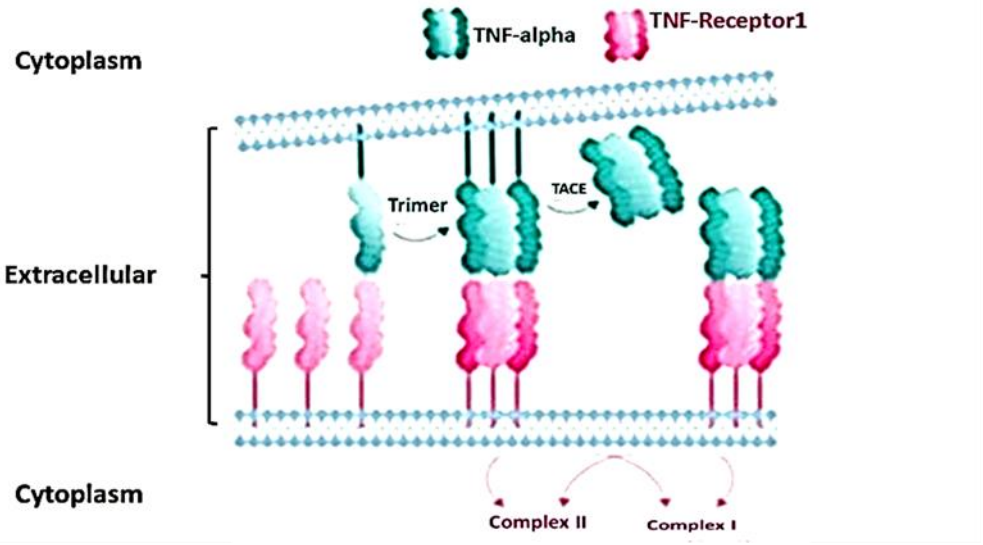


Figure 3-1 the valid aggregation state of TNFSF proteins and TNF-alpha receptors. The trimer-station of Tumour Necrosis Factor Receptor superfamily (TNFRSF) can only occur when TNF-α and Tumour Necrosis Factor Receptor 1 (TNFR1) bind to each other in the extracellular domine of TNFR1. This conformation effectively stimulates the downstream pathways to induce either cell death or survival pathways (Idriss HT and Naismith JH, 2000).

TNF-α induces a reduction of cellular Glutathione (GSH) levels, which might be responsible for the accumulation of reactive oxygen species (ROS) in mammalian cells; hence, TNF-α

induces oxidative stress in a ROS dependent fashion (Xue X, et al., 2005). However, TNF-α induced NF-KB activation can induce the activation of some NF-kB dependent signaling pathways (Sun S, et al., 2012). NF-KB following several activations of the signalling cascade translocate to the nucleus and activates genes with deoxyribonucleic acid (DNA) binding sites for NF-kB via c-myc intron binding protein1 (MIBP1) (Palazzo I, et al., 2022). Since CLIC4 promoters have been identified for containing multiple binding sites for MYC, NF-KB, and TGF, therefore, CLIC4 could be implicated with this stress signaling pathway through either upregulation or intracellular translocation, notably, translocation to the nucleus (Shu KS, et al., 2007), and to the ER from the cytosol and probably in the ER lumen due to the existence of a higher concentration of GSSG (Chakravarthi S, et al., 2007).

The ER is a significant place for post-translational modification, particularly for disulfide formation within folding proteins entering the mammalian secretory pathway (Bulleid N, et al., 2009). The consequence of oxidative stress in the ER is the accumulation of misfolded and unfolded proteins (Zeeshan H.M.A., et al., 2016). Hence, a family of oxidoreductases including PDI (Jessop C.E, et al., 2009) is located in the ER and is responsible for forming native disulfide bonds and editing any misfolded or non-native disulfide bonds (Poet G.J, et al., 2017). The formation and reduction of disulfide bonds not only required the accepting and donating of electrons but also required to exist oxidative and reductive pathways within the cell. As the oxidoreductase for forming a disulfide bond, must itself be oxidized to capable for accepting electrons and on the contrary, for isomerizing and reducing a non-native disulfide bond, must itself be reduced to be able to be donating electrons and be functional as an isomerase or reductase (Purich D.L, et al., 2020). Subsequent to each cycle of reduction or oxidation, the oxidoreductase must be returned to its original state, or active redox state before it can deliver further catalytic rounds (Griffith O. W., 1982, Moilanen A, et al., 2020).

For CLIC4 to act as an oxidoreductase (Alkhameci H, et al., 2015) for performing isomerization or reduction reactions, it must be preserved in a reduced redox state to remain active. Reduced and oxidized redox state of a protein have indicated with cysteine redox state that are markedly identified with post-translational modifications of protein (Wani R, et al., 2014). Consequently, there is a requirement for specific labelling followed by mass spectrometry (MS) to identify the abundance of cysteine residues (Pilo A.L, et al.,

2016), and an (MS/LC) to detect a relative comparison of protein expression levels in the redox states (Becher F, et al., 2017).

The soluble form of the CLIC4 protein has a distinct function from its ion channel activity and is recognized with the enzymatic catalytic activity. This enzymatic activity of CLIC4 resembles that of the glutaredoxin (Grx) (Valenzuela S, et al., 2013), owning to glutathione-dependent thiol transferase activity to catalyse the glutathione-dependent reduction of dehydroascorbate (DHA) (Warton K, et al., 2002). This enzymatic activity is structurally related to the thioredoxins in the N-terminal of the CLICs family and potentially is involved in the reduction of intracellular disulfide (Littler RD, et al., 2010). The catalytic reactions contribute to the redox modulation within cells and can be coupled with GSH, NADPH and glutathione reductase (GR) (Begara-Morales, et al., 2015, Couto N, et al., 2016, Van Lith M, et al., 2021), Like the GSTs, CLIC4, a member of the glutaredoxin family, contains a GSH binding site within its conserved thioredoxin domain known as the G-site containing a single cysteine residue [Cys-Gly-Phe-Ser] with (CXXS) motif and can act as monothiol oxidoreductase by forming mixed disulfides between GSH and target proteins, (Valenzuela SM, et al., 2013). This reaction can particularly apply to stress response proteins in oxidative stress conditions to isomer and reduce the non-native disulfide bonds to maintain homeostasis within the cells (Alkhameci H, et al., 2015).

In this chapter, I have established a stable cell line overexpressing a designed fusion protein with a V5 epitope tag in the C-terminal to identify the localization of CLIC4 protein in different subcellular fractions notably in the ER. To investigate whether the translocation of CLIC4 from the cytosol into the ER could be considered owning to its redox state in response to oxidative stress, I first identified the localization of CLIC4 in main subcellular compartments under optimization of two oxidative stress-inducers such as TNF-α and H_2O_2. This approach enables us to figure out that TNF-α with respect to concentration can induce translocation of CLIC4 from the cytosol into the ER and nucleus. Although sufficient information on nuclear translocation of CLIC4 exists (Shu K.S, et al., 2005 and 2007, 2012) and it can be considered owning to NLS (nuclear localization signals) of CLIC4 (*K*VVA*KKY*R*:* residues 199–206) at the C-terminal of helix 6 (Littler DR, et al., 2005), there is still a lack of information about the re-localization of CLIC4 into the ER.

I assume that this translocation can be due to cysteine 35 (CXXS) motif in N-terminal of CLIC4 (Littler RD, et al., 2010), which could be involved with reductase and isomerase

activity of CLIC4 depending on glutathione. To understand the possibility of redox activity of Cys35 in the reduction and isomerization of CLIC4, I designed a mutant. To create a CLIC4 mutant in (CXXS) motif, Cysteine 35 was replaced with Alanine because Alanine may cause oxidative inactivation by preventing either intra/or intermolecular disulfide bond formation (Nolan S.M, et al., 2000). It was proven that Cys35 is easily accessed in the active site surface of the CLIC4 three-dimensional structure (Singh H, et al., 2007). In the mutant form(C-A), it is clear that the active site of CLIC4 was not able to form disulfide bonds with glutathione in mammalian cells and made a redox signaling response either by oxidoreductase or membrane-binding protein activity (Singh H, et al., 2007). Subsequently, it was assumed that the other Cys100, Cys189, and Cys234 probably make a non-native disulfide bond under oxidizing conditions and resulted in the inhibition of redox activity of CLIC4 in response to oxidative stress and might be inhibit the translocation of CLIC4 toward the ER from the Cytosol.

In this study to uncover the functional role of CLIC4 in mammalian cells, I first determine the expression and localization of CLIC4 to several subcellular organelles with specific organelle markers; then, I optimize two oxidative stress inducers such as TNF-α and H_2O_2 on translocation and expression of CLIC4. The result illustrated that under TNF-α induction CLIC4 can re-localized from the cytosol to the ER. To uncover the functional role of CLIC4 in the ER we prepared the SP cells as a source of the ER (Wilson R, et al., 1995) and examined the redox state of CLIC4 under TNF-α treatment. To determine the expression of CLIC4 under a redox state in a human cell line, we have modified the cysteine residue with subjecting to several alkylation reagents (Oka O, et al., 2013), suggesting causing a shift in mobility when the protein is separated by SDS-PAGE and demonstrating the possibility of the existence of a robust reductive pathway in the mammalian cells to maintain CLIC4 in a reduced state.

Since the active site of CLIC4 is either partially or completely reduced, suggesting CLIC4 appears in a reduced position as a soluble form with either oxidoreductase or isomerase activity and could be involved in disulfide bond reduction to eliminate the oxidative stress effect. I therefore assume that the presence of a reductive pathway to recovering of CLIC4 from the addition of TNF-α to return to a reduced state and re-establish free thiol. Hence, to investigate if this assumption can be occurred, I detect the redox state at the various time points after removal of TNF-α by resuspending in the fresh medium, demonstrating

that a strong reductive pathway exists to recover and maintain CLIC4 in a reduced state and CLIC4 might be able to participate in this pathway with its both physiological functions either with the catalytic enzyme activity or with its ion channel activity in a reduced position.

Moreover, to identify the abundance and redox state of CLIC4 cysteine residues, particularly cysteine 35 as a catalytic cysteine with the ability for modification, we markedly demonstrate the redox status of CLIC4 via post-translational modifications with several alkylation reagents including IAA and 4VP within a standard mass spectrometry protocol (Coscia F, et al., 2020), demonstrating CLIC4 in both untreated samples and reduced samples were placed in a reduced state, suggesting that a robust reductive pathway has located in mammalian cells to maintain CLIC4 in a reduced state. In addition, we demonstrated that CLIC4 under oxidative stress induced by TNF-α can translocate from the cytosol into the ER (Duncan R.R, et al., 1997), due to gradual depletion along with reduction of CLIC4, which could be resulted from its ion channel activity that, in turn, can regulate its reductase activity (Valenzuela SM, et al., 2016).

Finally, to examine the effect of the ion channel activity on the reduction of CLIC4, I subjected wild type of stable humane cell lines to IAA-94 as an ion channel inhibitor, our result indicated that the inhibition of ion channel activity of CLIC4 can abolish the reduction of CLIC4. In addition, to make sure the functional role of CYS35 in ion channel function of CLIC4 and possibility to regulate the oxidoreductase activity of CLIC4, I designed a mutant of redox motif of CLIC4 with replacing of Cysteine with Alanine, indicating that reduction of CLIC4 again was abolished and no evidence of cytosolic mis-localization of CLIC4 toward ER was observed.

3.2 Results

3.2.1 Expression of CLIC4-V5 in mammalian cells

To establish a system to monitor the expression and localization of CLIC4 in the mammalian cells, we fused the V5 tag at the C-terminus of the CLIC4 protein and in the N-terminal with a poly-A for initiate the translation. The resulting CLIC4-V5 construct was transiently transfected into HT1080 and HEK293Tcells. The transiently overexpressed CLIC4-V5 was analyzed with western blotting and following immunofluorescence microscopy to indicate the transfection efficiency of CLIC4-V5 in the human cell. To identify CLIC4-V5 expression level, cells were seeded in three confluences, including 80%, 60%, and 40%, respectively, and lysates were separated by reducing SDS-PAGE and CLIC4-V5 visualized by western blotting, three distinct species were separated. All samples were fully reduced when (50mM) DTT was added to the samples prior to electrophoresis (Figure 3-2 C, lanes 1-3). To detect the overexpressing of transfected cells, coverslips were included in the dish that can be fixed by methanol to check the transfection by fluorescence microscopy (Figure 3-2 A, panel a). Meanwhile, we designed a negative control (Figure 3-2 A, panel b) to distinguish un-transfected or mock-transfected cells and the other one as a positive control (Figure 3-2 A, panel c) to identify the staining efficiency. Note: the exogenous CLIC4-V5 means that I made the construct of PcDNA3.1(+)-CLIC4-V5 and then transfected into the HT1080 as an exogenous CLIC4 that is totally different with endogenous CLIC4 that translated by cells.

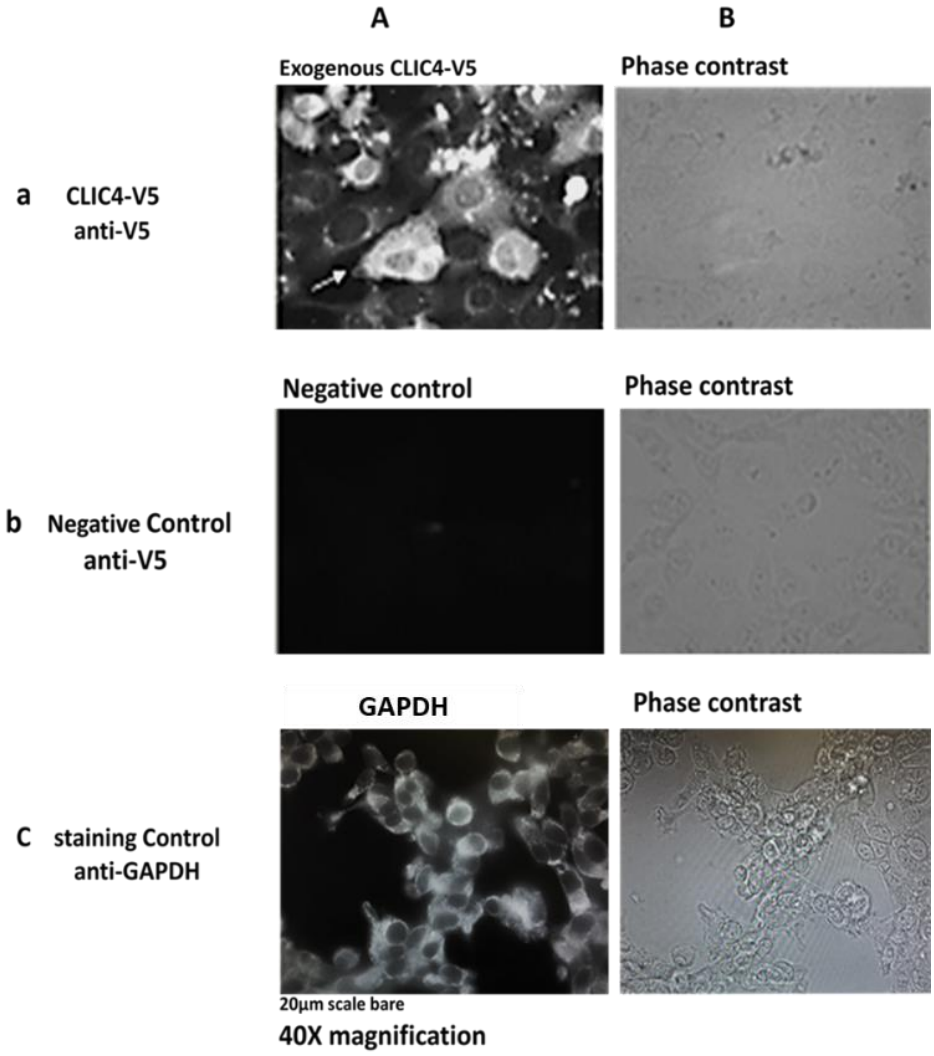
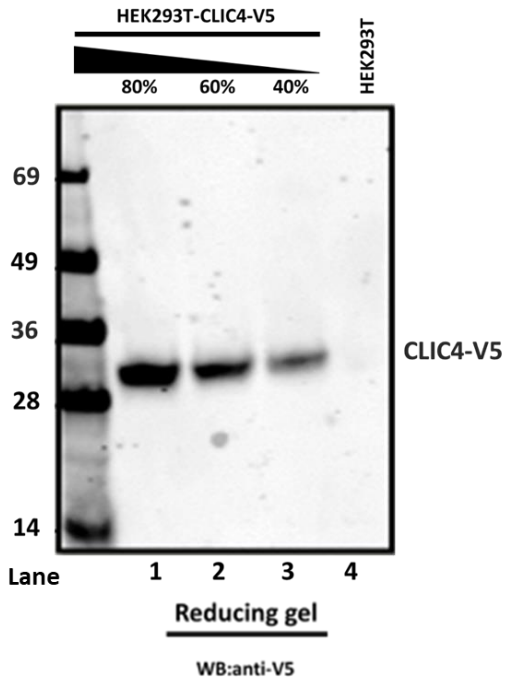


Figure 3-2 Expression of CLIC4-V5 in mammalian cells A. HT1080 cells were fixed and imaged by Epi-immunofluorescence microscopy with x40 magnification. HT1080 cells were either transiently transfected and fixed in methanol to immunostaining with an anti-V5 antibody (panel a) or un-transfected as a negative control (panel b) and incubated with a GAPDH antibody to detect the endogenous GAPDH as a positive control or staining control (panel c). Scale bar: 20µm. **B.** The phase contrast is an optical contrast technique for making unstained phase and cells can be visible in high contrast (the fluorescence imaging was performed by Dr Marcel Van Lit).



C. CLIC4-V5 construct was transiently transfected into HEK293Tcells. The transiently overexpressed CLIC4-V5 was analyzed with western blotting and following immunofluorescence microscopy to indicate the transfection efficiency of CLIC4-V5 in the human cell. To identify CLIC4-V5 expression level, cells were seeded in three confluences, including 80%, 60%, and 40%, respectively, and lysates were separated by reducing SDS-PAGE and CLIC4-V5 visualized by western blotting, three distinct species were separated. All species were fully reduced when (50mM) DTT was added to the samples prior to electrophoresis. These results indicated that recombinant protein CLIC4-V5 was transiently transfected and successfully overexpressed in mammalian cells.

3.2.2 Expression of CLIC1-V5 in mammalian cells

To establish a system to monitor the expression and localization of CLIC1 to the mammalian cells, I fused the V5 tag at the C-terminus of the CLIC1 protein and in the N-terminal with a poly-A. The resulting CLIC1-V5 construct was transiently transfected into HT1080 and HEK293Tcells. The transiently overexpressed CLIC1-V5 was analyzed with western blotting and following immunofluorescence microscopy to indicate the transfection efficiency of CLIC1-V5 in the human cell. To identify CLIC1-V5 expression level, cells were seeded with 80%, confluency, and lysates were separated by reducing SDS-PAGE and CLIC1-V5 visualized by western blotting with anti-V5. The sample was fully reduced when (50mM) DTT was added to the samples prior to electrophoresis (Figure 3-3, C, lanes 1-2). To detect the overexpressing of transfected cells, coverslips were included in the dish that can be fixed by methanol to check the transfection efficiency by fluorescence microscopy (Figure 3-3, A, panel a). Meanwhile, we designed a negative control (Figure 3-3, A, panel b) to

distinguish un-transfected or mock-transfected cells and the other one as a positive control (Figure 3-3, A, panel c) to identify the staining efficiency.

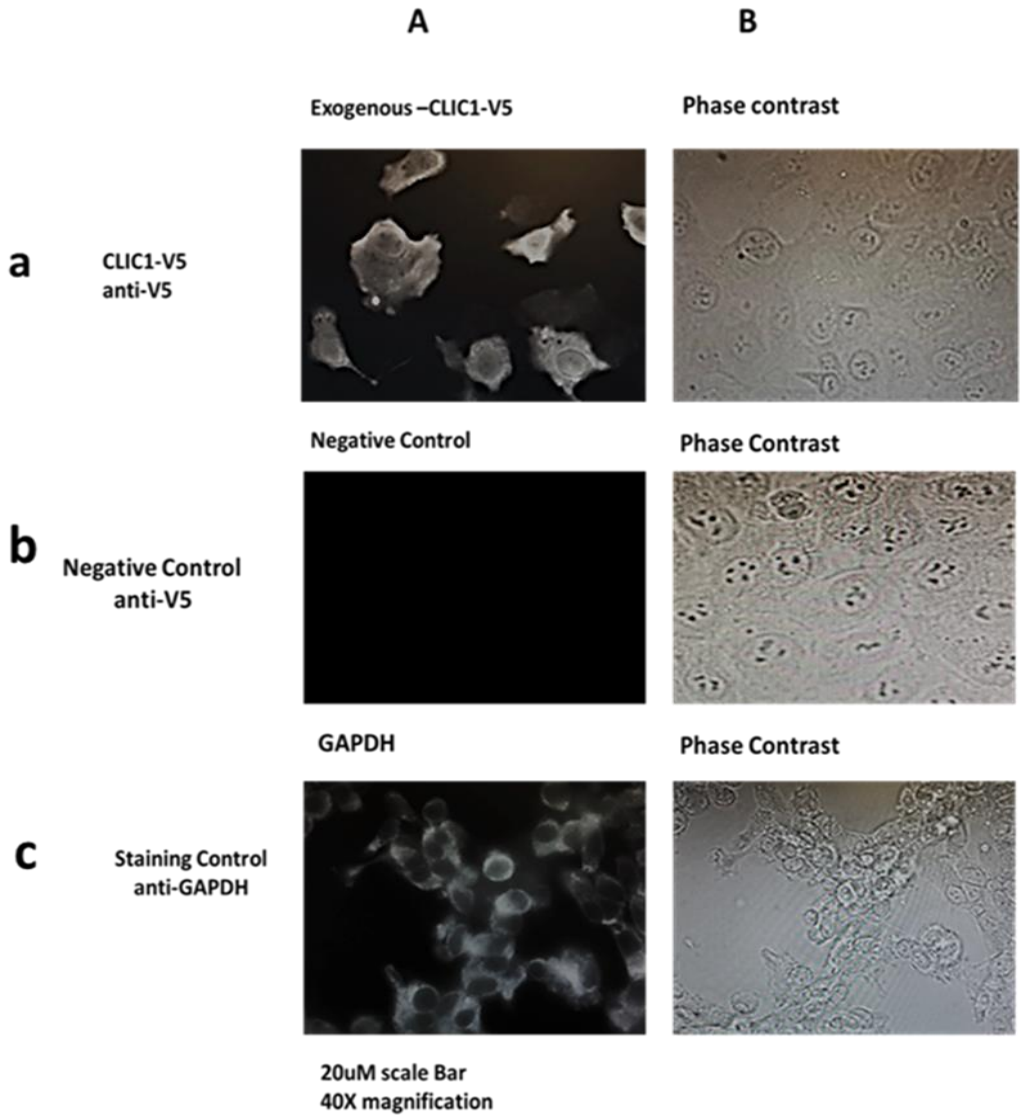
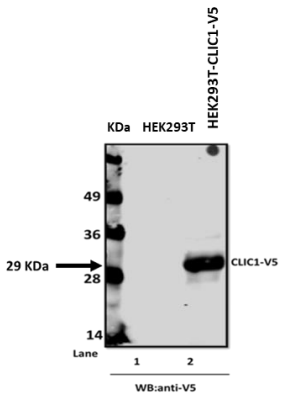


Figure 3-3 Expression of CLIC1-V5 in mammalian cells. A.HT1080 cells were fixed and imaged by Epi-immunofluorescence microscopy with x40 magnification. HT1080 cells were either transiently transfected and fixed in methanol to immunostaining with an anti-V5 antibody (panel a) or un-transfected as a negative control (panel b) and incubated with a GAPDH antibody to detect the endogenous GAPDH as a positive control or staining control (panel c). Scale bar: 20µm. B. the phase contrast is an optical contrast technique for making unstained phase and cells can be visible in high contrast (Image was performed by Dr Marcel Van Lit).

C.



C. CLIC1-V5 construct was transiently transfected into HEK293Tcells. The transiently overexpressed CLIC1-V5 was analyzed with western blotting. To identify CLIC1-V5 expression level, cells were seeded in the 80% confluency and lysates were separated by reducing SDS-PAGE and CLIC1-V5 visualized by western blotting, the samples were fully reduced when (50mM) DTT was added to the samples prior to electrophoresis. These results indicated that recombinant protein CLIC1-V5 was transiently transfected and successfully overexpressed in mammalian cell lines. For control of transfection efficiency, the un transfected cells were included (lane 1).

3.2.3 Cell lines

Following examinate the expression of designed construct of CLIC4-V5 and CLIC1-V5 by immunofluorescent imaging and western botting, we established the stable cell line. To generate the stable cell line, I examined two types of human cells including HEK293T and HT1080. We selected the HEK293T cells for ability to overexpress the wild ranges of proteins at a high level including CLIC4.

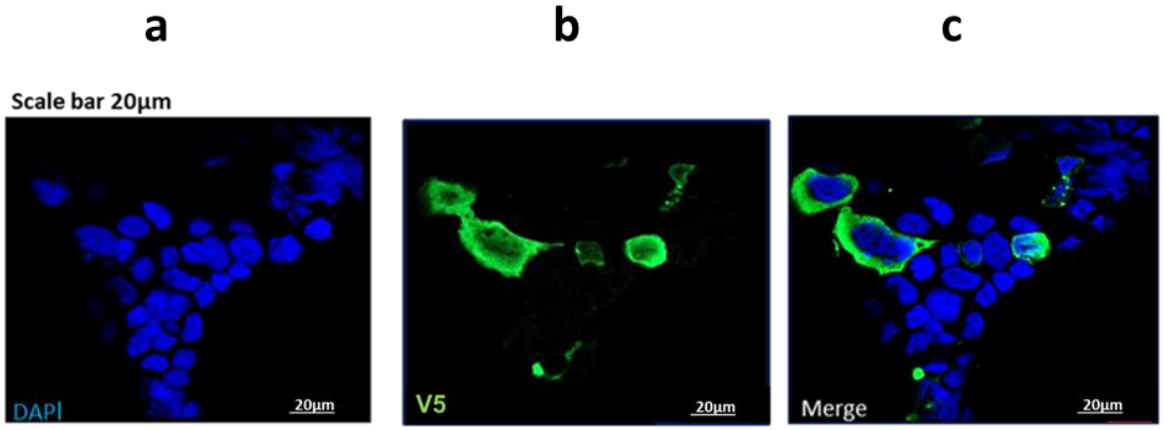
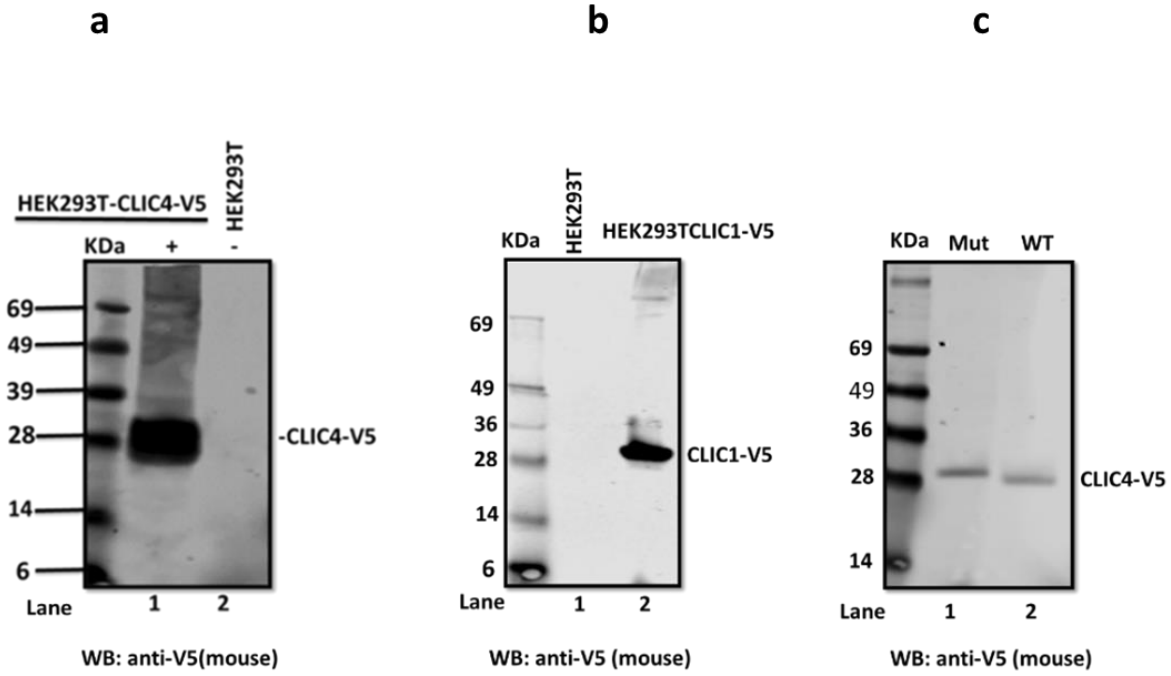


Figure 3-4 The expression of CLIC4 in HEK293T cells (A) Stably transfected HEK293T cells were simultaneously fixed in methanol and immunostained for labeling with (a) DAPI (Nucleus-marker/blue), (b) anti-V5 (Rabbit) to recognize the transfected cells with green Alexa fluor fluorescent dye in 480 waves light, (c) Merged images show the total staining. The image was taken by (CLSIM) confocal laser scanning immunofluorescence microscopy and the visualization was carried out with 63X magnification and Scale bar was 20µm.



B. HEK293T individually stably transfected with CLIC4-V5, CLIC1-V5 and mutant CLIC4-V5 and overexpressed CLIC4-V5(A), CLIC1-V5(B), and mutant CLIC4-V5(C) to design HEK293T-CLIC4-V5, HEK293T-CLIC1-V5, and HEK293T-mutant CLIC4-V5 (cysteine 35 to Alanine) stable cell line respectively. Each cell line was tested with western blotting. To determine the transfection efficiency of CLIC4-V5, and CLIC1-V5, the HEK293T cells were either transfected in %80 confluences or un-transfected as a control (a, lane 2), (b, lane 1). The cells were washed in PBS, before lysis. The lysates were analysed by reducing SDS-PAGE and western blotting (WB) with anti-V5. (Figure 3-4, a, b, and c lanes 1–2). To compare the expression wild type and mutant of CLIC4-V5, stable HEK293T-CLIC4-V5 (wild type) and mutant (cysteine 35 to Alanine) were tested with western blotting and visualized with anti-V5 (c, lane 1-2), the samples were fully reduced when (50mM) DTT was added to all samples prior to electrophoresis.

The result indicated that CLIC4-V5, CLIC1-V5, and mutant CLIC4-V5 (C-A) were stably transfected and overexpressed with over 80% in HEK293T, the transfection efficiency was confirmed with western blotting and confocal microscopy.

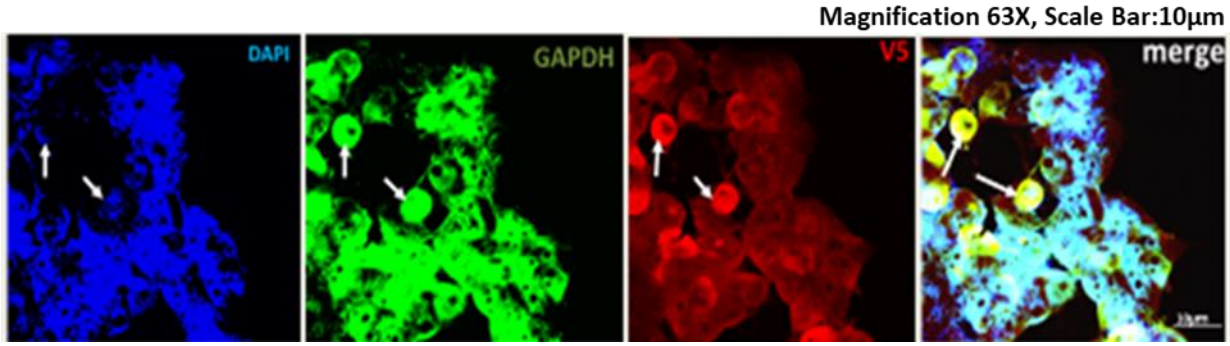
3.2.4 Expression and localization of CLIC4 to intracellular organelles in mammalian cells

To determine the localization and expression of CLIC4 under oxidative stress, we generated the transiently transfected HEK293T cell line expressing a V5-tagged CLIC4 to identify the localization of CLIC4-V5 in the cytosol, mitochondria, nucleus, ER, and plasma membrane of the mammalian cells. To improve the imaging for a clear visualization of CLIC4 localization, I included the 0.02% triton to increase the permeabilization of the cell membrane to qualify the staining (Van Lit M, et al., 2011). To detect the localization of CLIC4 to the cytosol, mitochondria, nucleus, ER, and plasma membrane, we have utilized specific organelle antibodies such as GAPDH, ATP-5A, $HDAC_2$, CNX, and NA/K ATPase following immunofluorescence microscopy, respectively (Figure 3-5, 3-6, 3-7, and 3-9 in the upper panel). In addition, as CLIC4 is known as a Mito-CLIC with 60% mitochondrial localization (Shu KS, et al., 2005), we examined CLIC4 expression and localization in HT1080 cells by immunostaining using anti-V5 antibody and a Mito tracker, as a functional mitochondria marker (Figure 3-6). To generate the oxidative stress condition, HEK293T cells were treated with either (2mM) H_2O_2 (Cao Z, et al., 2014) and (10pM, 10nM, 10uM), TNF-α (Shu KS, et al., 2005) or left untreated as a control for normal conditions. One sample was neither transfected nor treated and only included to control transfection. To prepare subcellular organelles at once, fractions were isolated from the transfected HEK293T cells with 80% confluency through standard protocol for fractionation without using detergent as described in (Moniruzzaman, M, et al., 2018). The procedure has been optimized to provide the extraction with high protein recovery and low cross-contamination in less than 2 hours, the fractions were resuspended in (100 ul) isolating buffer. The samples for each fraction were separated by reducing SDS-PAGE and being visualized by western blotting (WB) via anti-GAPDH, ATP-5A, $DACH_2$, CNX, Na/K ATPase, and anti-V5 to separate different species. All species were fully reduced once (50mM) Dithiothreitol (DTT) was added to the samples prior to electrophoresis.

3.2.4.1 Expression and localization of CLIC4-V5 in mammalian cytosol

To determine the cytosolic localization and expression of CLIC4 under oxidative stress, we generated the transiently transfected HEK293T cell line expressing a V5-tagged CLIC. To generate the oxidative stress condition, HEK293T cells were treated with either (2mM H_2O_2) (Cao Z, et al., 2014) and (10pM TNF-α) (Shu KS, et al., 2005) or left untreated as a control for normal physiological conditions. One sample was neither transfected nor treated and only included to control transfection. To prepare cytosolic organelles, following treatment cells, the cytosolic fractions were isolated from the transfected HEK293T cells with 80% confluency, and the cytosolic fractions were resuspended in (100ul) isolating buffer. The samples were separated by reducing SDS-PAGE and being visualized by western blotting (WB) via anti-GAPDH (Rabbit), and anti-V5 (mouse) to separate different species. All species were fully reduced once (50mM) Dithiothreitol (DTT) was added to the samples prior to electrophoresis.

(A)



(B)

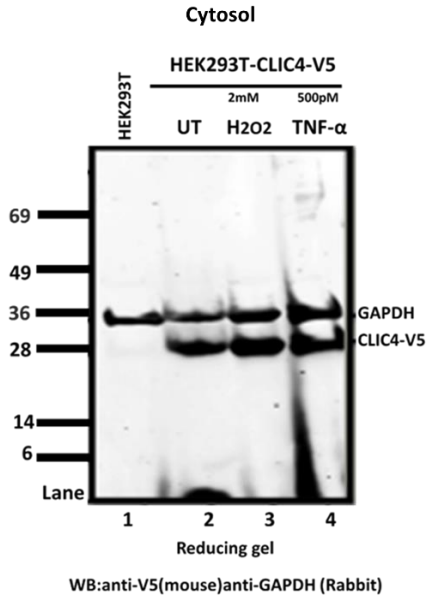


Figure 3-5 Expression and localization of the CLIC4-V5 to mammalian cytosol. (A) HEK293T cell lines transiently transfected with a plasmid driving expressed CLIC4-V5, and simultaneously were fixed in methanol for immunostaining and labelling with (a), DAPI (Nucleus-marker/blue) (b), GAPDH (Cytosolic-marker/Alexafluor488/green) and (C) V5 (transfected cells/alexafluor568/red) and imaged by (CLSIM) confocal laser scanning immunofluorescence microscopy. (d) Merged images are shown on the right with the yellow color indicated by white arrows, which reaffirms co-localization. The scale bar was 10µm and images was carried out with 63X magnification. **(B)** Cells were mock-treated or treated with (2mM) H2O2 for 60 minutes or with (500 pM) TNF-α for 15 minutes (lanes 2–4). Un-transfected HEK293T cells were included as a control (lane 1). Prior to cell lysis, cytosolic fractions were isolated from HEK293T cells, and then the pellet was resuspended in (100ul) of the cytosolic buffer. The lysates were resolved by reducing (R) SDS-PAGE (lanes 1–4) and western blotting (WB) with anti-GAPDH (top) or anti-V5 (bottom) antibodies. The excepted bands for GAPDH as a cytosolic marker is 36KDa and for CLIC4 is 28KDa.

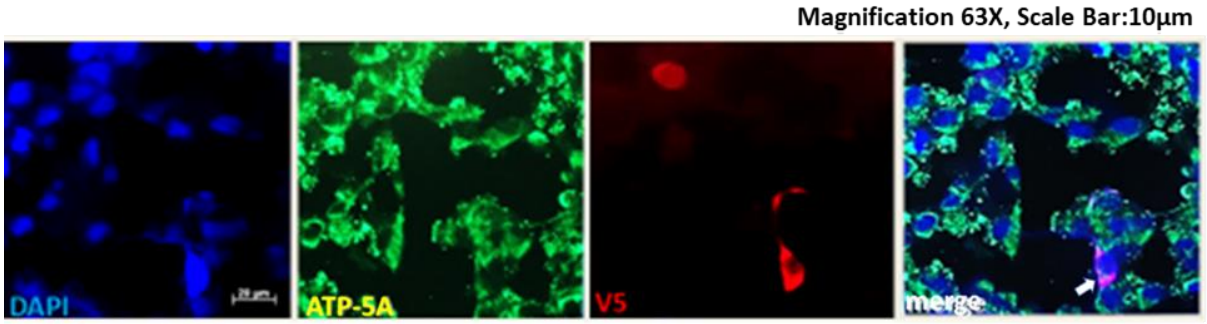
The results indicated that CLIC4 was localized to the mammalian cytosol due to co-localization with GAPDH and expressed in the normal physiological and oxidative conditions (Figure 3-5, B, lane 1-4). The expression levels of recombinant CLIC4-V5 during oxidized conditions with (2mM) H_2O_2 was the same as the normal condition (Figure 3-5, B, lane 2 and 3), suggesting that due to the high concentration of GSH (up to 10 mM) in the cytosol (Chakravarthi S, et al., 2004), the protein might be set in a stable form. On the other hand, the concentration of H_2O_2 could be insufficient to generate an alteration in the redox balance during oxidative conditions. By comparing TNF-α treated samples, the expression of CLIC4 increased, demonstrating TNF-α induces a reduction of cellular GSH levels, being probably responsible for the accumulation of ROS within the cells (Xue X, et al., 2005), which in turn targets the upregulation of CLIC4 expression. CLIC4 expression in response to oxidative stress could be monitored through either gene expression regulation or redox translocation to other subcellular compartments (Singh H, et al., 2007) (Figure 3-5-1, lane 4). Overall, CLIC4 was expressed and localized to the cytosolic mammalian cells in normal and oxidative stress induced conditions. By comparing H_2O_2, CLIC4 has shown a stronger response to TNF-α at the level of the expression.

3.2.4.2 Expression and localization of CLIC4-V5 in mitochondria of mammalian cells

To determine the mitochondrial localization and expression of CLIC4 under oxidative stress, we generated the transiently transfected HEK293T cells expressing a V5-tagged CLIC4. To generate the oxidative stress conditions, HEK293T cells were treated with either (2mM)

H_2O_2 (Cao Z, et al., 2014), and (10pM) TNF-α (Shu KS, et al., 2005) or left untreated as a control for normal physiological conditions. One sample was neither transfected nor treated and only included to control the transfection efficiency. To prepare mitochondria fraction (Tokatlidis K, et al., 2018), following treatment cells, mitochondria fractions were isolated from the transfected HEK293T cells with 80% confluency and the mitochondria fractions were resuspended in (100ul) isolating buffer. The samples were separated by reducing SDS-PAGE and being visualized by western blotting (WB) via anti-ATP-5A (Rabbit) and anti-V5 (mouse) to separate different species. All species were fully reduced once (50mM) Dithiothreitol (DTT) was added to the samples prior to electrophoresis.

(A)



(B)

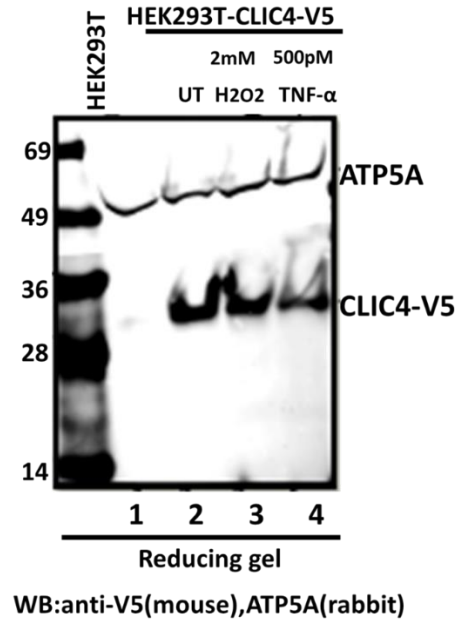


Figure 3-6 Expression and localization of the CLIC4-V5 to mitochondria in mammalian cells (A) HT1080 cell lines transiently transfected with a plasmid driving CLIC4-V5 expressed were simultaneously fixed in methanol and immunostained for labeling with (a) DAPI (Nucleus-marker/blue), (b) anti-ATP-5A (Mitochondria-marker, with green Alexa fluor fluorescent dye in 480 waves light), (c) anti-V5 (to recognize transfected cells with red

Alexa fluor fluorescent dye in 568 wave light) then imaged by (CLSIM) confocal laser scanning immunofluorescence microscopy. (d) Merged images are shown on the right with white color narrow reaffirming co-localization. 63X magnification and Scale bar:10µm. (B) HEK293T cells expressing were mock-treated or treated with (2mM) H_2O_2 for 60 minutes or with (500pM) TNF-α for 15 minutes (lanes 2–4). Untransfected HEK293T cell lysates were included as a control (lanes 1). Prior to cell lysis, mitochondria fractions were extracted from HEK293Tcells, then pellets were resuspended in (100ul) of mitochondria buffer. The lysates were resolved by reducing SDS-PAGE (lanes 1–4) and western blotting (WB), which were hybridized with anti-ATP-5A (top) or anti-V5 (bottom) antibodies. The excepted band for mitochondria markers ATP-5A is 50KDa and for CLIC4 is 28KDa.

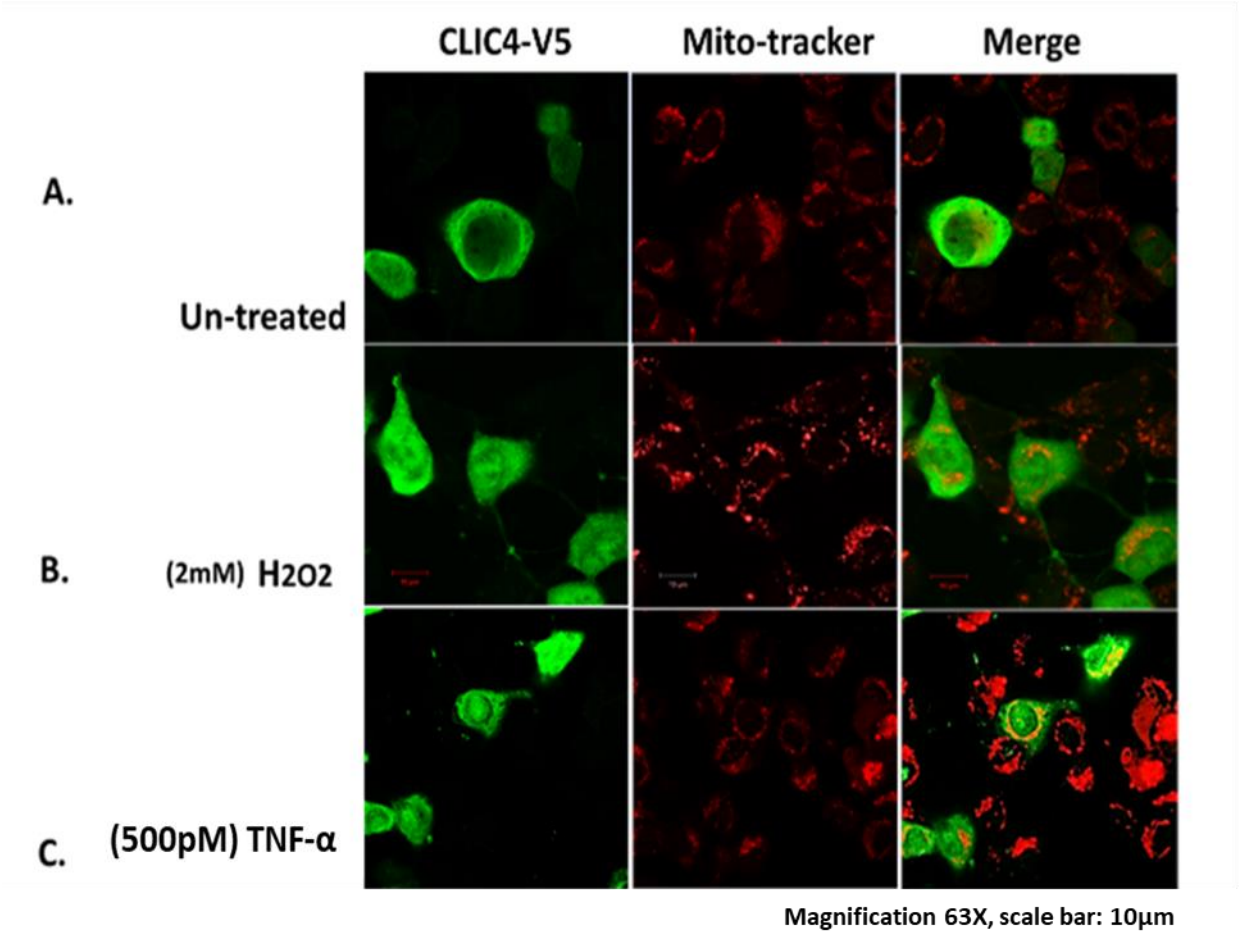


Figure 3-7 The localization of CLIC4-V5 to mitochondria in HT1080 cells. The HT1080 cells were transfected with plasmid driving expressed CLIC4-V5 either treated with (500 pM) TNF-α and (2mM) H_2O_2 or left untransfected as a staining control and incubated with Mito-tracker (a specific marker for detecting functional mitochondria) then fixed with cold methanol. Next, the slides were incubated with anti-V5 antibody and secondary antibodies. The image shows the co-localization of CLIC4 and mitochondria with magnification (63X) and scale bar:10µm in three conditions (Xiao B, et al., 2016). The confocal imaging confirmed CLIC4 localized to the mitochondria and expressed in oxidative stress conditions.

To investigate whether oxidative stress can stimulate expression and localization of CLIC4 in the mitochondria, transiently transfected HEK293T with CLIC5-V5 tagged were treated

in the absence or presence of oxidative stress inducers including (2mM) H_2O_2 for 1 hour and (500pM) TNF-α for 15 minutes. The confocal microscopy at three excitation wave lengths (448nm, 480nm and 568nm for blue, green, and red, respectively) were set for differential channels to obtain merge image to confirm mitochondrial localization of CLIC4 to mammalian cells (Figure 3-6).

In addition, to evaluate the expression of CLIC4 during the oxidative stress in mitochondria of human cells, following treatment, HT1080 was incubated with Mito-tracker (final concentration 0.5µM) for 40 min into the 6 well plate at 20 °C (RT). After the fluorescence emission saturated, cells were washed with PBS containing Triton (final concentration 0.1%) and the staining will be followed as described in chapter 2. Mito-tracker is chemically reactive and linking to thiol groups in the mitochondria. The dye becomes permanently bound to the mitochondria and remains thus after the cell were died or fixed (Chazotte B, et al., 2011). The result of mito-traker staining demonstrated that CLIC4 was localized to the mitochondrial and expressed in normal and oxidative conditions.

To evaluate the expression and localization of mitochondrial CLIC4, HEK293T cells transiently transfected with construct driving CLIC4-V5, and prior to cell lysis, mitochondria fractions were extracted from HEK293Tcells, then pellets were resuspended in (100ul) of mitochondria buffer. The lysates were resolved by reducing SDS-PAGE (Figure 3-6, B, lanes 1–4) and western blotting (WB). Mitochondrial CLIC4-V5 was visualized with anti-V5 (bottom), and an anti-ATP-5A (top) antibodies.

The results above demonstrated that CLIC4 was expressed and localized to mitochondria, but a decrease in expression was observed in samples under TNF-α treatment (Figure 3-6, B, lane 4), indicating there is some alteration in the redox status of CLIC4 in response to TNF-α- induced oxidative stress in the mitochondria. The amount of CLIC4 expression in the presence of TNF-α would be decreased to a lower level than in the absence of TNF-α in samples 2 and 3 (untreated and H_2O_2 treated samples) (Figure 3-6, B, lanes 2, and 3), which indicated more reduction of cellular GSH levels, which might happen in the presence of TNF-α than in the absence of TNF-α (Xue X, et al., 2005).

However, the samples treated with H_2O_2 placed in a steady state same as untreated samples (Figure 3-6, B, lanes 2 and 3), suggesting the concentration of H_2O_2 was not affect CLIC4 expression in response to oxidative stress (Figure 3-6 lane 3). The results suggested that CLIC4 expressed and localized to the inner mitochondria membrane due to

colocalization with ATP-5A and is possibly involved in oxidative response by regulation of gene expression and protein translocation. The reduction of cellular GSH level under TNF-α induction led to alteration of redox balance in the cells and, in turn, target CLIC4 gene expression via survival signaling pathway (Abdul-Salam VB, et al., 2019), and multiple binding sites of CLIC4 promoter (Huang m, et al., 2018), which would be derived gene regulation through intermediated transcription factors c-myc, and p53 (Shu KS, et al., 2007). However, the dissipation of mitochondrial membrane potential and nuclear translocation of CLIC4 from the mitochondria (Xu Y, et al., 2013) can be occurred following upregulation of CLIC4 gene expression via H_2O_2 in neuron cells (Ye X, et al., 2015); hence, both TNF-α and H_2O_2 have a significant effect on CLIC4 expression in mitochondria and relocalization from the cytosol into intracellular organelles. Nuclear translocation of CLIC4 from the mitochondria in response to oxidative stress can occur through releasing of cytochrome c and activating Caspase 9 and 3, respectively (Shu KS, et al., 2005) (Figure3-8).

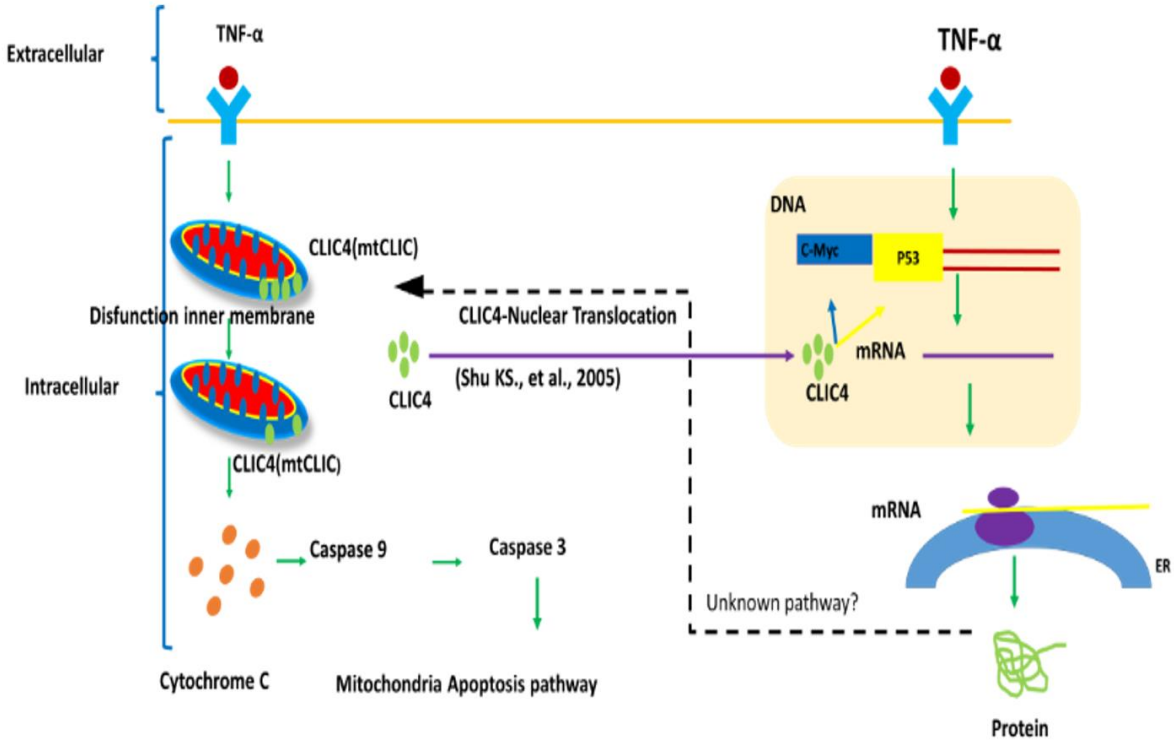
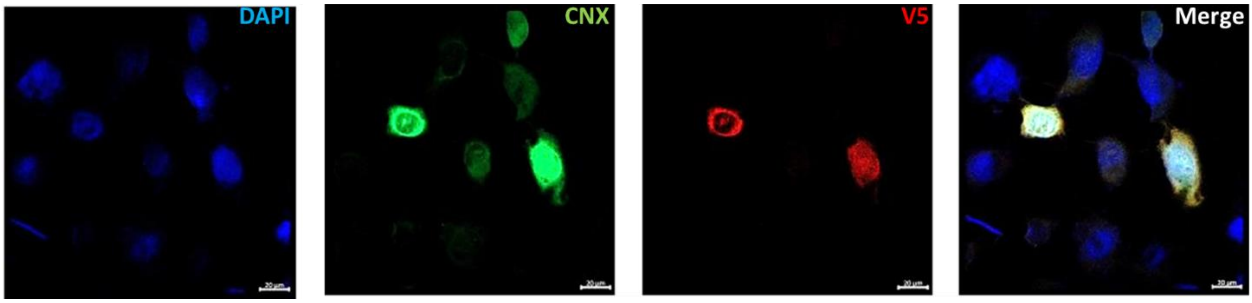


Figure 3-8 mtCLIC/CLIC4 is a TNF-α-regulated, cytoplasmic, and mitochondrial protein that belongs to the CLIC family of intracellular chloride channels. mtCLIC associates with the inner mitochondrial membrane. Dual regulation of mtCLIC by two stress response pathways of TNF-α and H_2O_2 suggested that this chloride channel protein might contribute to the cellular response to oxidative stress. Overexpression of mtCLIC by transient transfection reduces mitochondrial membrane potential, releases cytochrome *c* into the cytoplasm, activates caspases, and induces apoptosis (Shu KS, et al., 2007). TNF-α can induce nuclear translocation in response to nuclear apoptotic pathways through c-myc and P53 as well (Shu KS, et al., 2005).

3.2.4.3 Expression and localization of CLIC4-V5 in mammalian ER

To determine the localization and expression of CLIC4 under oxidative stress, we generated the transiently transfected HEK293T cell line expressing a V5-tagged CLIC4. To generate the oxidative stress condition, HEK293T cells were treated with either (2mM) H_2O_2 (Cao Z, et al., 2014), and (10pM) TNF-α (Shu KS, et al., 2005) or left untreated as a control for normal physiological conditions. One sample was neither transfected nor treated and only included to control the transfection efficiency. To prepare the membrane fraction (Oka O, et al., 2018), following treatment cells, the membrane fractions were isolated from the transfected HEK293T cells with 80% confluency, and the membrane fractions were resuspended in (100ul) isolating buffer. The samples were separated by reducing SDS-PAGE and being visualized by western blotting (WB) via anti-CNX (Rabbit), and anti-V5 (mouse) to separate different species. All species were fully reduced once (50mM) Dithiothreitol (DTT) was added to the samples prior to electrophoresis.

(A)



(B)

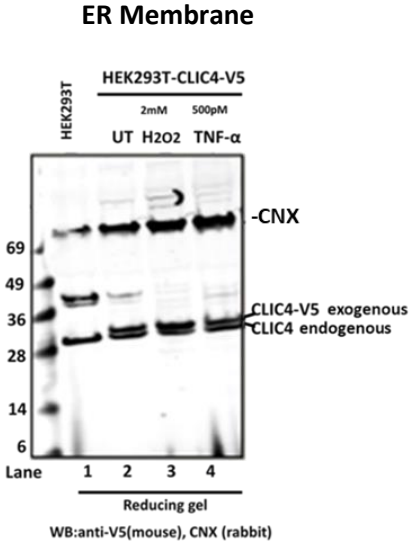


Figure 3-9 Expression and localization of the CLIC4-V5 in mammalian ER (A) Transiently transfected HT1080 cells were simultaneously fixed in methanol and immunostained for labeling with (a) DAPI (Nucleus-marker/blue), (b) anti-CNX (ER-marker, with green Alexa fluor fluorescent dye in 480 waves light), (c) anti-V5 (to recognize transfected cells with red Alexa fluor fluorescent dye in 568 wave light) then imaged by (CLSIM) confocal laser scanning immunofluorescence microscopy. (d) Merged images are shown on the right with white color reaffirming co-localization. Visualization was carried out with 63X magnification and Scale barwas 10µm. **(B)** HEK293T cells expressing CLIC4-V5 were either left untreated or treated with (2mM) H_2O_2 for 60 minutes and (500pM) TNF-α for 15 minutes (lanes 2–4). Un-transfected HEK293T cell lysates were included as a control (lanes 1). Prior to cell lysis, membrane fractions were isolated from transfected HEK293T cells and then pellets were resuspended in 100ul of the membrane buffer. The lysates were resolved by reducing SDS-PAGE (lanes 1–4) and western blotting (WB), which proteins were visualized with anti-CNX, anti-V5, and anti-CLIC4 for recognizing endogenous CLIC4. The excepted band for CNX is 70KDa and for CLIC4 is 28KDa.

To determine the expression and localization of CLIC4 to the ER compartment under oxidative stress conditions, following transiently transfected HEK293T cells with CLIC4-V5 construct, HEK293T cells were treated with (2mM H_2O_2) for 60 min, and (10PM TNF-α) for 15 min before isolating membrane fractions and were lysed after isolating in membrane buffer. Lysate samples were denatured and separated by SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose blotting membrane followed by blocking with blocking agent, 3% milk. The membranes were incubated with different primary antibodies (anti-CNX, anti-V5, and anti-CLIC4). After incubation with rabbit 800 and mouse 680 secondary antibody, the membranes were scanned by Li-Cor Odyssey 9260 Imager to obtain image (Figure 3-9, B).

The result above indicated that CLIC4 localized to the ER membrane of mammalian cells due to co-localization with CNX as a protein resident in the ER membrane (Leach, M.R., et al., 2002), and CLIC4 was expressed either in normal or oxidative stress conditions induced by (2mM) H_2O_2 and (500 pM) TNF-α. The level of CLIC4 expression in all species was the same level as the normal condition(Figure 3-9, B, lanes 1-4), suggesting that CLIC4 could be either placed in a steady sate or the concentration of both oxidative stress inducers were not sufficient to make an alteration on the redox balance to induce the oxidative stress response for CLIC4, which might appear with the translocation, mis-localization, and ER accumulation, therefore, a higher concentration needs to be applied.

To compare the expression of the exogenous to endogenous CLIC4 (Figure 3-9, B, lane 1-4), western blot was carried out with both anti-V5 and anti-CLIC4. We found the migration of two bands for each of the samples in different redox state except un transfected

(Figure3-9, B, lanes 1-4). The levels of expression of three samples were presented in a similar mode and no significant differentiation has been seen, indicating TNF-α and H_2O_2 concentrations were not sufficient to induce oxidative stress to promote differential expression and translocation of CLIC4 to other subcellular compartments. Our result demonstrates that both endogenous and exogenous CLIC4 proteins were expressed and localized to the ER membrane under normal and oxidative stress conditions. Localization of CLIC4 in the ER membrane could result from transferring of cytosolic equivalent from the cytosol to the ER due to its ion channel function and oxidoreductase activity.

3.2.4.4 Expression and localization of CLIC4-V5 in the mammalian nucleus

To determine the localization and expression of CLIC4 to the nucleus of mammalian cells under oxidative stress, we generated the transiently transfected HEK293T cell line expressing a V5-tagged CLIC4. To generate the oxidative stress condition, HEK293T cells were treated with either 2mM H_2O_2 (Cao Z, et al., 2014), and 10pM TNF-α (Shu KS, et al., 2005) or left untreated as a control for normal physiological conditions. One sample was neither transfected nor treated and only included to control the transfection efficiency. To detect the nuclear localization of CLIC4, following treatment cells, the nuclear fractions were isolated from the transfected HEK293T cells with 80% confluency, and the membrane fractions were resuspended in (100ul) isolating buffer. The samples were separated by reducing SDS-PAGE and being visualized by western blotting (WB) via anti-$HDAC_2$ (Rabbit), and anti-V5 (mouse) to separate different species. All species were fully reduced once (50mM) Dithiothreitol (DTT) was added to the samples prior to electrophoresis.

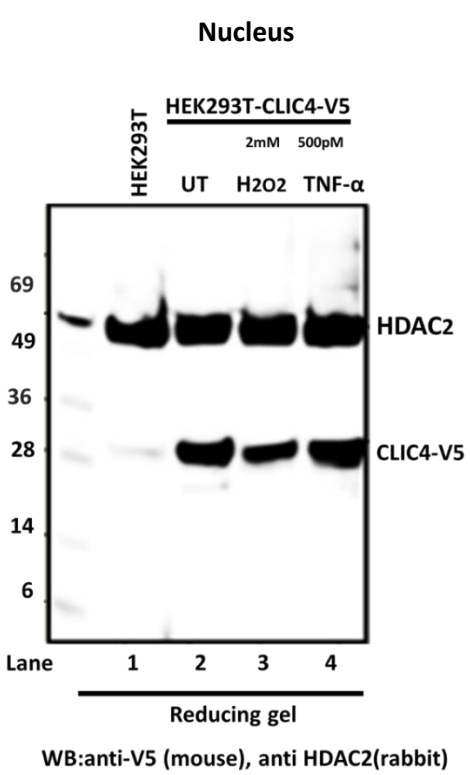


Figure 3-10 Expression and localization of CLIC4-V5 to the mammalian nucleus. HEK293T cells expressing CLIC4-V5 were either treated with 500pM TNF-α for 15 minutes with 2mM H_2O_2 for 60 minutes (lanes 3, and 4) or left untreated (lane 2). One sample was no treated nor transfected as a control for transfection efficiency (lane 1). The cells were washed in PBS that was supplemented with an EDTA-free protease inhibitor tablet before nucleus extraction. The isolated nucleus pellet was resuspended in 100ul nucleus buffer and then analysed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and $HDAC_2$ (lanes 1–4). The excepted band for HDAC2 is 50KDa and for CLIC4 is 28KDa. The endogenous CLIC4 is recognized with anti-clic4 (rabbit)and the exogenous clic4 is recognized with anti-V5(mouse antibody).

To investigate the existence of CLIC4 in the nucleus, we first isolated nucleus fractions before and after treatment. To optimize the oxidative response of CLIC4 to oxidative stress, two oxidative inducers with highlighted concentration were tested. HEK293T cells were transiently transfected with plasmid driving CLIC4-V5 treated either in the present or in the absence of 2mM H_2O_2 for 60 minutes and 500 pM TNF-α for 15 minutes before fractionation upon described protocol in (Kim S, et al., 2017). HEK293T cells were washed with PBS supplemented with an EDTA-free protease inhibitor tablet and centrifuged for 3 min at 300 rpm, then cells were resuspended in 1 ml ice-cold fractionation buffer for 10 min. Cells were lysed by passing through 1 ml syringe, 30 times. The remaining sample was spun at 1000 g, for 7 min at 4 °C. The pellet was a nuclear fraction. The nuclear fraction was resuspended in 100ul nucleus extraction buffer and incubated for 60 min on a 4 °C rocker, then it was spun at 100,000 g for 30 min at 4 °C and was transferred supernatant to the fresh tube; this is the nuclear extract. The nucleus fraction was lysed after isolated in

the nucleus buffer and the resulting lysate was denatured and separated by SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose blotting membrane followed by blocking with blocking agent, milk. The nucleus was incubated with different primary antibodies (anti-HDAC2, anti-V5) (Figure 3-10).

The results above demonstrated that CLIC4 was localised to the nucleus due to co-localization with HDAC2 and expressed before and after oxidative stress-induced by H_2O_2 and TNF-α. However, the expression level of CLIC4 under TNF-α treatment was more than H_2O_2 treatment (Figure 3-10, lane 3, and 4), demonstrating that TNF-α could be considered to induce nuclear CLIC4 accumulation, which might result from either gene expression regulation with mediating the signalling pathway via p53 (Shu KS, et al., 2007), or inducing cytosolic CLIC4 translocation to the nucleus (Shu KS, et al., 2005). In the untreated samples, CLIC4 expression appeared more than the H_2O_2 treated sample, indicating oxidative stress induced by H_2O_2 can be significant effect on decreasing the CLIC4 expression (Figure 3-10, lanes 2 and 3).

The result show that TNF-α have the potential to induce CLIC4 expression in the nucleus with compare to H_2O_2, suggesting TNF-α can able to induce the expression of CLIC4 not only through intermediate transcription factors (Shu KS, et al., 2005) but also through alteration of redox balance in the cells via reduction of GSH level (Xeu X, et al., 2005), which might be responsible for ROS accumulation and, in turn, lead to redox variation in the cells and target the hemostasis response for CLIC4 by increasing the expression and cytosolic depletion towards nucleus. Hence, in the next experiment to figure out the re-localization of CLIC4 towards nucleus in response to oxidative stress, we applied several concentrations of oxidative stress inducers.

3.2.5 Determination of CLIC4 nuclear translocation in respone to oxidative stress

An important aspect of this project is the determination re-localization of CLIC4 to intracellular compartment in response to alteration of redox balance induced by oxidative stress within mammalian cells. To evaluate the process of re- localization of CLIC4, we need to determine the optimal concentration of the oxidative stress inducers, which are considered as main factor for translocation. It has been previously demonstrated that TNF-α is required to nuclear translocation of CLIC4 with a low concentration in mice and keratinocyte prior to apoptosis (Shu KS, et al., 2007). To establish an assay for determining

the optimal concentration of oxidative reagents we sought to reproduce work carried out previously (Shu KS, et al., 2007). To evaluate which concentration of oxidative inducers can induce CLIC4 re-localization, we treated the intact cells with the lower and higher concentration of TNF-α or H_2O_2. We utilized two oxidative stress inducers to compare which of them capable to induce nuclear translocation of cytosolic CLIC4 and we isolated cytosolic and nuclear fractionation at the same time with the same purification pathway without any detergent (Kao O, et al., 2018).

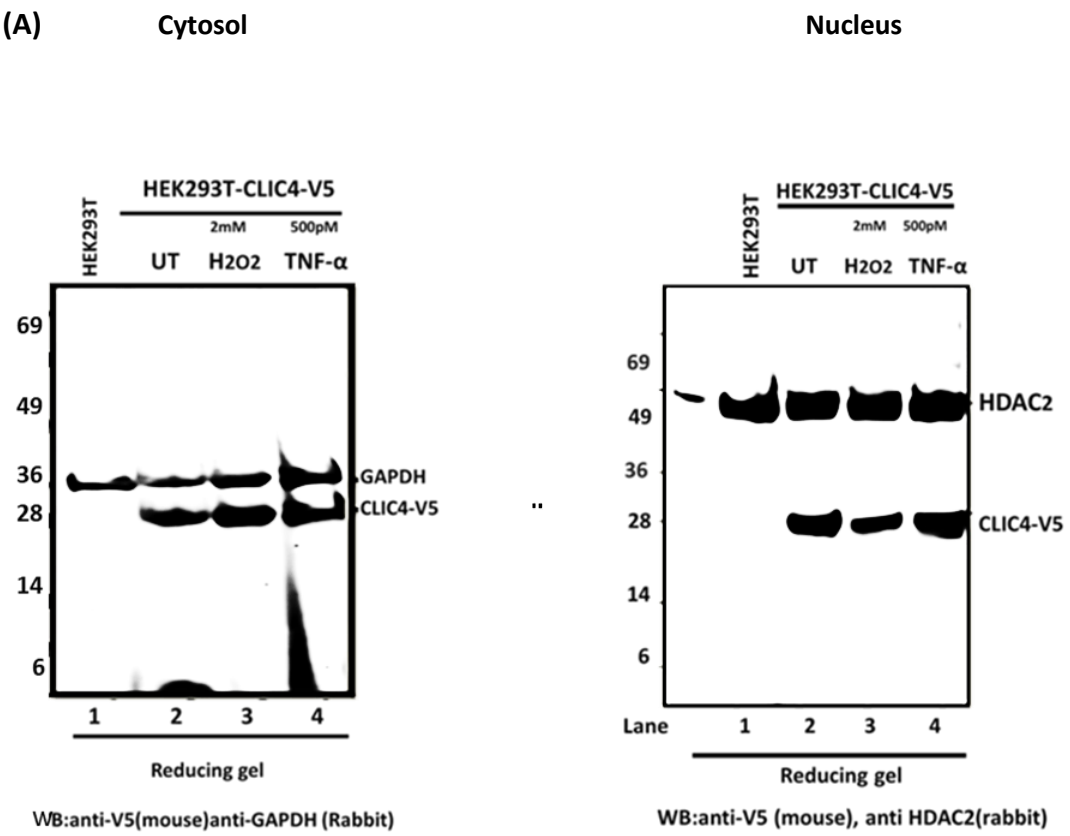


Figure 3-11-A TNF-α induce nuclear translocation of CLIC4 from the cytosol with respect to concentration. HEK293T cells expressing CLIC4-V5 either left as an untreated sample (lane 2) or treated with (2mM) H_2O_2 for 60 min and (500pM) TNF-α for 15 min (lanes 3, and 4). Following treatment, cells were washed with PBS supplemented with an EDTA-free protease inhibitor tablet and centrifuged for 3 min at 300 rpm, then cells were resuspended in 1ml ice-cold fractionation buffer for 10 min. Cells were lysed by passing through 1ml syringe, 30 times. The remaining sample was spun at 1000g, for 7 min at 4 °C. Pellet was a nuclear fraction; supernatant contains cytosol. The nuclear fraction was resuspended in 100ul nucleus extraction buffer and incubated for 60min on a 4°C rocker, then were spun at 100,000g for 30 min at 4°C and were transferred supernatant to a fresh tube, this is the nuclear extract. The supernatant from earlier step was spun at 100,000g for 30 min at 4°C. The supernatant was removed to a fresh 1.5ml tube that was the cytosol and precipitated with ice-cold acetone (5x volume). Pellet was cytosol fraction that was resuspended in 100ul specific cytosolic buffer in the presence of protease inhibitor and analysed by reducing SDS-PAGE and western

blotting (WB) with anti-V5 and anti-GAPDH to detect the cytosolic fraction (Left panel, Lanes 1-4), anti-V5 and anti-HDAC2 to detect the nuclear fraction as well (Right panel, lanes 1-4).

In the previous results, we showed that CLIC4 was expressed and localized to the cytosol and nucleus before and after treatment with 500pM TNF-α or 2mM H_2O_2. In addition, there was no evidence of the alteration in CLIC4 expression to induce either depletion or translocation towards different subunits (Figure3-11-A), suggesting the applied concentrations of oxidative reagents were not sufficient to make the redox response for CLIC4. Hence, to investigate nuclear CLIC4 translocation in response to oxidative stress, in the next experiment we applied a higher concentration of oxidative stress inducers to optimize the actual concentration of 2.5mM H_2O_2 for 60 min and 10nM TNF-α for 15 min, which can influence CLIC4 re-localization and expression (Figure 3-11-B).

(B)

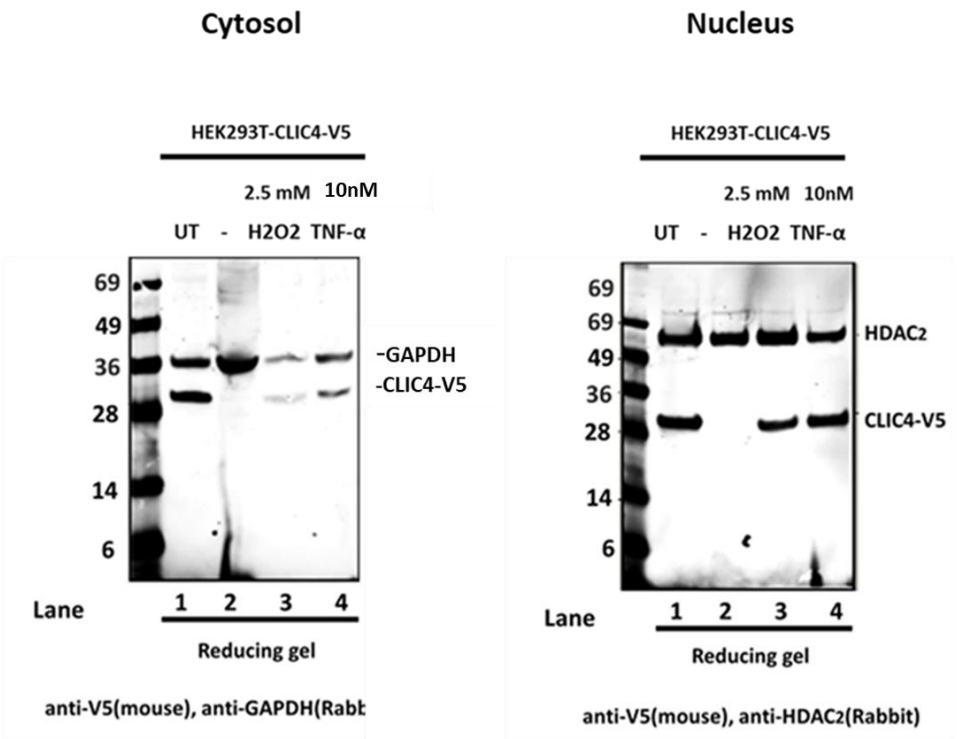


Figure 3-11-B TNF-α induces nuclear translocation of CLIC4 from the cytosol HEK293T cells expressing CLIC4-V5 either left as an un-treated sample (lane 2) or treated with (2.5mM) H_2O_2 for 60 min of 10nM TNF-α for 15 min (lane 3 and 4). Nuclear and cytosolic fractions were isolated as described above. The cytosolic and nuclear species analysed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and anti-GAPDH to detect the cytosolic fraction (lanes 1–4, left panel), anti-V5 and anti-HDAC2 to detect the nuclear fraction as well (lane 1-4, right panel).

To optimize the actual concentration of oxidative stress inducers to induce re-localization of CLIC4 into cytosol, following transiently transfection HEK293T cells with plasmid driving

CLIC4-V5, cells were treated with 2.5mM H_2O_2 for 60 min and 10nM TNF-α for 15 min (Figure 3-11-B, lane 3 and 4) or left as an untreated sample (lane1). One sample was included as a control neither transfected nor treated (lane2). Nuclear and cytosolic fractions were isolated as described above. The isolated cytosol and nucleus were lysed, and the resulting lysate was denatured and separated by SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose blotting membrane followed by blocking with milk. The membranes were incubated with different primary antibodies (anti-V5, anti-GAPDH, and anti-$HDAC_2$ for recognizing CLIC4-V5, cytosol and nucleus, respectively). After incubation with mouse 680 and rabbit 800 secondary antibody, the membranes were scanned by Li-Cor Odyssey 9260 Imager to obtain image (Figure 3-11-B left and right panels). Our result indicates that CLIC4 was expressed and localized to the nucleus and cytosol before and after treatment, and through increasing the concentration of both oxidative reagents appearing a nuclear translocation of CLIC4 in the nucleus and a re-localization in the cytosol of the human cells (Figure 3-11-B, left and right panels).

To determine the efficient concentration and incubation time for relocation of CLIC4 from the cytosol into nucleus in human cell line, in the next experiment, we applied a higher concentration of (5mM) H_2O_2 for 10min (Cao Z, et al., 2013) and 10µm TNF-α for 15min (Shanmugam, et al., 2016) concerning to restriction of the incubation time (Figure 3-11-C).

(C)

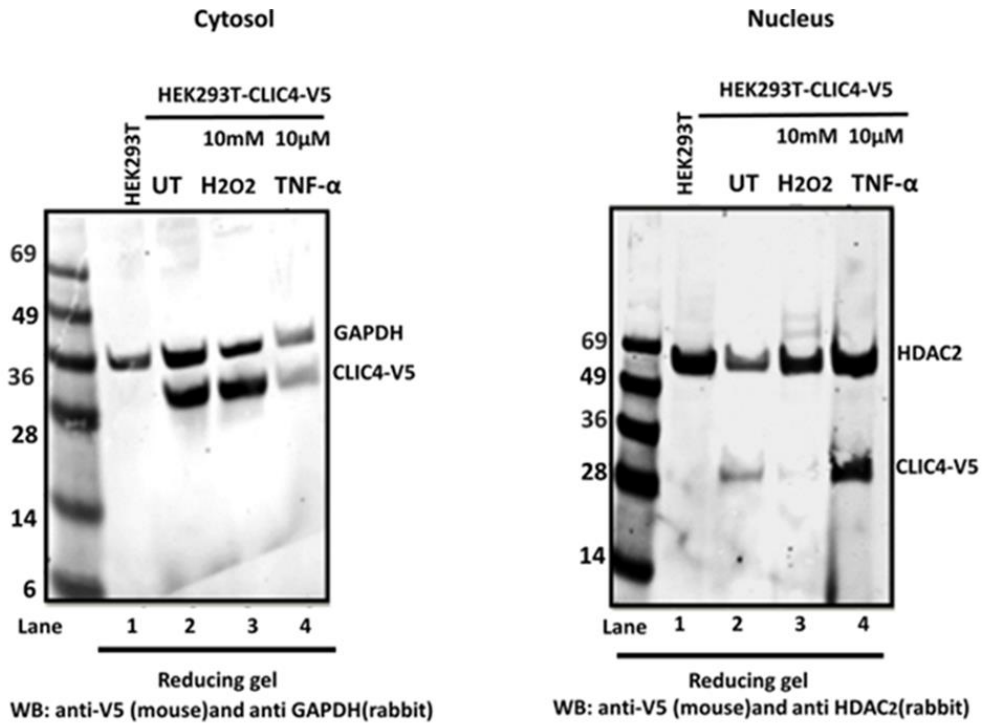


Figure 3-11-C TNF-α induces nuclear translocation of CLIC4 from the cytosol (A) HEK293T cells were transiently transfected with CLIC4-V5 construct and were treated with 10μmTNF-α for 15 minutes (Shanmugam, et al., 2016) and 5mM H2O2 for 10 minutes (Cao Z, et al., 2013) (Figure3-10, B, lane 4). We used the approach described for TNF-α concentration to detect the nuclear translocation (Li H, et al., 2015). Following isolation of nucleus and cytosol through the same purification pathway at the same time as described in the last experiment, cytosolic and nucleus fractions were resolved on the reduced SDS-PAGE and visualized with anti-V5, anti-HDAC2, and GAPDH, respectively. In the cytosolic fraction, the lowest expressed species had a relative stress-induced corresponding to TNF-α (Left panel, lane 4). The two highest and similar expressed species had a relative to un-treated and oxidized samples, corresponding to normal physiological condition, and oxidative stress induced by H2O2, respectively (Left panel, Lanes 2, and 3). All species were fully reduced when DTT (50mM) was added to the samples prior to electrophoresis. In the left panel, the nucleus fraction, the highest expressed species had a relative to stress-induced corresponding to TNF-α (Right panel, lane 4). The lowest expressed specie had relative to untreated and oxidative samples corresponding to normal and H2O2, respectively (Lanes 2, and 3). Mock transfected HEK293T cell lysates were included as a control for transfection efficiency (Left and right panel, lane 1).

The experiments with the high concentration of TNF-α provided us with more information to understand the ability of the redox response of CLIC4 to oxidative stress. Our results indicated that with an increase in TNF-α concentration, a translocation appeared in the cytosol; conversely, a dramatical CLIC4 expression level can arise in the nucleus, suggesting that TNF-α can initiate a signaling transduction pathway after binding with two receptors and leading to the activation of the redox signaling mediators (Li Z, et al., 2016).

Excessive activation of TNF-α signaling is associated with the concentration of TNF-α and led to reducing cellular GSH levels that can eventually proceed to the development of intermediate factors such as TGF-beta (Liu R.M, et al., 2021). There is clear that the interrelationship and overlap between the actions of TNF-α and TGF-β1 in human cells; therefore, it is postulated that TNF-α might play a significant role in regulating TGF-β1 expression through activating the extracellular regulated kinase (ERK) resulting in stabilization of TGF-β1 mRNA and increased expression of TGF-β1 (Sullivan DE, et al., 2009). Thus, increasing TGF-β1 expression in mice and consequently suppressing the expression of both catalytic and modifier subunits of glutamate-cysteine ligase (GCL), resulting in decreasing the GSH concentration in mouse lungs (Liu R.M, et al., 2021). Together, the data suggested that the high concentration of TNF-α can activate a specific extracellular kinase to promote the TGF-β1 expression, and consequently reduction of GSH, driving nuclear

CLIC4 localization and again binding to TGF-beta for renewing this pathway via phosphor-Smad 2 and 3. The nuclear localization of CLIC4 under TGF-beta induction was associated with and dissociated thereafter (Shukla A and Yuspa S.H, 2010), (Figure 3-12). Hence, our result can confirm the nuclear translocation of CLIC4 in response to oxidative stress upon TNF-alpha induction in the mammalian cells.

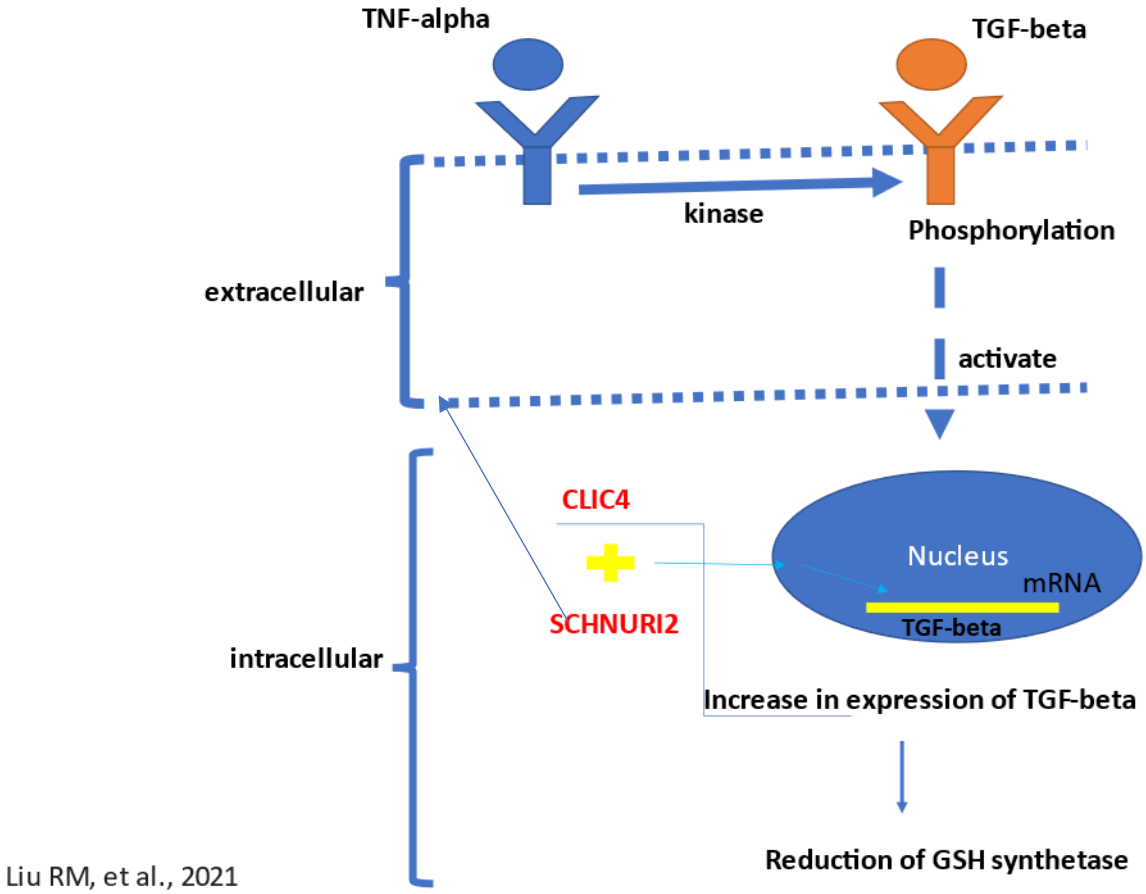


Figure 3-12 Schematic signaling pathways of transduction of CLIC4 to the nucleus: Excessive activation of TNF-α signaling is associated with the concentration of TNF-α led to reducing cellular GSH levels and can eventually proceed to the development of intermediate factors such as TGF-beta (Liu R.M, et al., 2021). There are clear interrelationships and overlap between the actions of TNF-α and TGF-β1 in human cells, therefore, we postulated that TNF-α might play a significant role in regulating TGF-β1 expression through activating the extracellular regulated kinase (ERK) resulting in stabilization of TGF-β1 mRNA and increased expression of TGF-β1 (Sullivan DE, et al., 2009). Thus, increasing TGF-β1 expression in mice and consequently suppressing the expression of both catalytic and modifier subunits of glutamate-cysteine ligase (GCL), decreased GSH concentration in mouse lungs (Liu RM, et al., 2021). However, accumulation of CLIC4 in the nucleus makes it the best substrate for CDK5 to induce apoptotic pathways via p53 (Shu KS, et al., 2005).

3.2.6 CLIC4 responses to TNF-α-induced the oxidative stress by relocalising from the cytosol into the ER.

An important part of my project will be determining the relocalization of CLIC4 to intracellular organelles in response to the alteration of redox balance, which is induced by oxidative stress within mammalian cells. To evaluate the process of relocalization of CLIC4, we need to determine the optimal concentration of the oxidative stress inducers, which are considered as main factor for relocalization. In previous experiment we indicated that the nuclear translocation of CLIC4 from the cytosol in a high concentration of TNF-α. In this experiment to establish an assay for determining the optimal concentration of oxidative reagents to induce relocalization of CLIC4 from the cytosol into the ER, we utilized two concentrations of oxidative stress inducers to compare which of them capable to induce relocalization of CLIC4 from the cytosol into the ER (Figure 3-12, A and B).

(A)

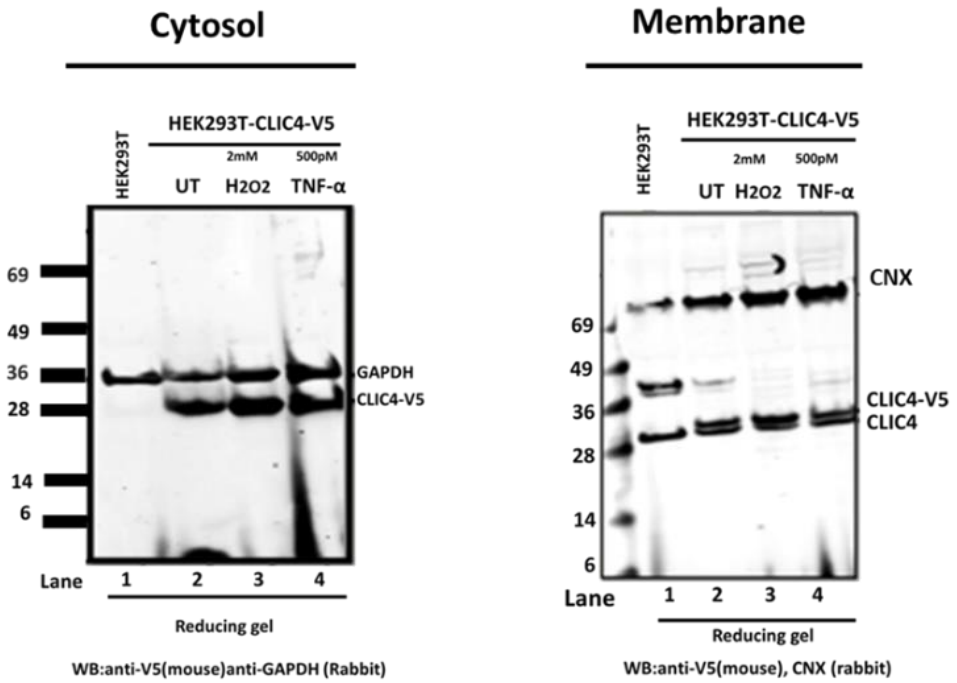


Figure 3-13-A TNF-α concentration play a significant role on CLIC4 translocation into the ER. HEK293T cells expressing CLIC4-V5 either left as an untreated sample (lane1) or treated with 2mM H_2O_2 for 60 min and 500pM TNF-α for 15 min (lanes 3 and 4). Following treatment, cells were washed with PBS supplemented with an EDTA free protease inhibitor tablet and centrifuged for 3 min at 300 rpm; then, cells were resuspended in a 1ml ice-cold buffer A for 10 min. Cells were lysed by passing through 1ml syringe, 30 times. The remaining sample was spun at 1000g, for 7 min at 4 °C. Pellet was a nuclear fraction; supernatant contains cytosol and membrane. The supernatant was spun at 100,000g for 30 min at 4°C. The supernatant was

removed to a fresh 1.5ml tube that was the cytosol and precipitated with ice-cold acetone (5x volume) and the cytosolic fraction was resuspended in 100ul specific cytosolic buffer in the presence of protease inhibitor and analysed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and anti-GAPDH (lanes 1–4, left panel). The pellet from the last ultracentrifuges was the membrane fraction and resuspended in 100ul specific membrane buffer in the presence of protease inhibitor and analysed by reducing SDS-PAGE and western blotting (WB) with anti-CNX antibody (top) or anti-V5(mouse) antibody to recognize the exogenous CLIC4-V5 and anti-CLIC4 (rabbit) antibody to detect the endogenous CLIC4.

Our result indicated that CLIC4 in response to oxidative stress induced by (2mM) H_2O_2 for 60 min and (500pM) TNF-α for 15 min was expressed at the same level as before and after treatment with the low concentration of oxidative stress inducers, no evidence of either depletion from the cytosol or ER translocation observed (3-13-A), suggesting the concentration of both H_2O_2 and TNF-α were not sufficient to make a significant redox change in the cells to trigger the level of CLIC4 expression or translocation in response to oxidative stress. Hence, in the next experiment, we need to examine a higher concentration of oxidative stress inducers with respect to restriction incubation time for H_2O_2 (Figure 3-13-B).

(B)

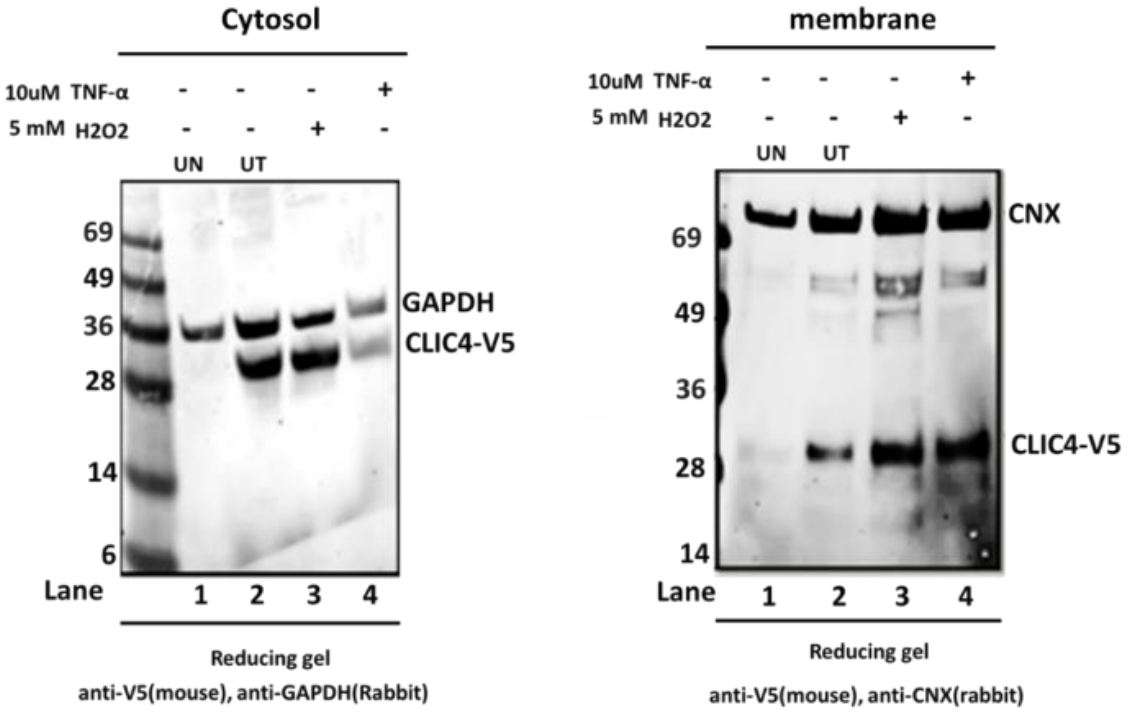


Figure 3-13-B CLIC4 in response to TNF-α by relocalising into the ER from the cytosol. HEK293T cells transiently transfected with CLIC4-V5 construct were either left as an untreated sample (Left and right panels, lane2) or treated with 5mM H_2O_2 for 10 min and 10uM TNF-α for 15 min (Lanes 3, and 4), respectively.

Following treatment and prior to cell lysis, cytosolic fractions were isolated from HEK293T cells as described above, and then the pellet was resuspended in (100ul) of the cytosolic buffer. The lysates were resolved by reducing SDS-PAGE (Left panel lanes 1–4) and western blotting (WB) with anti-GAPDH (top) or anti-V5 (bottom) antibodies. In the (Right panel), the pellet from the last ultracentrifuges was the membrane fraction and resuspended in 100ul specific membrane buffer in the presence of protease inhibitor tablet and analysed by reducing SDS-PAGE (Lanes 1–4), and western blotting (WB). The expression and the ER membrane localization of CLIC4-V5 were visualized with anti-CNX antibody (top) or anti-V5 antibody (bottom) before (Lane2) and after treatment (lanes 3, and 4). One Sample neither transfected nor treated are shown in (Left and right panels, lane1) as a control for transfection efficiency. All species were fully reduced with (50mM) DTT.

To optimize the efficient concentration of H_2O_2 and TNF-α to induce CLIC4 translocation from the cytosol into the ER, we tested a high concentration of H_2O_2 and TNF-α in mammalian cells. The transiently transfected cells were either treated with (10uM) TNF-α for 15 min (Xue X, et al., 2005) and (5mM) H_2O_2 for 10 min (Cao et al., 2013) or left untreated. Following treatment, the fractionation of cytosol and membrane were isolated as described above, and samples were resolved on the SDS-PAGE (Figure 3-13-B) and visualized with anti-GAPDH, anti-CNX, and anti-V5 to detect cytosolic, ER membrane CLIC4-V5.

Under the high concentration of oxidative stress inducers, the CLIC4 expression level in response to TNF-α induction was dramatically increased in the ER membrane (Right panel, lane 4), and conversely, a translocation of cytosolic CLIC4 under TNF-α treatment (Left panel, lane 4), suggesting TNF-α concentration was sufficient to induce re-localization of CLIC4 from the cytosol into the ER. While species after and before treatment with (5mM) of H_2O_2 were expressed at the same level in the cytosol (Left panel, lanes 2, and 3), and no cytosolic mislocalization was observed (Left panel, lanes 2, and 3), suggesting TNF-α particularly capable to induces CLIC4 re-localization from the cytosol into the ER membrane. However, although translocation and insertion of CLIC4 was asigned in 1997 by Duncun through N-terminal domaine (Figure 3-14), there is no evidence until now for this translocation in response to oxidative stress. Our result for the first time indicate relocalization of CLIC4 from the cytosol into the ER in respons to TNF-α, suggesting CLIC4 could be had a functional role in the ER.

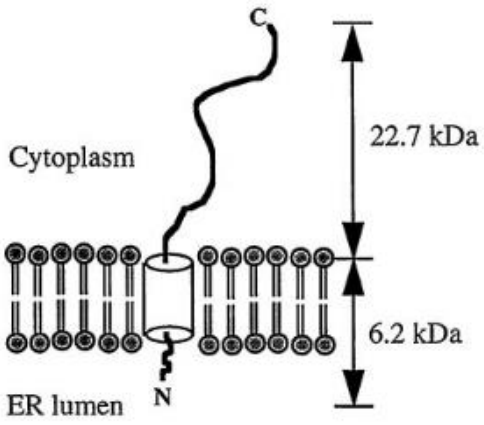


Figure 3-14 Schematic illustration showing the predicted membrane topology of CLIC4. The localization and insertion of CLIC4 into the ER membrane through N-terminal domain that included ER luminal and membrane domains with 6.2 KDa, and a cytoplasmic domain with 22.7KDa that remine in the cytosol (Duncan RR, et al., 1997).

The functional role of CLIC4 in the ER compartment was indicated in the Knockdown CLIC4 (Xue H, et al., 2016). By downing regulation of CLIC4 expression through siRNA, the apoptotic ER pathway was appeared via increasing the CHOP protein, cleavage of caspase 4, and intermediating ATF-4 in mammalian ER (Chen Y, et al., 2013). Furthermore, inhibition of expression of CLIC4 during starvation can be contributed to the ER stress (Zhong J, et al., 2012), suggesting CLIC4 could be had a crucial role in the ER of mammalian cells (Figure 3-15).

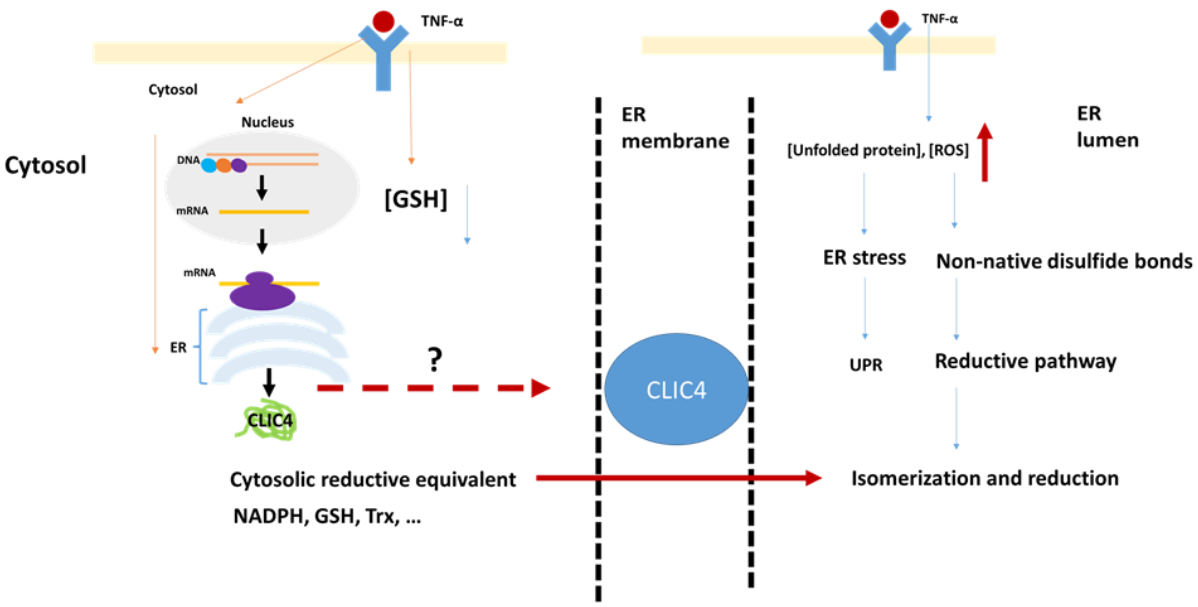


Figure 3-15 TNF-α is responsible for inducing the reduction of cellular GSH level. The schematic representation indicated that TNF-α induces the reduction of cellular GSH, which might be cause to ROS accumulation and accumulation unfolded and mis-folded proteins in the ER. The accumulation of unfolded proteins in the endoplasmic reticulum (ER) causes to ER overload, resulting in ER stress (Xue x et al 2005). To cope with ER stress, mammalian cells trigger a specific response known as the unfolded protein response (UPR) (Cubero F, et al., 2008). The accumulation ROS can be resulted from mis-folded proteins via non-native disulfide bonds formation (Zhang Z, et al., 2019). Hence, the reductive pathway is required for removing the mis-folded proteins in the mammalian cells, respectively (Lindholm D, et al., 2017).

Our result indicated that CLIC4 in response to TNF-α induced-oxidative stress translocated to the ER from the cytosol, suggesting this translocation dependent on alteration of redox balance (GSSG/GSH) in the mammalian cells. Hence, in the next experiment, to investigate the main reason for this re-localization, we first need to examine the redox status of CLIC4 in the intact cells and SP cells.

3.2.7 CLIC4 is retained in a Reduced State in intact and SPcells

To assess the requirement for a reductive pathway, we first examined the redox state of CLIC4 within mammalian cells grown in culture, which is an important aspect of this project. To evaluate the process of reduction we will need to determine the redox status of the two CLICs family members (CLIC1 and CLIC4) which are most likely to be involved in reductive pathway. It has previously been demonstrated that CLIC4, rather than ion channel function, is an oxidoreductase enzyme with isomerization and reduction ability to catalyze disulfide bonds and monitor its ion channel activity (Alkhameci H, et al., 2015).

To establish an assay for determining the redox status of CLIC4, we were required to reproduce work carried out previously (Oka O, et al., 2013). To evaluate the redox state under physiological conditions, we treated intact cells with the membrane permeable alkylating agent NEM to prevent disulfide exchange and freeze redox status by providing caps on free thiols. We then treated cell lysates with a second larger alkylating agent AMS that caused a shift in mobility when the protein is separated by SDS-PAGE. NEM alkylation prevented the modification of free thiols (-SH). The second alkylation step was carried out with AMS in the presence of a reducing agent (TCEP); therefore, proteins that contained disulfide bonds when present in the intact cells showed a fall asleep electrophoretic migration.

When the redox state of two members of CLICs family (CLIC1 and CLIC4) was assessed using this approach, a clear decrease in mobility was seen for the proteins from intact cells treated with the oxidizing agent (TNF-α) in comparison to cells treated with the reducing agent DTT (Figure 3-16-A, lanes 2, and 3). In untreated or normal physiological conditions, when no reducing or oxidizing agent was added to cells, both CLIC1 and CLIC4 migrated with mobility of the reduced protein (Figure 3-16-A, lane 1), demonstrating that they are reduced and placed in a steady state position. This is important to know although the ER lumen is characterized by the disulfide formation in secretory proteins to enter the secretory pathway in mammalian cells (Oka O, et al., 2013), this is a requirement that the active site of the ER oxidoreductase partially or completely be reduced. The reduced form provides the activated form for an oxidoreductase to be functional.

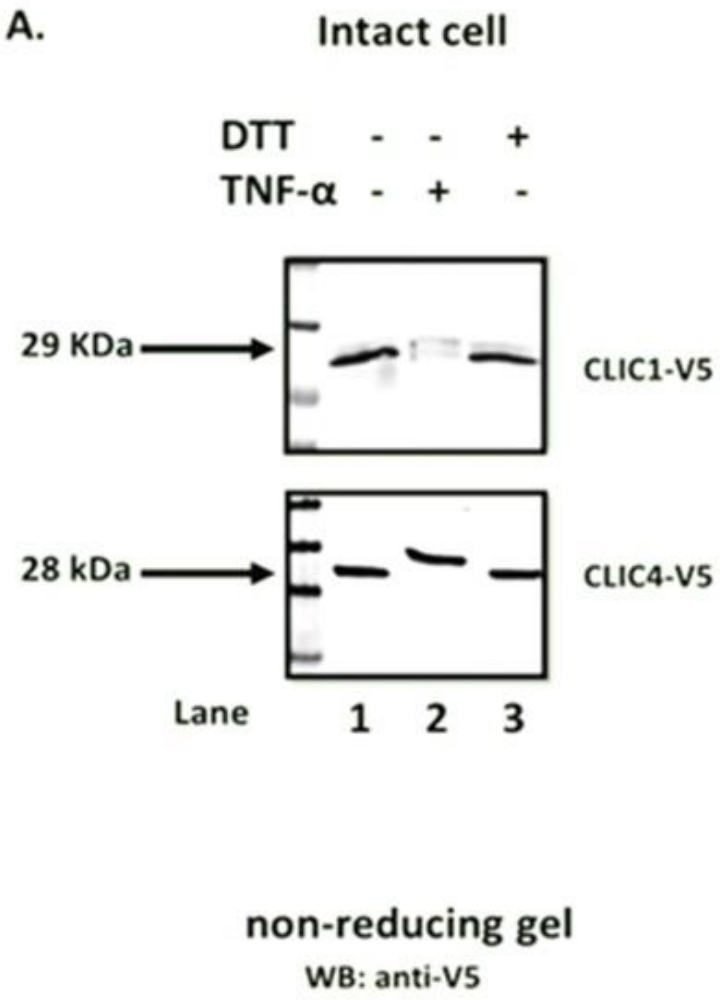


Figure 3-16-A CLIC4 predominantly is located in a reduced state and quickly recovered from oxidative stress induced by TNF-α. HEK293T cells (a human embryonic kidney cell line) in suspension were either left untreated (lane 1) or treated with TNF-α (10uM) (lane 2) or DTT (1mM) (lane 3). The samples were subjected

to modification with AMS to increase the mass in the oxidized samples, SDS-PAGE, and western blots probed with antibody to anti-V5. Oxidized species appeared a decrease in migration or electrophoresis shift.

To address the redox state of CLIC4 in the intact cells (in vivo condition), the intact cells were untreated (UT), treated with DTT for reducing or TNF-α for oxidizing samples at room temperature. The samples were then treated with (25 mM) NEM to block free thiols followed by 2 times PBS wash to remove excess NEM. Redox state of CLIC4 was determined as above (Figure 3-16-A). Samples were then separated by SDS-PAGE and visualized by western blot. Two bands for CLIC1 and one single band for CLIC4 were observed in all three samples no matter if the sample was untreated, oxidized or reduced (Figure 3-16-A, lanes 1-3).

For CLIC4 to act as an oxidoreductase, its active site must be completely or partially reduced as well. Such a reduced state or activated form would be required if considered CLIC4 is involved in disulfide bond reduction or isomerization. Even if CLIC4 is presented as an ion channel in the ER membrane for transferring of cytosolic equivalent, assuming must be reduced for transferring or isomerization. To recover from extra oxidizing agents and re-established free thiols, the presence of a reductive pathway should be considered as a requirement to enable CLIC4 to recover from the addition of oxidative stress inducer (TNF-α).

For CLIC1, in untreated intact cells, two bands were observed: an upper band and a lower band (Figure 3-16-B, Lane 1, upper panel). The lower band migrated with the band in sample reduced by DTT (Figure 3-16-B, lanes, 1 and 3, upper panel) indicating reduced form of CLIC1 and the upper band migrated at the same position with the band in oxidized sample (Figure 3-16-B, lane 3) indicating oxidized form of CLIC1. This result suggested CLIC1 can form disulfide bond between the catalytic cysteine (Cys24) within its redox motif (CXXS) and a non-catalytic cysteine (Cys 59) (Littler R.D et al., 2010) under redox condition (Figure 3-16-B).

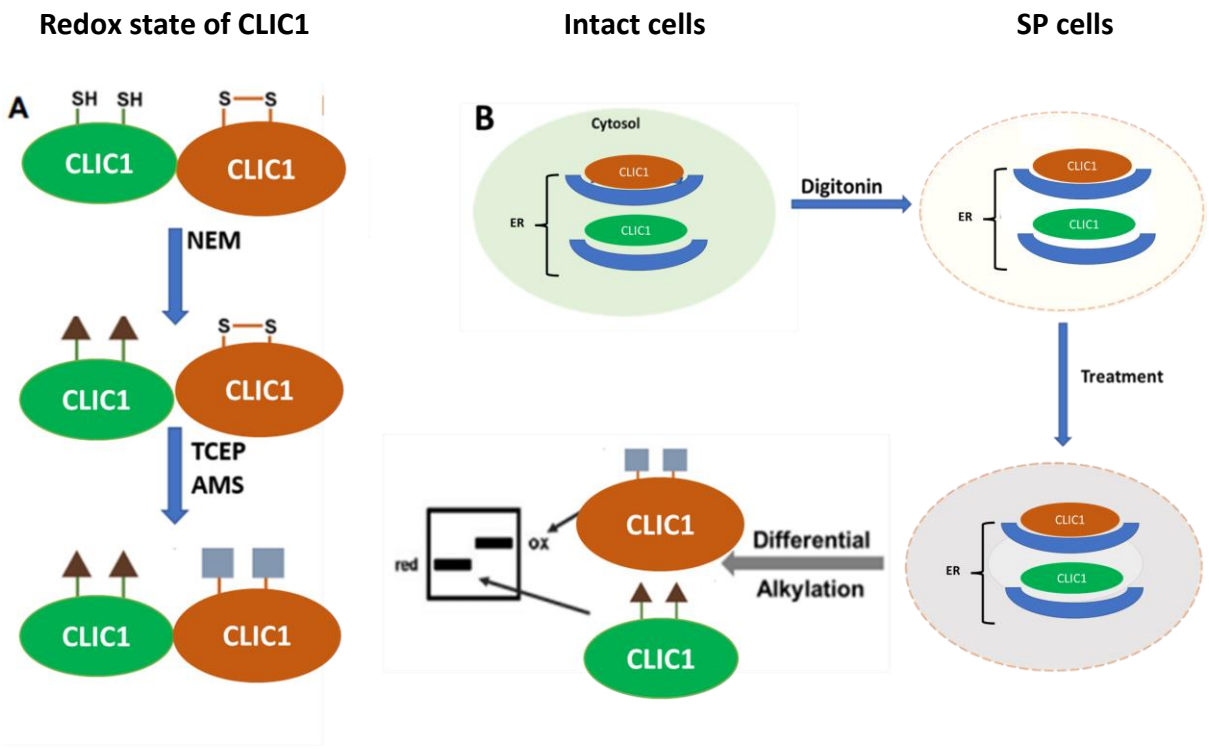


Figure 3-16-B Determining the redox state of CLIC1. A. Schematic diagram of the protocol for determining the redox state of CLIC1. In intact or SP cells, there are two forms of CLIC1, reduced form (green oval) and oxidized form (orange oval). The free thiols in reduced CLIC4 will be blocked by alkylating agent NEM (molecular weight: 125.13) (brown triangle). After reduction by the reducing agent TCEP, existing disulfides in oxidized CLIC1 were broken followed by the alkylation by another alkylating agent AMS (molecular weight: 466.4) (blue square) which has larger molecular weight than NEM (that is why the reason of differentiation of mobility in the redox western blotting). Different redox states of CLIC1 will be identified by the electrophoresis mobility shift on gel because of the different molecular weight between NEM and AMS. B. Schematic diagram of determination of CLIC1 redox state in SP cells. SP cells were prepared from confluent flask of HEK293T cells. SP cells were untreated or treated with diamide or DTT in KHM buffer. Redox state of CLIC1 was then determined by differential alkylation which can be visualized on SDS-PAGE gel by the different mobility. Blue squares indicate alkylating agent AMS; brown triangles indicate smaller alkylating agent NEM. Note: In the case of CLIC4, CLIC4 only included one cysteine in its active site with (CXXS) redox motif and not able to make disulfide bands (oxidized and reduced), thus the only one band observed in the western blotting that we can compare with reduced or untreated band because observed the same migration on SDS-PAGE.

The result above indicated that two CLICs family members, CLIC1 and CLIC4, under redox conditions, appeared in a reduced position in the intact cells. CLIC4 is able to recover from addition of TNF-α to regenerate free thiols (-SH), suggesting a strong reductive pathway exists to recover and maintain CLIC4 in a reduced or activated state. In addition, we illustrated that CLIC4 appeared a re-localization from the cytosol to the ER in response to TNF-α induced oxidative stress. These results emphasize that although the ER lumen is

considered for the formation of disulfide bonds in secretory proteins for entry to secretory pathway in the mammalian cells, the active site of CLIC4 is either partially or completely reduced, suggesting CLIC4 placed in a reduced position as a soluble form with oxidoreductase activity and could be involved in disulfide bond reduction or isomerization to eliminate the oxidative stress effect.

C. **SP Cell**

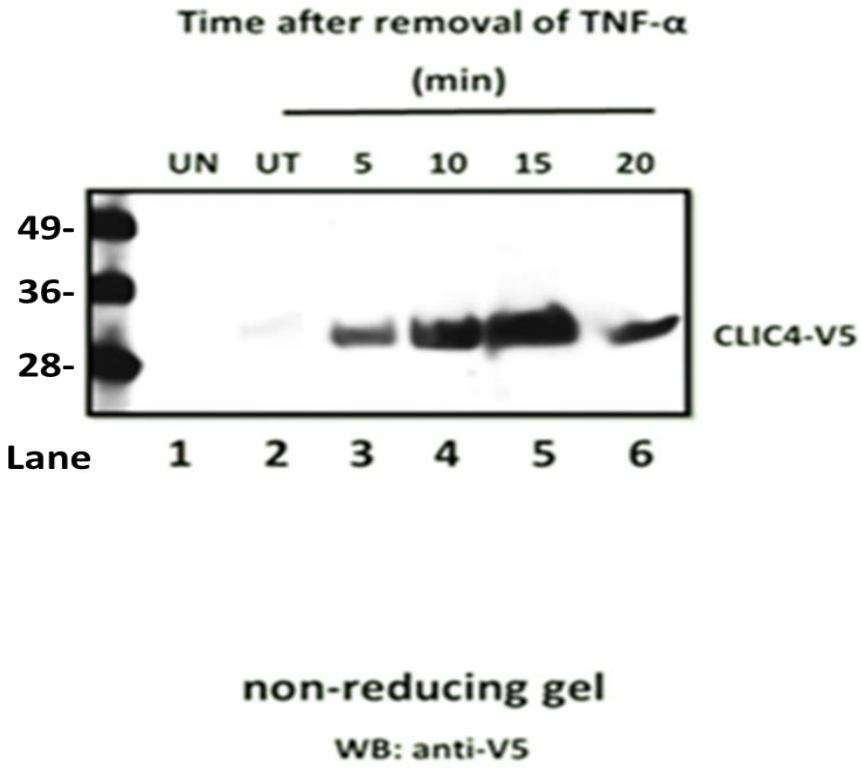


Figure 3-16-C reduction of CLIC4 in SP cells following oxidative stress. HEK293T cells were either left untreated (lane 2) or treated with TNF-α (10uM) for 5 min (lane3-6). Un transfected HEK293T cell lysates were included as a control (lanes 1), TNF-α was removed with fresh medium, and then incubated at 37 ℃ at various time points following oxidation, the redox state of CLIC4 was determined by alkylation with AMS and western blotting with an anti-V5 antibody.

To investigate this hypothesis “the recovery of CLIC4 after removal of TNF-α can occur owning to the existence of a reductive pathway in mammalian cells”, we treat HEK293Tcells that transiently transfected with CLIC4-V5 with TNF-α and detect the redox state of CLIC4 after removal of TNF-α by resuspending in the fresh medium in several times point, and then prepare SP cells as a source of the ER (Wilson R, et al., 1995). (Figure 3-16-C, lane 1-

6), CLIC4 was recovered 20 min following removal of TNF-α induced oxidative stress, demonstrating that in SP cells as a source of the ER, a vigorous reductive pathway exists to recover and maintain CLIC4 in a reduced state. Moreover, reduced state of CLIC4 is an activated position to be able to work as an oxidoreductase enzyme (Alkhameci H, et al., 2015) for reduction and isomerisation of non-native disulfide bonds in the ER to cope with oxidative stress induced by TNF-α. These results indicated the possibility of existence of a robust reductive pathway in the mammalian cells to maintain CLIC1 and CLIC4 in a reduced state. To confirm CLIC4 predominantly in placed in a reduced state, we applied a mass spectrometry to analysis the redox state of CLIC4.

3.2.8 Mass Spectrometry aid to identify site and abundance of CLIC4 cysteine residues in mammalian cells

In the last experiment we indicated that CLIC4 in response to oxidative stress after removal of TNF-α immediately oxidized and then reduced, demonstrating that there is a reductive pathway in the mammalian cells to maintain CLIC4 in a reduced state. To confirm the redox state of CLIC4 in the mammalian ER, there is a requirement to apply the mass spectrometry (MS/MS) to provide us with more information about site, abundance, and redox state of cysteine residues of CLIC4 in mammalian cells (Figure 3-17). To identify the cysteine sites within CLIC4 protein, after purification of CLIC4 from mammalian cells through immunoprecipitation with anti-V5 agarose beads antibody (Cao X, et al., 2019), and Coomassie blue staining, the mass spect experiments was carried out upon the mass-to-charge ratio (m/z) of molecules that are present within a sample (Tiedge, M, et al., 2014). To determine the relative abundance, the samples need to be ionized to generate a mass spectrum peak with respect to mass-to-charge ratio (m/z), and to detect a efficiency coverage of cysteine residues a correct amount of loading peptide for fragmentation of each peptide need to be applied. The mass spectrometry analysis was carried out at St. Andrew University.

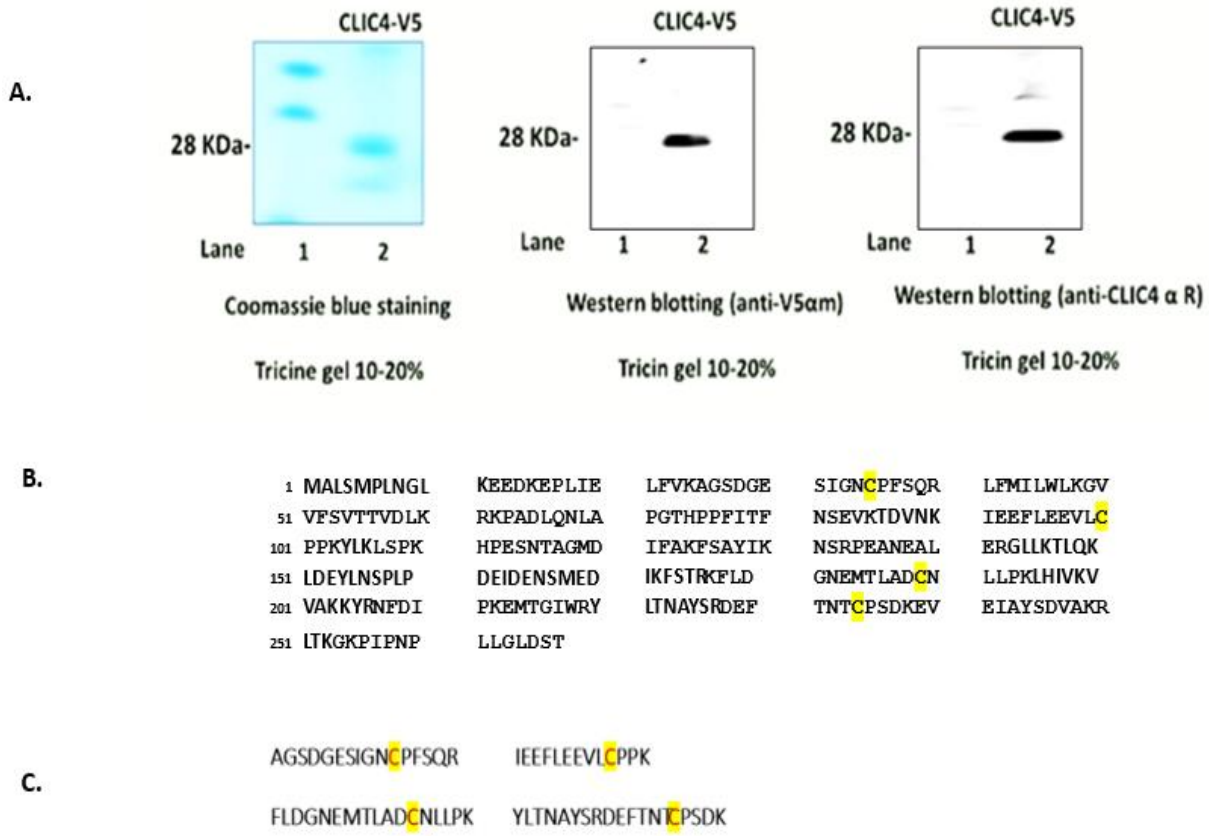


Figure 3-17 Mass Spectrometry analysis of CLIC4 redox state in mammalian cells **A**. To purify CLIC4, HEK293T cell lines which stably overexpress CLIC4-V5, were first precleared by incubation with 50ul of 10% Protein A Sepharose (PAS) for 30 minutes at 4°C, before incubation with anti-V5-conjugated agarose beads for 16 hours at 4°C. The beads were washed three times with lysis buffer supplemented with 0.5% SDS and then incubated with (10mM) DTT for 5 minutes to elute the cross-linked complexes. Then samples were boiled for 5 min to separate the immunocomplex from the beads and centrifuged at 13,000 × g for 1 min at 4°C to recover sample from beads and run on the gel for Coomassie blue staining **(I)** and CLIC4 was visualized with western blotting by subjecting to both anti-V5 and anti-CLIC4 antibodies **(II and III)** to find a right place of CLIC4 for gel extraction. **B**. To determine the abundance of cysteine residue sites, CLIC4 exposed to the mass spectrometry to analyse sample on ESI instrument and searched the data through BMS internal database and NCBI. The cysteines to be potentially modified with either 4-vinylpyridine (4VP), N-ethylmaleimide (NEM), or IAA (carbamidomethyl) in the database search for identify reduced and oxidized modified cysteine, respectively. **C**. The analysis identified 4 peptides of CLIC4 as being presented 4 cysteine residues separately in each peptide and this can be considered as an advantage for CLIC4 to mass spectrometry analysis to avoid any disulfide bridge between cysteine residues.

To determine the redox state of CLIC4 via mass spectrometry, there is a requirement to identify the cysteine sites of CLIC4 upon NCBI protein sequencing as a control sample. The results indicated that 4 cysteines are all in regions of sequence that produced peptides that were in the mass spec detectable range. They are: AGSDGESIGN**C**PFSQR, IEEFLEEVL**C**PPK,

FLDGNEMTLAD**C**NLLPK, and YLTNAYSRDEFTNT**C**PSDK. It is obvious that this peptide arrangement provides a great advantage for CLIC4, which can be detected in all 4 cysteines without having to use the alternative enzymes to trypsin (Samodova D, et al., 2020).

To prepare the samples within the standard mass spec protocols, following a gentle alkylation with (4-Vinyl Pyridine) 4VP, the protein samples always were reduced with DTT and alkylated with IAA so that all cysteines are 'capped' and not free and therefore not able to form disulfide bridges with other free cysteines. This vital step was performed for two main reasons. The first, to help denature the protein as the trypsin enzyme can access as much of the structure and digest it into peptides, and secondly, no disulfide bridged 'crosslinked' peptides exist, as these do not sequence simply in the mass spec (Woods A.G, et al., 2012).

To address the redox state of cysteine residues of CLIC4, the modification of cysteines is the essential step. Cysteine (Cys) is a major target for redox post-translational modifications (PTMs) that occur in response to redox changes in the cellular environment. upon the potential variable modifications on the cysteine through modifications/mass additions, the abundance and correct place for cysteine can be found, e.g., +57 as an extra mass for the carbamidomethyl formed by alkylating with IAA when we search the data for oxidized cysteine (Rookyard A, et al., 2020). Our results indicated that CLIC4 detected with 4 peptide fragmentation by full coverage of 4 individual cysteines without any cross-link between each other, suggesting we can identify the redox state with full coverage of four cysteine residues (Figure 3-17, A, B, C and D).

1	**MALSMPLNGL**	**KEEDKEPLIE**	**LFVKAGSDGE**	**SIGNCPFSQR**	**LFMILWLKGV**
51	**VFSVTTVDLK**	**RKPADLQNLA**	**PGTHPPFITF**	**NSEVKTDVNK**	**IEEFLEEVLC**
101	**PPKYLKLSPK**	**HPESNTAGMD**	**IFAKFSAYIK**	**NSRPEANEAL**	**ERGLLKTLQK**
151	**LDEYLNSPLP**	**DEIDENSMED**	**IKFSTRKFLD**	**GNEMTLADCN**	**LLPKLHIVKV**
201	**VAKKYRNFDI**	**PKEMTGIWRY**	**LTNAYSRDEF**	**TNTCPSDKEV**	**EIAYSDVAKR**
251	**LTKGKPIPNP**	**LLGLDST**			

Figure 3-17 A coverage of peptides.

B.

AGSDGESIGNCPFSQR

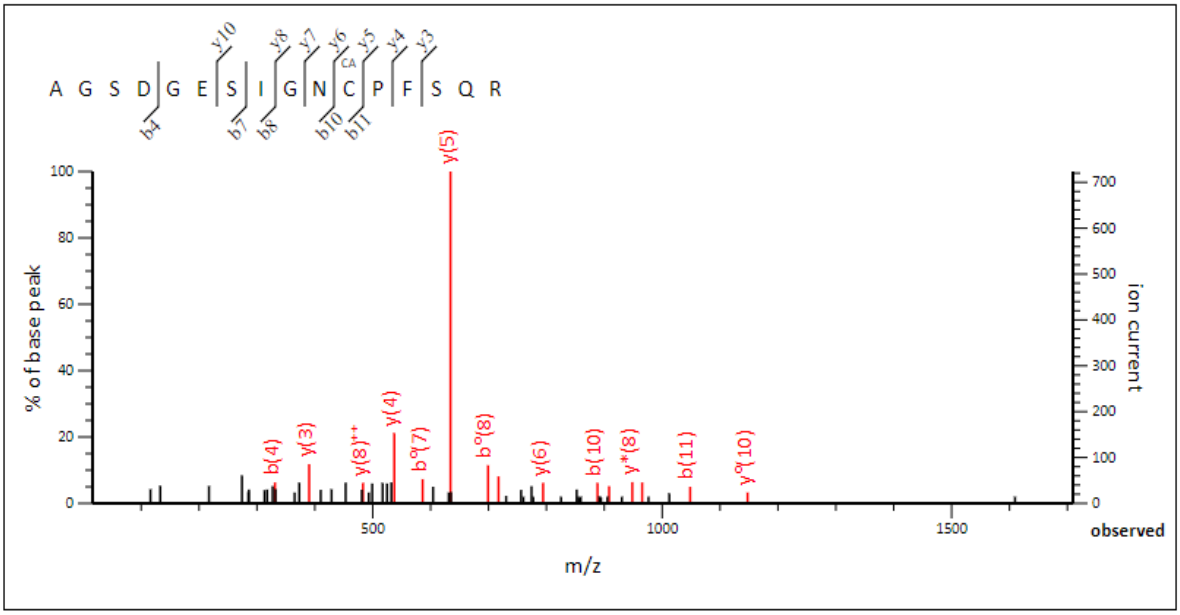


Figure 3-17-B1 Mass spectrometry analysis of control sample indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (lysine) **K**. via ion chromatogram peaks and identify the modified cysteine 35 via variable modification: Carbamidomethyl (C), ions score:39 and Expect:0.00012, matches: 15/154 fragmentations and using 21 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS.

IEEFLEEVLCPPK

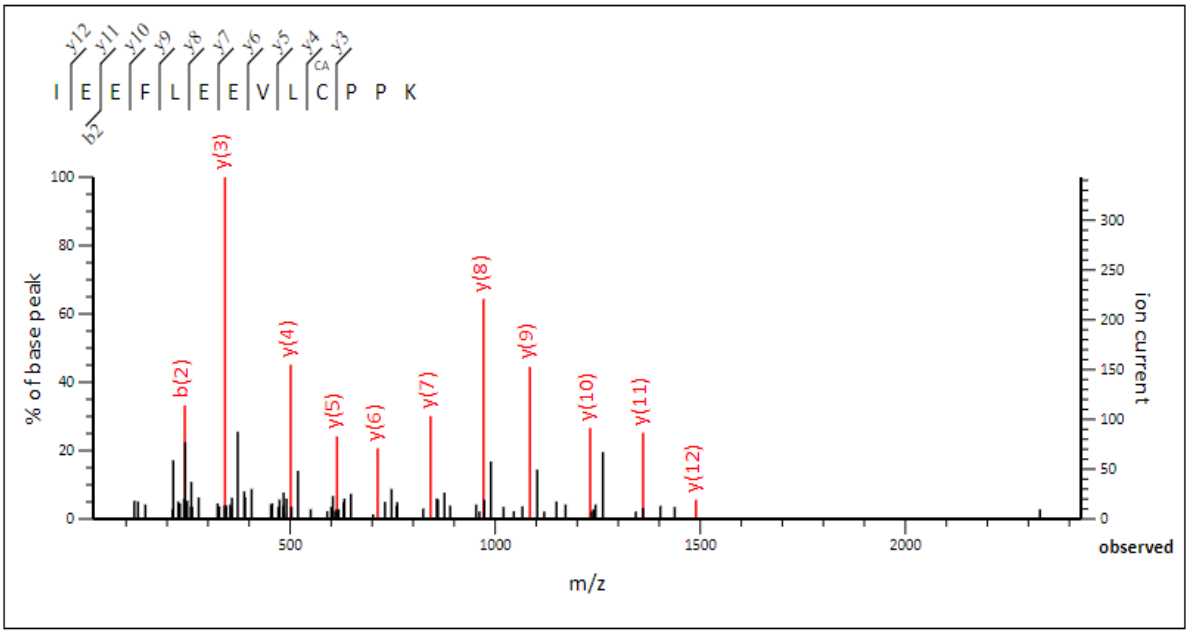


Figure 3-17-B2 Mass spectrometry analysis of control sample indicating the MS/MS fragmentation IEEFLEEVLCPPK following trypsin digestion from (lysine) **K**. via ion chromatogram peaks and identify the modified cysteine 100 via variable modification: Carbamidomethyl (C), ions score:93 and Expect:2.2e-09, matches: 15/106 fragmentations and using 14 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS.

KFLDGNEMTLADCNLLPK

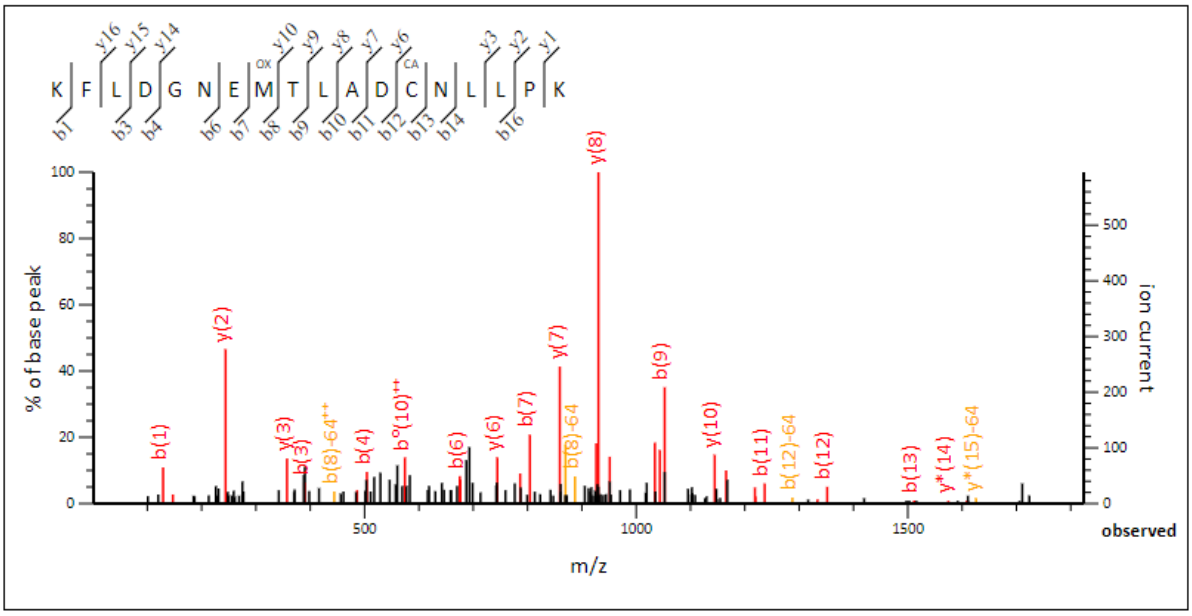


Figure 3-17-B3 Mass spectrometry analysis of control sample indicating the MS/MS fragmentation KFLDGNEMTLADCNLLPK following trypsin digestion from (lysine) **K**. via ion chromatogram peaks and identify the modified cysteine 189 via variable modification: Carbamidomethyl (C), and oxidation of methionine (M),

ions score:74 and Expect:1.2e-07, matches: 40/288 fragmentations and using 64 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS.

DEFTNTCPSDK

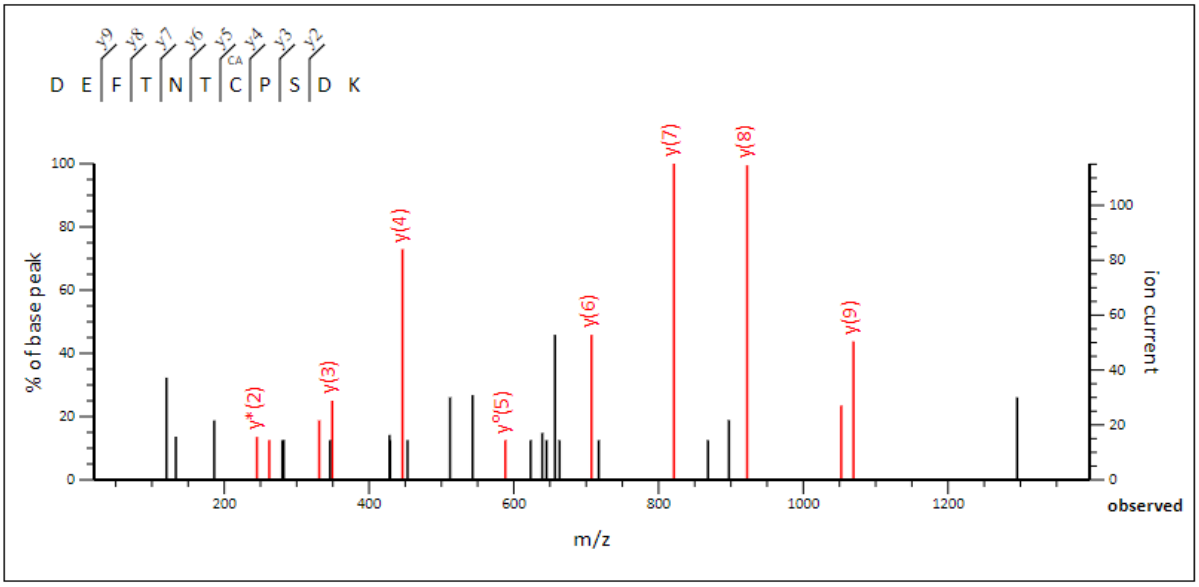


Figure 3-17-B4 Mass spectrometry analysis of control sample indicating the MS/MS fragmentation DEFTNTCPSDK following trypsin digestion from (Arginine) **R**. via ion chromatogram peaks and identify the modified cysteine 234 via variable modification: Carbamidomethyl (C), ions score:53 and Expect:4.5e-06, matches: 13/110 fragmentations and using 18 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS.

C.

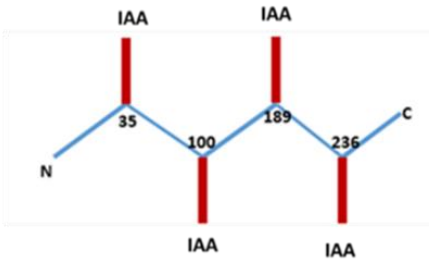


Figure 3-17-C- Mass Spectrometry detects 4 cysteine sites for CLIC4 A. mass spectrometry data is located in-house Mascot Server to identify and characterize CLIC4 from the sequence databases. The data is searched against CLIC4 sequence upon NCBI. The search in the Mascot server set the cysteines to be potentially modified with either 4VP(4-vinilpridin) for reduction or IAA (carbamidomethyl) for oxidation with respect to increase the molecular weight for each modifying alkylation reagent in the database search. The data for

CLIC4 was indicated with 70% protein sequence coverage, which was highlighted with red color. **B.** ion chromatogram upon fragmentation of peptides including AGSDGESIGN**C**PFSQR, IEEFLEEVL**C**PPK, FLDGNEMTLAD**C**NLLPK, and YLTNAYSRDEFTNT**C**PSDK with detecting coverage of 4 cysteines (C35, C100, C189, C234). The analysis identified CLIC4 as being presented with 4 oxidized cysteines. **D**. The cartonice diagram shows the schematic CLIC4 protein with 4 cysteines that all modified with IAA indicating 4 cysteine residues in the absence of NEM alkylation were in placed in oxidized state.

To indicate the redox state of CLIC4, following immunoprecipitation of CLIC4 by anti-V5 agarose beads, and staining with Coomassie blue, the gel bands (in 50% CAN/H_2O) were subjected to be alkylated with 30mM 4VP (4-vinil pyridine) for 10 minutes in the dark at 20 ̊c Room temperature (RT) to modify any free cysteines in the sample. Then the sample was reduced with 10mM (DTT) in 50mM Ammonium Bicarbonate for 1 hours at 60̊C, then cool down in RT. This will break any cysteines in disulfide bonds. Then alkylate with 100mM (IAA) in Ammonium Bicarbonate, which will modify the newly broken cysteines. Then digest the protein into peptides with trypsin (1:50)(w/w) to gel (e.g 2.0ug of trypsin per 100 ug of protein), then incubate overnight at 37̊C. Take off supernatant which now contains peptides. For extracting more peptide, we can use 5% Formic acid and combine with ACN. These peptides were dried with vac speed for 4-6 hours by resuspended in Acetonitrile (ACN) and 1% formic acid to increase the peptides and analyze them on the mass spectrometer. The loading samples (peptides) were 10ul the maximum amount that can be loaded because usually the loading peptide amount is 6ul per well to run a mass spect analysis. This amount of loading peptide can be a requirement for maximum coverage of CLIC4 protein sequencing. The peptides were searched using Mascot 2.4 software against a custom-built database of 5447 protein sequences, including the sequence of CLIC4. Settings included 4VP and IAA modifications as possible modifications of cysteine with respect to increasing the molecular weight. The search identified CLIC4 as a protein with 70% sequence coverage. 4 of the 4 cysteines in the protein sequence, all 4 cysteines were detected by the mass spectrometer and database search. All 4 peptides contained-cysteine were identified as modified with IAA (carbamidomethyl) indicating that these 4 cysteines must have been involved in disulfide crosslinks. The experiment doesn't reveal any information about oxidation but due to being modified with IAA suggesting all cysteine residues were involved with disulfide cross-linked and were in an oxidized position.

In the next experiment to identify the redox state of CLIC4 in the ER compartment, we designed a workfellow to identify the redox state of cysteine residues in several redox conditions in the intact and SP cells.

3.2.9 Evaluation of the redox state of CLIC4 in the mammalian ER

In the previous experiments, we illustrated that CLIC4 predominantly located in a reduced state in mammalian cells, suggesting possibility for existing a reductive pathway to maintain CLIC4 in a reduced form. Furthermore, to evaluate the redox state of CLIC4, the cysteine sites and abundance was indicated via mass spectrometry as a sample control. In this experiment, we have tried to determine the redox state of CLIC4 under several redox conditions in the intact cells and SP cells through AMS modification (Figure 3-19-A, and Figure 3-19-B).

In the mammalian cells, CLIC4 is located in a reduced or oxidized form. To determine the redox state of CLIC4 in physiological condition, HEK293Tcell line with overexpressing CLIC4-V5 was either treated with oxidizing reagents including (5mM) Diamide, (5mM) H_2O_2, (10µM) TNF-α and reducing reagent (5mM) DTT for 5min at 37℃ or left as an untreated sample. After removal of oxidized and reduced reagents with fresh medium, then cells were washed with PBS contained (25mM) N-ethylmaleimide (NEM) for trapping free thiols (-SH). Cells were lysed and subjected to modification with AMS to increase the mass in the oxidized samples, SDS-PAGE, and western blots probed with antibodies to anti-V5. Oxidized species appeared to a decrease in migration or electrophoresis shift (Figure 3-18-A). This experiment repeated for reducing gel via incubating samples with (50mM DTT) (Figure 3-18-B).

A.

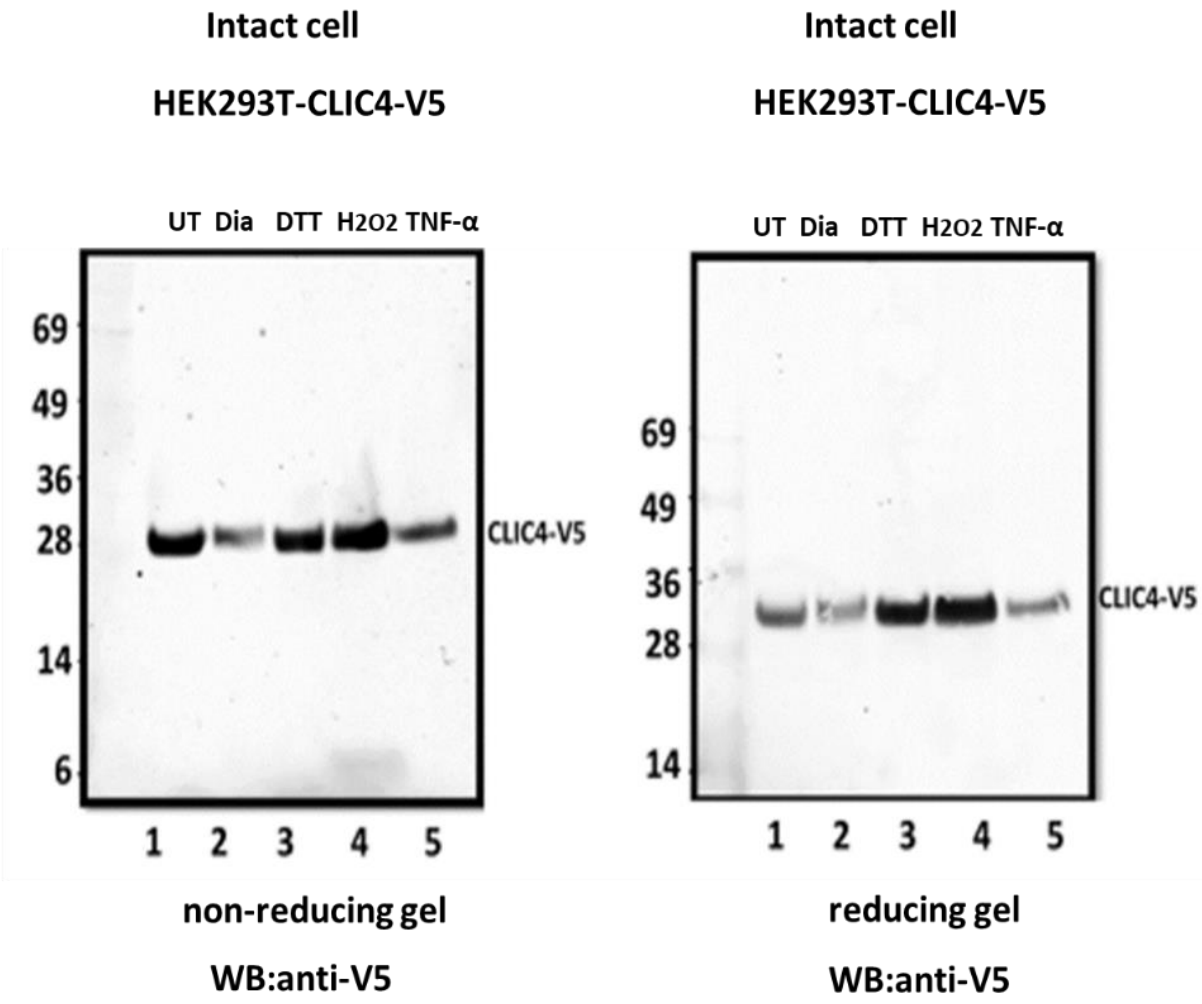


Figure 3-18-A Determination of redox state of CLIC4 under several redox condition in intact cells. The HEK293T cell line which stably overexpresses CLIC4-V5 were either treated with oxidizing reagents including (5mM) Diamide, (5mM) H_2O_2, (10uM) TNF-α, and reducing reagent (5mM) DTT or left as an untreated sample. The incubation time for all reagents was caried out for 5min at 37℃. Following extraction of proteins with exposing to IP-buffer, proteins were subjected to AMS modification to identify redox state by western blotting and proteins were visualized with anti-V5 antibody. The experiment was repeated for reducing gel by incubating samples with (50mM DTT).

Our result indicated that CLIC4 was expressed in several redox state in the mammalian cells. The migration of CLIC4 in untreated and reduced samples (Figure 3-18-A, lanes, 1 and 3) appeared at the same level, suggesting CLIC4 is located in a reduced state. By contrast the oxidized samples under diamide treatment illustrated a slower migration than untreated and reduced samples (Figure 3-18-A, lane 2 left and right panels), indicating oxidized samples were modified with AMS corresponding to the mass increasing. The migration of

samples under treatment of H_2O_2 was not illustrated a difference migration on SDS-PAGE (Figure 3-18-A, lane 4, left and right panels), suggesting the concentration of H_2O_2 was not sufficient to oxidize CLIC4. Samples under treatment of TNF-α were migrated at a different position corresponding to oxidized and reduced samples, demonstrating CLIC4 under TNF-α treatment was placed in an intermediate state (Figure 3-18-A, lane 5, left and right panels), suggesting CLIC4 partially oxidized and partially reduced. The migration of CLIC4 on both non-reducing and reducing conditions appeared at the same levels (3-19-A, left and right panels, lanes 1-5).

These results illustrated that in the normal physiological condition CLIC4 was located in a reduced state, suggesting there is a robust reductive pathway in the mammalian cells to maintain CLIC4 in a reduced state.

B.

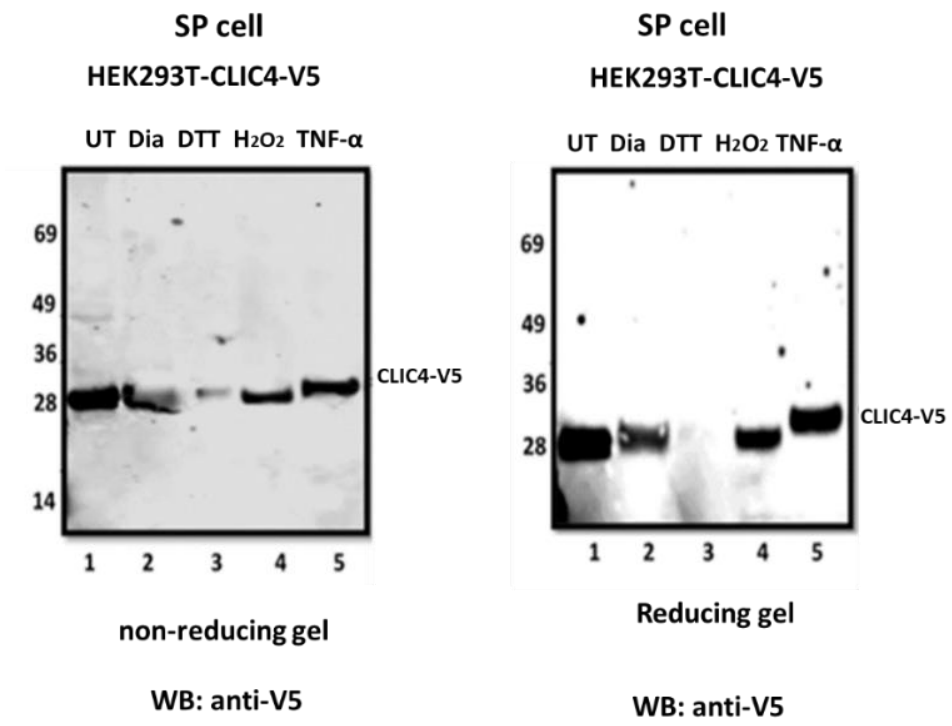


Figure 3-18-B Determination the redox state of CLIC4 under several redox condition in SP cells. The HEK293T cell lines which stably overexpresses CLIC4-V5 were either treated with oxidizing reagents including (5mM) Diamide, (5mM) H_2O_2, (10uM) TNF-α, and reducing reagent (5mM) DTT or left as an untreated sample. The incubation time for all reagents was caried out for 5min at 37℃. Following extraction of proteins with exposing to IP-buffer, proteins either were subjected to AMS alkylation to identify redox status of CLIC4 by western blotting and visualizing with anti-V5 antibody or purified through anti-V5 agarose beads for mass spectrometry analysis. The western blotting experiment was repeated for reducing gel by incubation samples with (50mM) DTT.

In the previous experiment we showed the redox state of CLIC4 in the intact cells. Our result indicated that CLIC4 in the normal physiological condition was placed in a reduced state and migrated with a shift electrophoresis speed same as reduced samples, suggesting there is a reductive pathway in the mammalian cells to maintained CLIC4 in a reduced state. In the next experiment, we need to determine the redox state of CLIC4 in the ER to compare with intact cells before subjecting to mass spectrometry to identify the oxidized and reduced cysteine residues. Hence, HEK293T-CLIC4-V5 cell lines were either treated with oxidizing reagents including (5mM) Diamide, (5mM) H_2O_2, (10µM) TNF-α, and reducing reagent (5mM) DTT or left as an untreated sample. The incubation time for all reagents was caried out for 5min at 37℃. Then after removal of oxidizing and reducing reagents through replacing with fresh medium, cells were washed with PBS contained 25mM (NEM) before being prepared for SP cells. Note, this is an important step before starting the experiment because proteins will become oxidized during the process of making the SP-cells. cells were lysed with lysis buffer (IP-buffer) and either were purified with anti-V5 agarose beads for mass spectrometry analysis or subjected with AMS alkylation to identify the redox state by western blotting and visualizing with anti-V5 antibody. Western blotting was caried out on non-reducing and reducing gel when DTT (50mM) was added to the samples prior to electrophoresis (Figure 3-19-A, left and right panels). Our results indicated that the untreated samples migrated at the same level as reduced sample (Figure 3-19-B, lane 1 and 3 left panels), suggesting CLIC4 was in placed in a reduced state in the normal physiological condition when no oxidized and reduced agents were added.

By contrast, the oxidized samples under diamide and H_2O_2 treatment illustrated a slower migration than untreated and reduced samples (Figure 3-18-B, lanes 2 and 4 left and right panels), indicating oxidized samples were modified with AMS corresponding to the mass increasing. Samples under treatment of TNF-α were migrated at a difference position corresponding to oxidized and reduced samples, demonstrating CLIC4 under TNF-α treatment was placed in an intermediate state (Figure 3-18-B, lane 5, left and right panels), suggesting CLIC4 partially oxidized and partially reduced. The migration of CLIC4 on both non-reducing and reducing conditions appeared at the same levels (3-19-B, left and right panels, lanes 1-5). The migration of CLIC4 on both non-reducing and reducing conditions appeared at the same levels (3-19-B, left and right panels, lanes 1-5) but sometimes we can see even greater differences in mobility when the proteins are separated under non-

reducing conditions. These results illustrated that in the normal physiological condition CLIC4 was located in a reduced state in the ER, suggesting there is a robust reductive pathway in the mammalian ER to maintain CLIC4 in a reduced state.

These results emphasize that although the ER lumen is considered for the formation of disulfide bonds within folding proteins entering the mammalian secretory pathway (Bulleid N, et al., 2006), the active site of CLIC4 as a oxidoreductase (Alkhameci H , et al., 2015) is either partially or completely reduced, suggesting CLIC4 appeared in a reduced position as a soluble form with oxidoreductase activity and could be involved in disulfide bond reduction or isomerization to eliminate the oxidative stress for maintaining haemostasis through reductive pathway. To confirm the redox status of CLIC4 in the ER compartment, in the next experiment we prepared the SP cells as a source of the ER (Wilson R et al., 1995), and subjected them to Mass spectrometry.

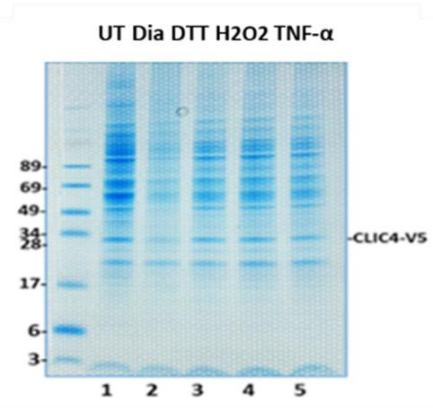
3.2.10 Mass Spectrometry confirms CLIC4 predominantly maintained in a reduced state.

Protein disulfide bonds rarely form in the cytosol because of the high concentrations of GSH (Chakravarthi S, et al., 2007). By contrast, the lumen of the endoplasmic reticulum (ER) contains a relatively higher concentration of oxidized glutathione (GSSG) (Hwang et al, 1992). This allows the formation of native disulfide bonds in the ER through a complex process involving not only disulfide-bond formation but also the isomerization of non-native disulfide bonds (Chakravarthi S, et al., 2007). Therefore, there is a requirement to indicate the redox state of CLIC4 in the ER through mass spectrometry (MS) to provide us more information about site, abundance, and redox state of cysteine residues of CLIC4 in the ER. The SP cells were prepared as a source of the ER (Wilson R, et al., 1995) and analysed in five different experimental conditions via modification of cysteine residues with N-ethylmaleimide (NEM) as an alkylation reagent to block any free thiols (-SH) and subsequent reduction.

The stable HEK293Tcell lines with overexpressing CLIC4-V5 were either treated with oxidized reagents including (5mM Diamide), (5mM H_2O_2), (10µM TNF-α), and reduced reagent (5mM DTT) for 5min at 37℃ or left untreated. After removal oxidized and reduced reagents through replacing with fresh medium, cells were washed with PBS contained 25mM (NEM) N-ethylmaleimide to block any free thiols (-SH) and subsequent reduction. Following preparing SP cells, cells were exposed to IP-buffer and purified with anti-V5 agarose beads through immunoprecipitation. The samples were subjected to SDS-PAGE and Coomassie blue staining (Figure 3-19-A) for mass spect analysis. 10ul of each sample was carried out for western blotting with subjecting to anti-V5 mouse (Figure 3-19-B), anti-V5 rabbit (Figure 3-19-C), to remove the background and anti-CLIC4 (Figure 3-19-D) to recognise CLIC4 protein to cut the gel band in a right place.

The redox state of CLIC4 in mass spectrometry was analyzed through a mass spect standard protocol with (30mM) 4VP. This will modify any free cysteines in the sample. Then samples were subjected to reduction with DTT. This will break any cysteines in disulfide bonds. Then alkylate with IAA, which will modify the newly broken cysteines. Then digest the protein into peptides with trypsin. Soak these peptides out and analyses them on the mass spectrometer (Figure 3-19, right panel).

HEK293T-CLIC4-V5

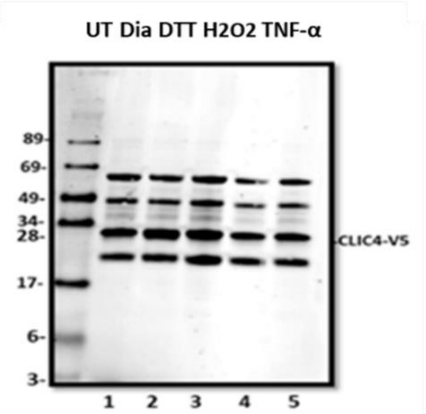


SDS-PAGE

Coomassie blue staining

A

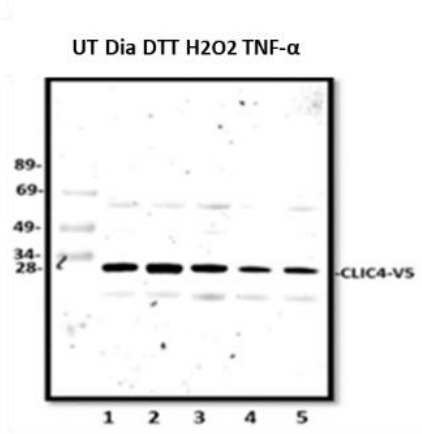
HEK293T-CLIC4-V5



WB: mouse anti-V5

B

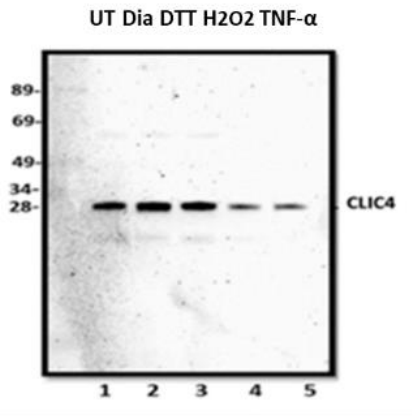
HEK293T-CLIC4-V5



WB: Rabbit anti-V5

C

HEK293T-CLIC4-V5



WB: Rabbi anti-CLIC4

D

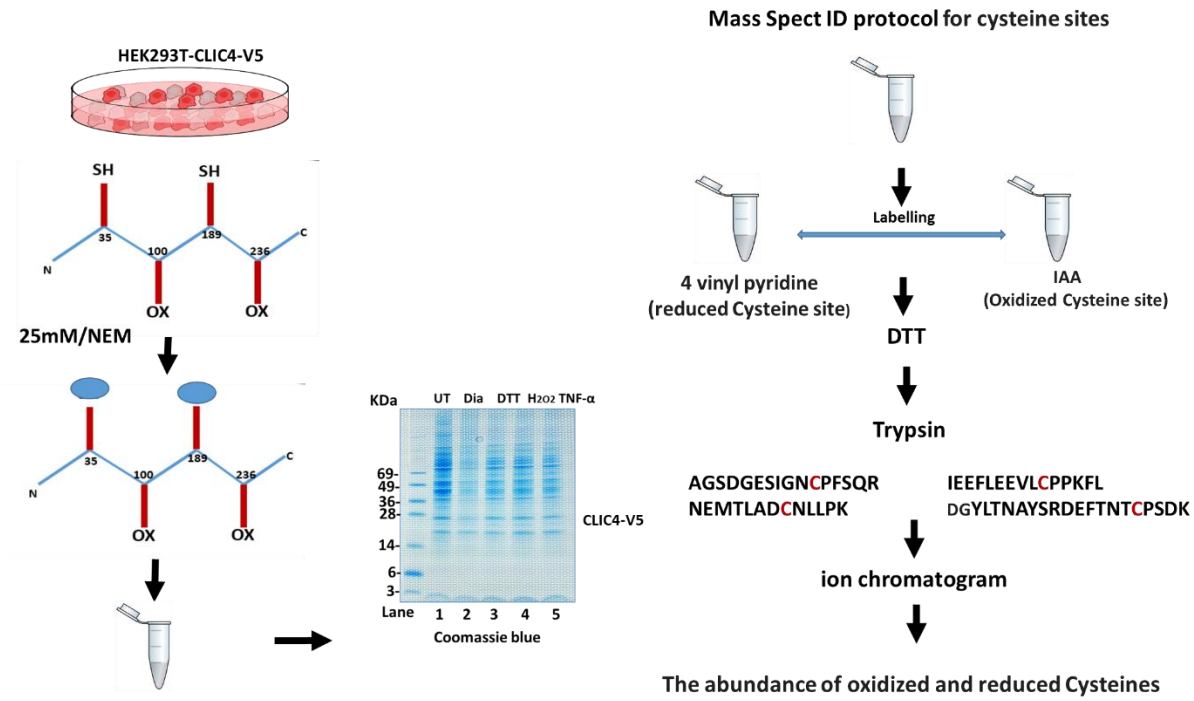


Figure 3-19 Mass spect analysis confirms CLIC4 is located in a reduced form in mammalian ER. To prepare the samples for mass spectrometry analysis to determine the redox state of CLIC4, HEK293T cells stably transfected with a plasmid driving overexpressed CLIC4-V5. Cells were either treated with oxidized reagents including (5mM Diamide, 5mM H_2O_2 and 10uM TNF-α) or reduced reagent (5mM DTT). Following making SP cells as a source of ER, the cells were treated with 25mM *N*-ethylmaleimide (NEM) to trapping the free thiols and cells exposed with IP- buffer. Cells either were purified with anti-V5 agarose beads via immunoprecipitation and subjected to SDS-PAGE and Coomassie blue staining for mass Spect analysis) or carried out to western blotting. Proteins were visualized with mouse anti-V5, rabbit anti-V5, and anti-CLIC4 antibody (upper panel). To purify CLIC4, treated samples with different redox reagents were isolated from HEK293Tcell line which stably overexpresses CLIC4-V5 and were precleared by incubation with protein A Sepharose (PAS) for 30 min at 4°C, before incubation with anti V5-conjugated agarose beads for 16 h at 4°C. The beads were washed three times with lysis buffer supplemented with 0.5% SDS and then incubated with 10 mM DTT for 5 min to elute cross-linked complexes. Then samples were boiled for 5 min to dissociate the immunocomplex from the beads and centrifuged at 13,000 × g for 1 min at 4°C to recover sample from beads and run on the gel for Coomassie blue staining (A) and western blotting with both anti-V5 for detecting with mouse and rabbit of secondary antibodies and anti-CLIC4 antibody (A, B, C, and D) to find a right place of protein to gel extraction. In the next step, after indicating the right bands to determine the abundance of cysteine sites, the gel bands contained-CLIC4 were subjected to a standard protocol for the mass spectrometry to analyse sample on ESI instrument and searched the data through BMS internal database and NCBI. The cysteines to be potentially modified either with 4-vinylpyridine (4VP), carbamidomethyl (IAA), and N-ethylmaleimide (NEM) in the database search (Figure 3-20, bottom panel).

To address the redox state of the CLIC4 in each sample the peptides were searched using Mascot 2.4 software against a custom-built database of 5447 protein sequences, including the sequence of CLIC4 from NCBI. The searches were carried out with variable potential modifications of oxidation cysteine (C) oxidation methionine (M) carbamidomethyl (C) N Ethylmaleimide (C). To determine the correct modification of cysteine residues and avoid any positive false, we consider two main indicators such as ion Score and Expect score. Hence, where the non-catalytic cysteine of a protein was modified with both NEM and IAA, the first thing is look at the peptide score/expect score. If one peptide (e.g., the IAA modified one) has a very high score/low expect probability, and the other has a very low score/high expect probability then we would assume that the one with the low score is maybe a false positive. However, if the two peptides have similar scores, and therefore are similarly confident then it looks like we have a mixed population of cysteines, usually it could be happened for catalytic cysteine with ability for intermediate or transiently modification (Perkins DN, et al., 2018). The result of BMS internal database obtained with 44% Protein sequence coverage for untreated samples, 27% for oxidized samples (under Diamide treatment), 80% for reduced samples (under DTT treatment) and 67% for oxidized sample (under H_2O_2 treatment). The mass spectrometry analysis identified CLIC4 was being presented in each sample. The coverage detected 4 cysteines (C35, C100, C189, C234) for untreated and reduced samples, 2 cysteines (C100, C189) for oxidized sample with Diamide, 3 cysteines (C35, C189, C234) for oxidized sample with H_2O_2, and detected 2 cysteine (C35, C189) for oxidized sample with TNF-α. These exist as modified by NEM/Carbamidomethyl (Panel a, b, c, d and e).

(Panel a)

A.

1	MALSMPLNGL	KEEDKEPLIE	LFVK**AGSDGE**	**SIGNCPFSQR**	LFMILWLK**GV**
51	**VFSVTTVDLK**	**RKPADLQNLA**	**PGTHPPFITF**	**NSEVK**TDVNK	**IEEFLEEVLC**
101	**PPK**YLKLSPK	HPESNTAGMD	IFAKFSAYIK	NSRPEANEAL	ERGLLKTLQK
151	LDEYLNSPLP	DEIDENSMED	IKFSTRK**FLD**	**GNEMTLADCN**	**LLPK**LHIVKV
201	VAKKYRNFDI	PKEMTGIWRY	LTNAYSR**DEF**	**TNTCPSDKEV**	**EIAYSDVAK**R
251	LTK**GKPIPNP**	**LLGLDST**			

Figure 3-20 A untreated sample

B.

AGSDGESIGNCPFSQR

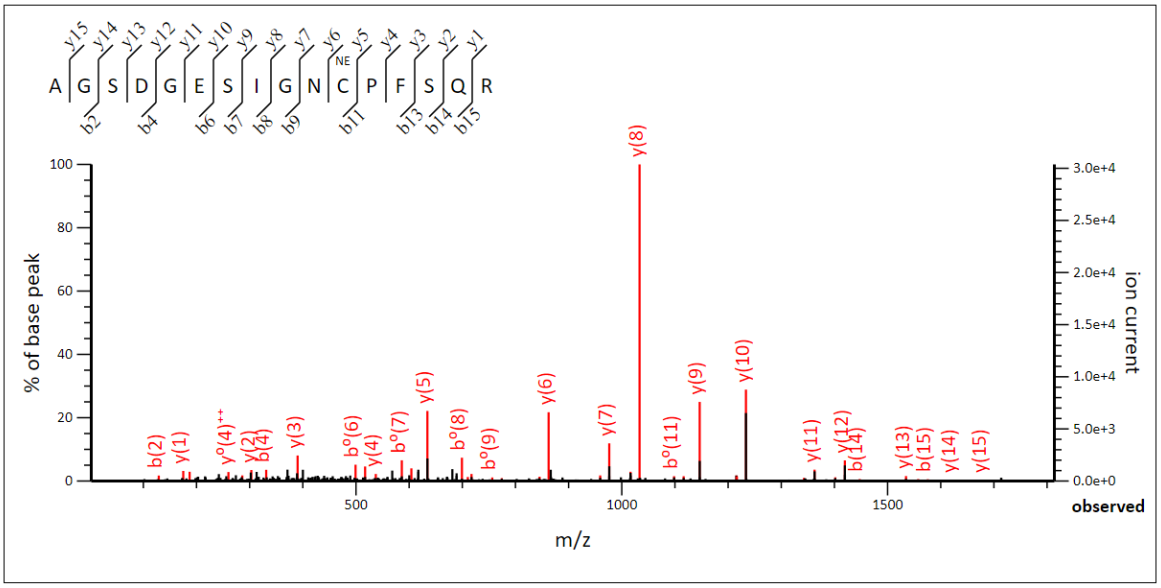


Figure 3-20-B1 Mass spectrometry analysis of untreated sample indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (lysine) **K**. via ion chromatogram peaks and identify the modified cysteine 35 via variable modification: N-ethylmaleimide (C), ions score: 142 and Expect: 1.8e-14, matches: 43/154 fragment ions and using 79 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 615

IEEFLEEVLCPPK

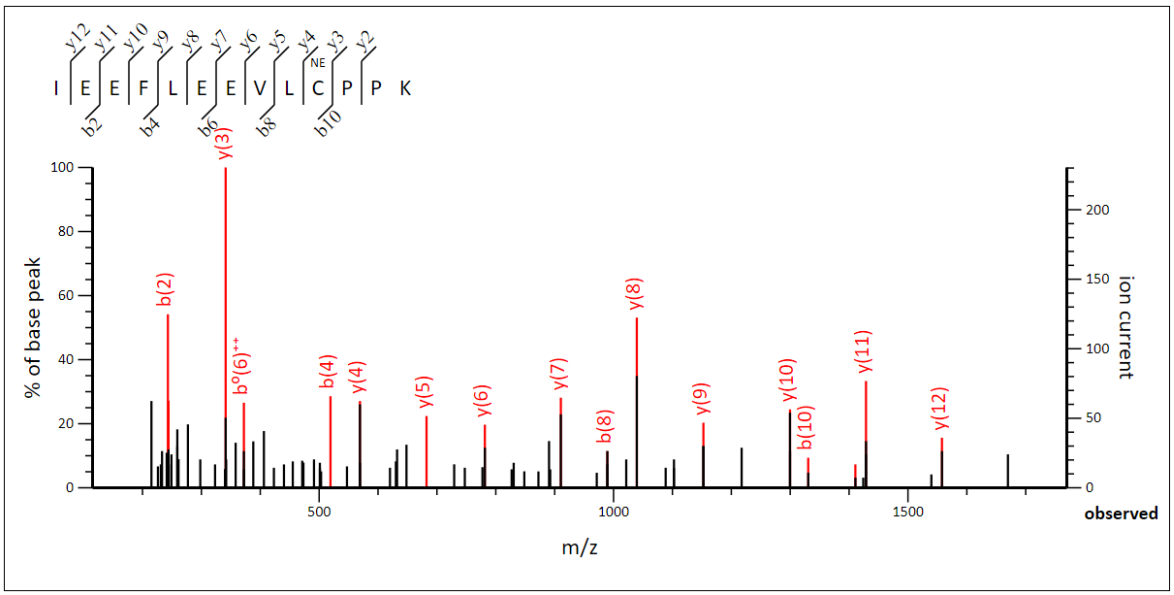


Figure 3-20-B2 Mass spectrometry analysis for untreated sample: indicating the MS/MS fragmentation IEEFLEEVLCPPK following trypsin digestion from (lysine) **K**. via ion chromatogram peaks and identify the modified cysteine 100 via variable modification: N-ethylmaleimide (C), ions score: 104 and Expect: 1.5e-10,

matches: 18/106 fragment ions and using 29 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query **598** .

FLDGNEMTLADCNLLPK

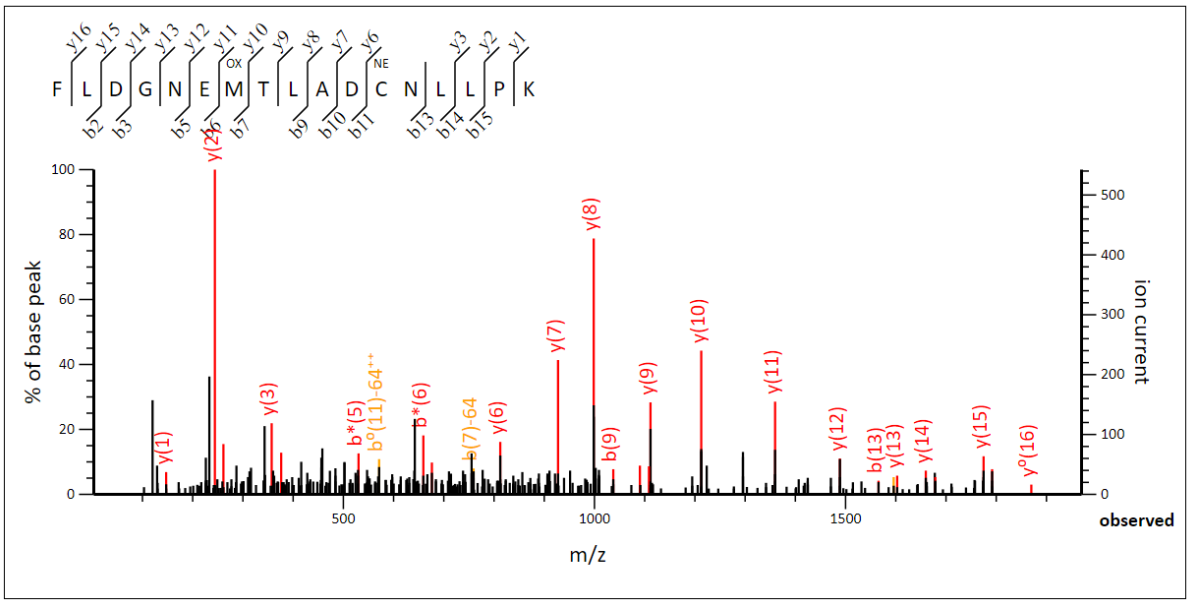


Figure 3-20-B3 Mass spectrometry analysis for untreated sample indicating FLDGNEMTLADCNLLPK the MS/MS fragmentation following trypsin digestion from (lysine) K. via ion chromatogram peaks and identify the modified cysteine 189 via variable modification: N-ethylmaleimide (C), ions score: 99 and Expect: 4.6e-10, matches: 29/264 fragmentations and using 52 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 645

DEFTNTCPSDKEVEIAYSDVAK

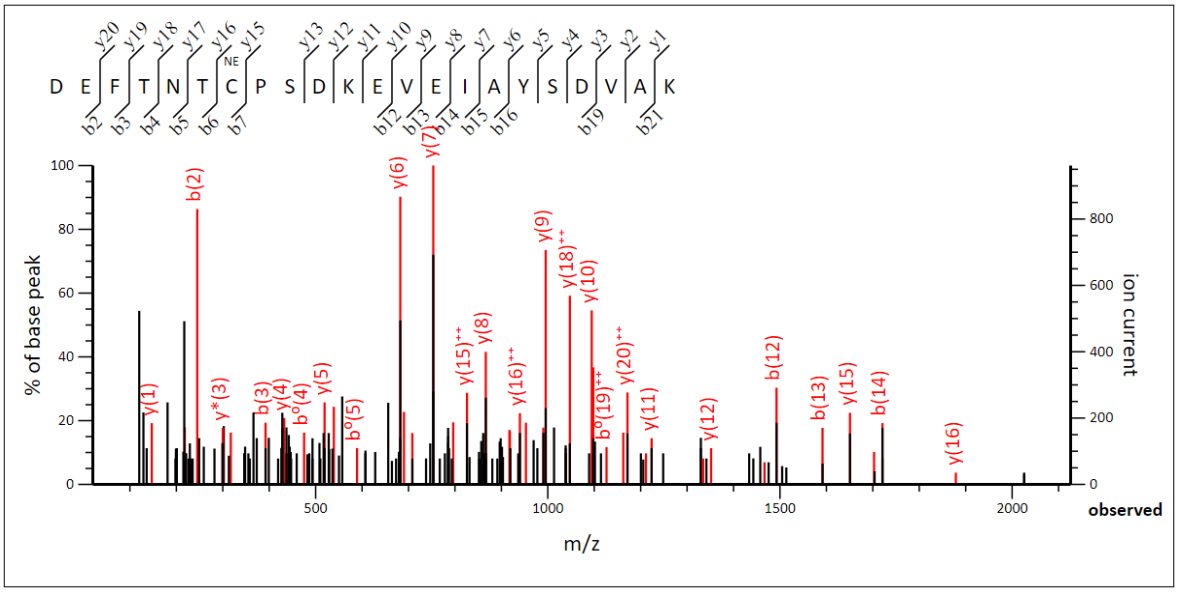


Figure 3-20-B4 Mass spectrometry analysis for untreated sample: indicating the MS/MS fragmentation DEFTNTCPSDKEVEIAYSDVAK following trypsin digestion from (Arginine) R. via ion chromatogram peaks and identify the modified cysteine 236 via variable modification: N-ethylmaleimide (C), ions score: 92 and Expect: 2.2e-09, matches: 42/238 fragmentations and using 92 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 670

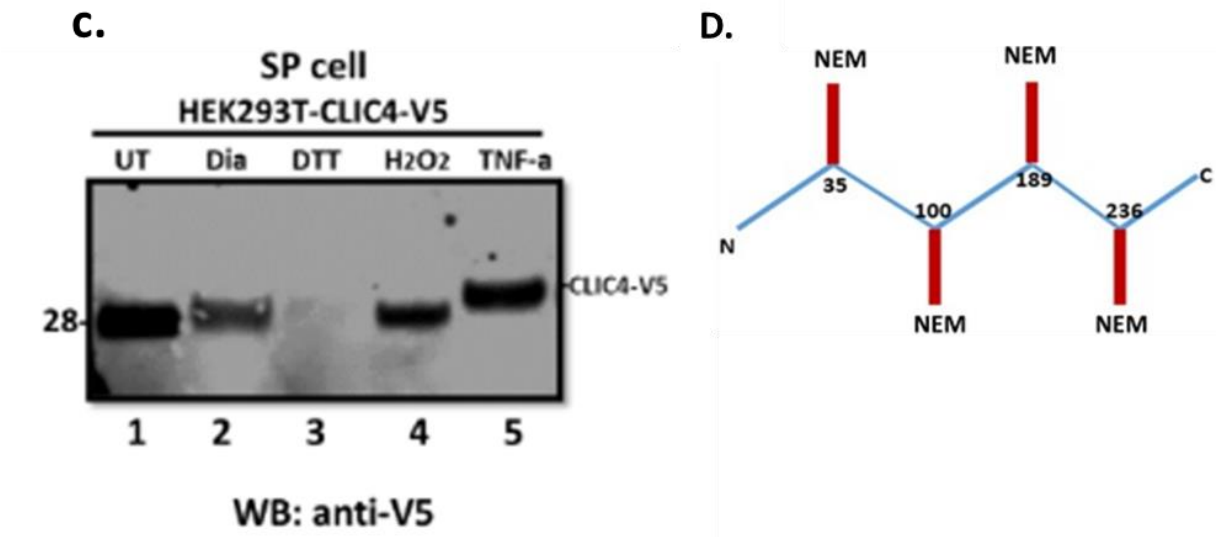


Figure 3-20-C-D the mass spectrometry analysis confirmed CLIC4 was located in a reduced position under normal physiological condition. MS/MS was carried out for CLIC4 and identified upon the abundance of CLIC4 (human) custom sequence in "house BMS database". The research carried out with variable potential modifications of oxidation (C), oxidation (M), carbamidomethyl (C), N-Ethylmaleamide (C), and some other more standard mods that all provided by mass spectrometry analysis at St Andrew mass spect centre **A.** The analysis identified CLIC4 as being presented and obtained protein sequences coverage for human CLIC4 in

untreated samples with 44%. The percentage of peptides coverage was identified with bold red colour. **B.** The MS/MS fragmentation indicated the coverage of 4 peptides that data is found in BMS CLIC4 (Humane). The coverage was included AGSDESIGN**C**PFSQR, IEEFLEEVL**C**PPK, KFLDGNEMTLAD**C**NLLPK, and DEFTNT**C**PSDK with 4 cysteine residues (C35, C100, C189, and C236) in each peptide, respectively. These exist as modified by NEM, suggesting all cysteine residues were placed in a reduced position in untreated sample that was able to be modified with NEM as an alkylation reagent. **C.** The untreated sample was exposed to modification with AMS, SDS-PAGE, and western blot probed with antibody to anti-V5. The untreated samples were migrated as the same electrophoresis shift with reduced samples. **D.** the cartoon shows the schematic diagram for CLIC4 protein with 4 cysteines that all were modified with NEM, indicating CLIC4 located in a reduced form and all cysteines trapped with NEM after treatment.

Protein sequence coverage in diamide treated samples (Panel b) indicated with two peptides TDVNKIEEFLEEVLCPPK and KFLDGNEMTLADCNLLPK, which both modified with NEM for cysteine 35 and cysteine 189. Therefore, due to lack of coverage of other cysteines the redox state of CLIC4 in this sample is not cleared. But when the migration of this sample on SDS-PAGE be compared with untreated sample, it looks like located in a oxidized state, suggesting other cysteine residues might be modified with IAA.

(Panel b)

A.

```
  1  MALSMPLNGL  KEEDKEPLIE  LFVKAGSDGE  SIGNCPFSQR  LFMILWLKGV

 51  VFSVTTVDLK  RKPADLQNLA  PGTHPPFITF  NSEVKTDVNK  IEEFLEEVLC
101  PPKYLKLSPK  HPESNTAGMD  IFAKFSAYIK  NSRPEANEAL  ERGLLKTLQK
151  LDEYLNSPLP  DEIDENSMED  IKFSTRKFLD  GNEMTLADCN  LLPKLHIVKV
201  VAKKYRNFDI  PKEMTGIWRY  LTNAYSRDEF  TNTCPSDKEV  EIAYSDVAKR
251  LTKGKPIPNP
```

Figure 3-21 oxidized sample (Diamide)

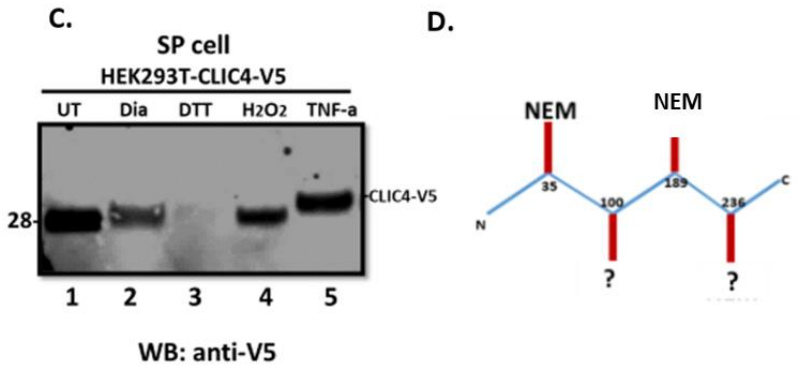


Figure 3-21 the mass spectrometry analysis indicated CLIC4 was located in an oxidized position under Diamide treatment. The research carried out with variable potential modifications of oxidation (C) oxidation (M) carbamidomethyl (C) N Ethylmaleamide (C) and some other more standard mods that all provided by mass spectrometry analysis. **A.** The analysis identified CLIC4 as being presented and obtained protein sequences coverage for human CLIC4 in oxidized sample with 27% for diamide treated sample. The percentage of peptides coverage was identified with bold red color. **B.** The MS/MS fragmentation indicated the coverage of 2 peptides that data is found in BMS with this address BMSO 500705 shaghayegh (CLIC4/Humane) against in BMS CLIC4 (Humane). The coverage included two fragmentations (AGSDESIGN**C**PFSQR, and DEFTNT**C**PSDK) for 2 cysteine residues (C35, and C189) in two peptides, respectively. These exist as modified by NEM, suggesting two cysteine residues were modified with NEM, suggesting due to lack of coverage for other cysteine residues the redox state of CLIC4 was not clear **C.** The oxidized samples under treatment with (5mM) Diamide at 37˚C for 5 minutes were exposed to modification with NEM to trap the free thiols (-SH) and modified with second alkylated reagent (AMS) to increase the mass for highlighting the electrophorese shift on the SDS-PAGE and a western blot was carried out with anti-V5 antibody. **D**. The cartoon shows the schematic diagram for CLIC4 protein with 2 cysteines including (Cys35) and (Cys236) that were modified with NEM, indicating although cysteines 35 and 189 were modified with NEM, CLIC4 might be located in an oxidized form when be compared to its migration on SDS-PAGE and we assume that other cysteine residues could be modified with IAA.

(Panel c)

A.

```
  1   MALSMPLNGL  KEEDKEPLIE  LFVKAGSDGE  SIGNCPFSQR  LFMILWLKGV
 51   VFSVTTVDLK  RKPADLQNLA  PGTHPPFITF  NSEVKTDVNK  IEEFLEEVLC
101   PPKYLKLSPK  HPESNTAGMD  IFAKFSAYIK  NSRPEANEAL  ERGLLKTLQK
151   LDEYLNSPLP  DEIDENSMED  IKFSTRKFLD  GNEMTLADCN  LLPKLHIVKV
201   VAKKYRNFDI  PKEMTGIWRY  LTNAYSRDEF  TNTCPSDKEV  EIAYSDVAKR

251   LTKGKPIPNP  LLGLDST
```

Figure 3-21 reduced samples (DTT)

AGSDGESIGNCPFSQR

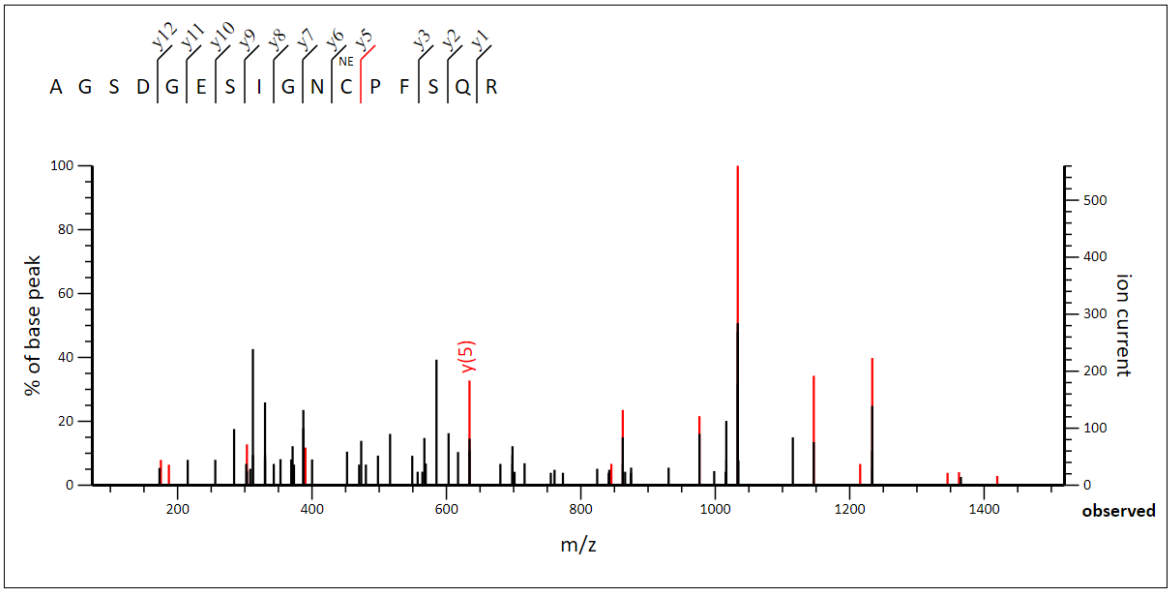


Figure 3-22-B1 Mass spectrometry analysis for reduced sample: indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 35 via variable modification: N-ethylmaleimide (C), ions score: 62 and Expect: 7e-07, matches: 15/154 fragmentations and using 55 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 652

IEEFLEEVLCPPK

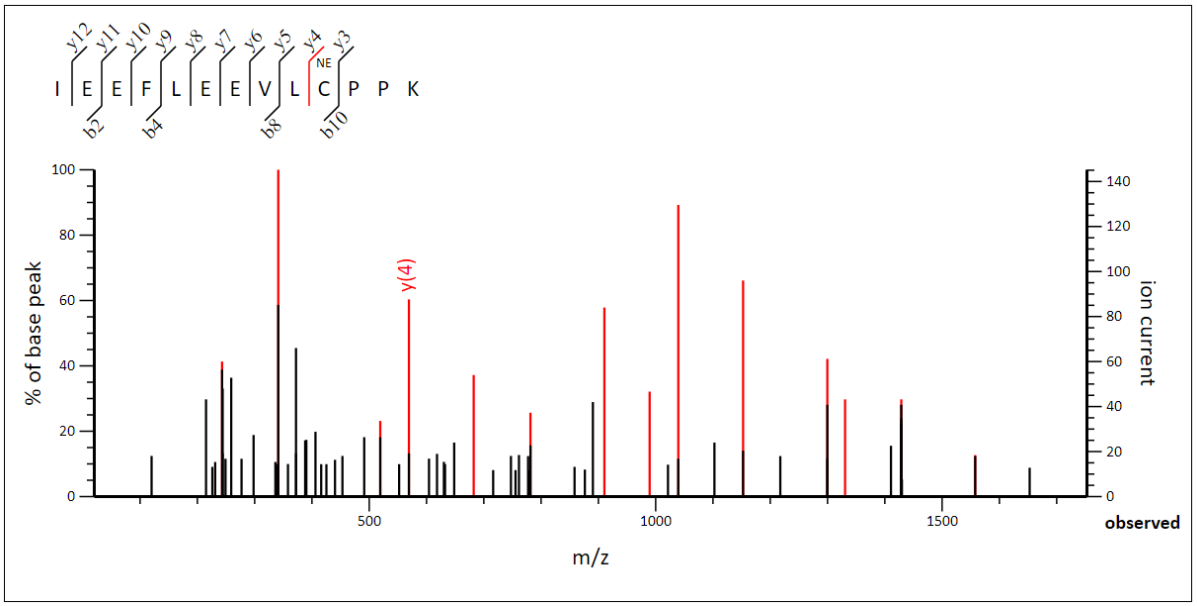


Figure 3-22-B2 Mass spectrometry analysis for reduced sample: indicating the MS/MS fragmentation IEEFLEEVLCPPK following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 100 via variable modification: N-ethylmaleimide (C), ions score: 62 and Expect: 3.5e-10, matches: 14/106 fragmentations and using 16 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 635.

FLDGNEMTLADCNLLPK

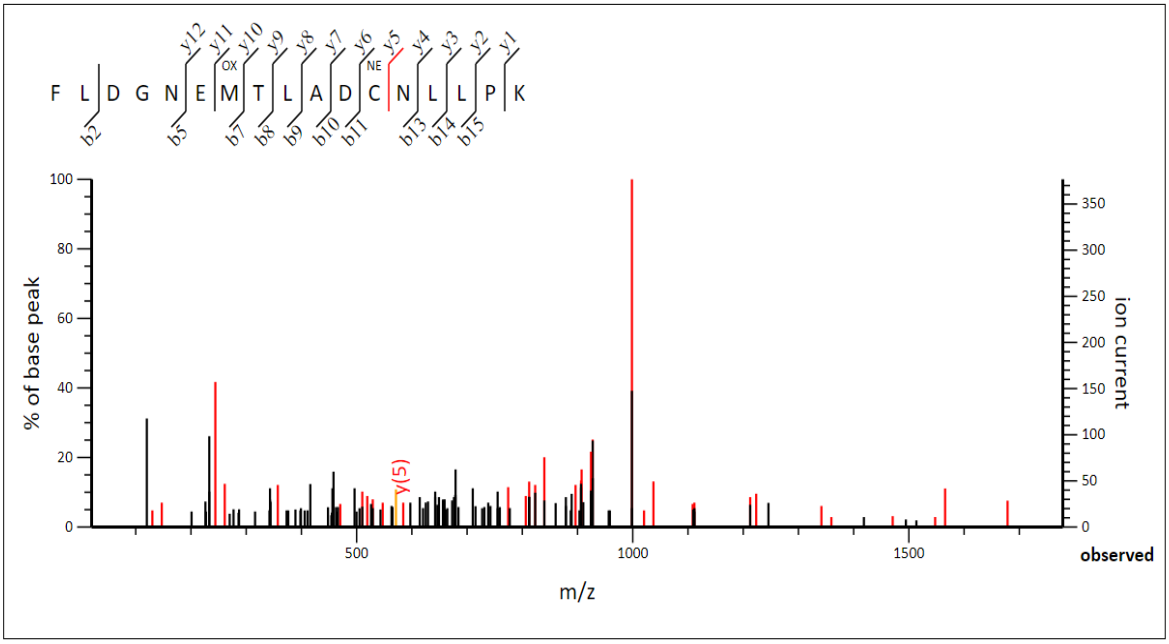


Figure 3-22-B3 Mass spectrometry analysis for reduced sample: indicating the MS/MS fragmentation FLDGNEMTLADCNLLPK following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 189 via variable modification: N-ethylmaleimide (C), ions score: 62 and Expect: 1.8 e-06, matches: 36/264 fragmentations and using 71 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 696

DEFTNTCPSDKEVEIAYSDVAK

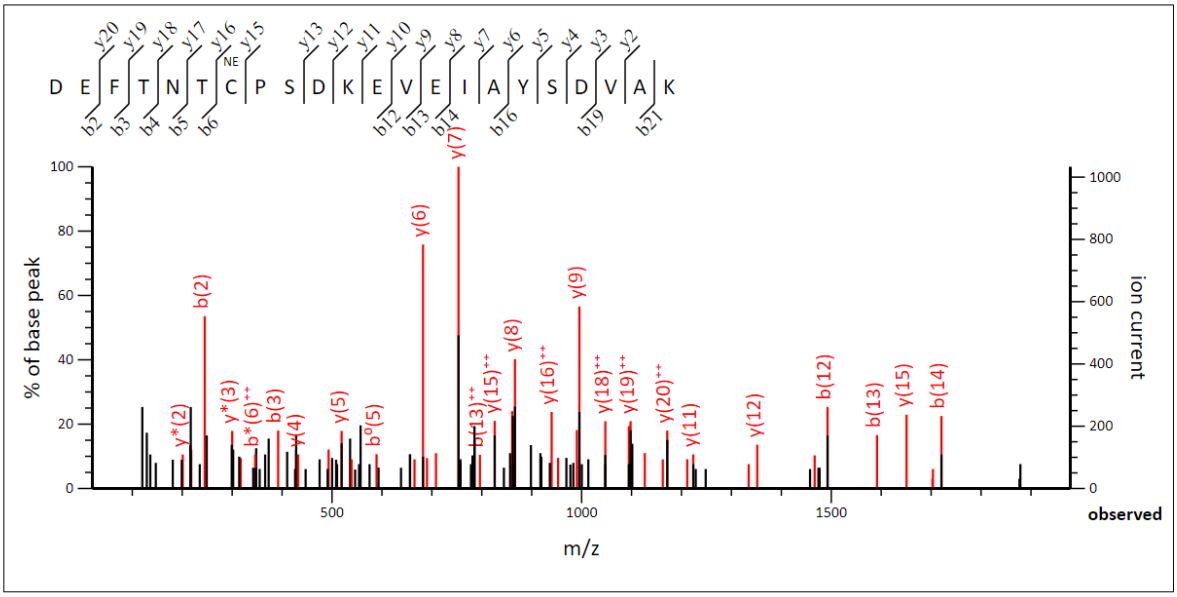


Figure 3-22-B4 Mass spectrometry analysis for reduced sample: indicating the MS/MS fragmentation DEFTNTCPSDKEVEIAYSDVAK following trypsin digestion from (Arginine) R. via ion chromatogram peaks and identify the modified cysteine 236 via variable modification: N-ethylmaleimide (C), ions score: 73 and Expect: 1.1 e-07, matches: 44/238 fragmentations and using 96 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 723

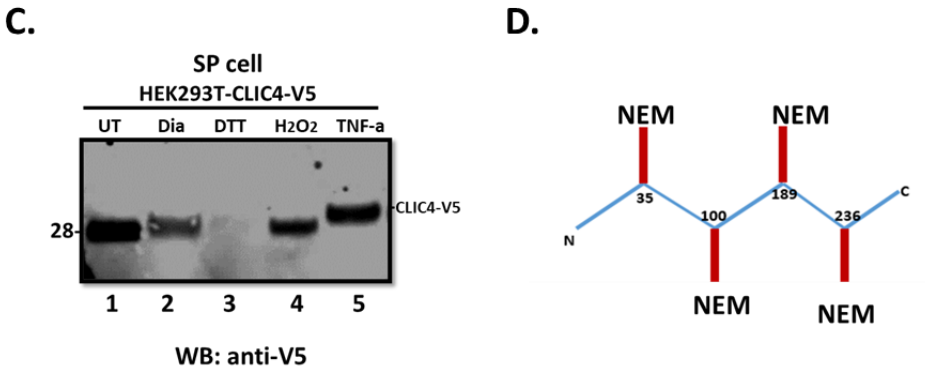


Figure 3-22 Panel (c), Mass spect analysis of redox state of CLIC4 under reduced condition in the mammalian ER. MS/MS was carried out and identified upon the abundance of CLIC4 (human) custom sequence in "house BMS database". The research carried out with variable potential modifications of oxidation (C) oxidation (M) carbamidomethyl (C) N Ethylmaleimide (C) and other standard mods that all provided by mass spectrometry analysis. **A.** The analysis identified CLIC4 as being presented and obtained protein sequences coverage for human CLIC4 with 80% for reduced samples. The percentage of peptides coverage was identified with bold red colour. **B.** The MS/MS fragmentation indicated the coverage of 4 peptides that data is found in bms with this address BMSO 500705 shaghayegh (CLIC4/Humane) in BMS CLIC4 (Humane). The coverage was included AGSDESIGN**C**PFSQR, IEEFLEEVL**C**PPK, KFLDGNEMTLAD**C**NLLPK, and DEFTNT**C**PSDK with 4 cysteine residues (C35, C100, C189, and C234) in each peptide, respectively. These exist as modified by NEM, suggesting all cysteine residues were placed in a reduced position in both untreated and reduced samples that were able to be modified with NEM as an alkylation reagent. **C.** The reduced samples were exposed to modification with AMS, SDS-PAGE, and western blots probed with antibody to anti-V5. Reduced samples were migrated in a similar speed or electrophoresis shift with untreated samples. (MS) results demonstrating CLIC4 exists in a reduced-redox state in the mammalian ER. **D.** the cartoon shows the schematic diagram for CLIC4 protein with 4 cysteines that were all modified with NEM, indicating CLIC4 located in a reduced form and all cysteines trapped with NEM after treatment.

(Panel d)

A.

```
  1  MALSMPLNGL  KEEDKEPLIE  LFVKAGSDGE  SIGNCPFSQR  LFMILWLKGV
 51  VFSVTTVDLK  RKPADLQNLA  PGTHPPFITF  NSEVKTDVNK  IEEFLEEVLC
101  PPKYLKLSPK  HPESNTAGMD  IFAKFSAYIK  NSRPEANEAL  ERGLLKTLQK
151  LDEYLNSPLP  DEIDENSMED  IKFSTRKFLD  GNEMTLADCN  LLPKLHIVKV
201  VAKKYRNFDI  PKEMTGIWRY  LTNAYSRDEF  TNTCPSDKEV  EIAYSDVAKR
251  LTKGKPIPNP  LLGLDST
```

Figure 3-23 oxidized (H_2O_2 treated) sample

B. AGSDGESIGNCPFSQR

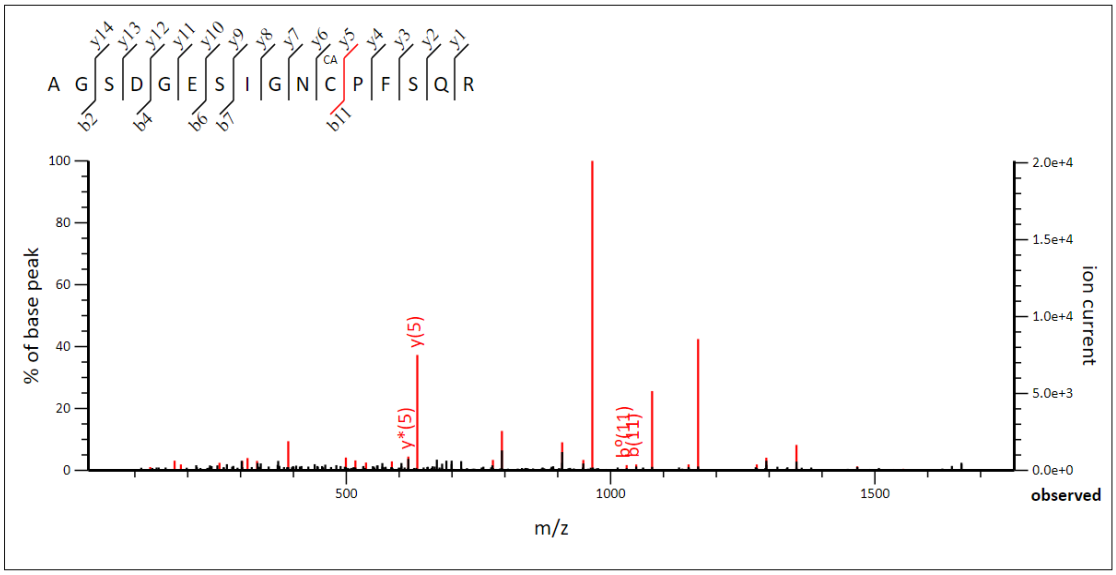


Figure 3-23-B1 Mass spectrometry analysis for H_2O_2 oxidized sample: indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 35 via variable modification: Carbamidomethyl (C), ions score: 148 and Expect: 2.8e-15, matches: 31/154 fragmentations and using 48 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 560

KFLDGNEMTLADCNLLP

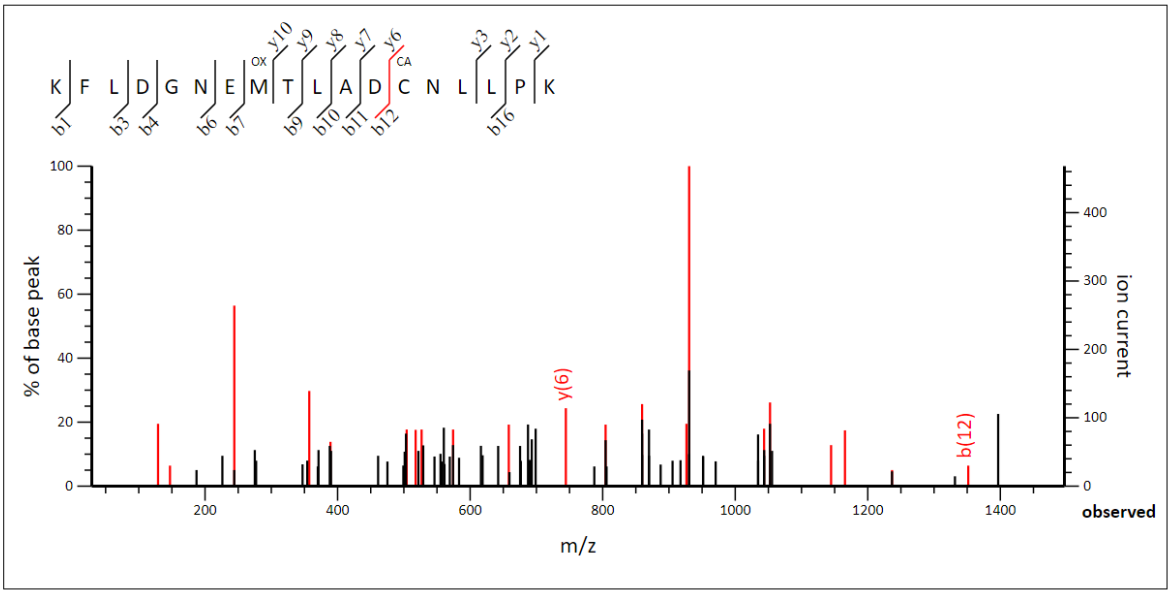


Figure 3-23-B2 Mass spectrometry analysis for H_2O_2 Oxidized sample: indicating the MS/MS fragmentation KFLDGNEMTLADCNLLPK following trypsin digestion from (Arginine) R. via ion chromatogram peaks and identify the modified cysteine 189 via variable modification: Carbamidomethyl (C),and Methionine oxidation (M), ions score: 71 and Expect: 2.8e-07, matches: 23/288 fragmentations and using 37 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 596

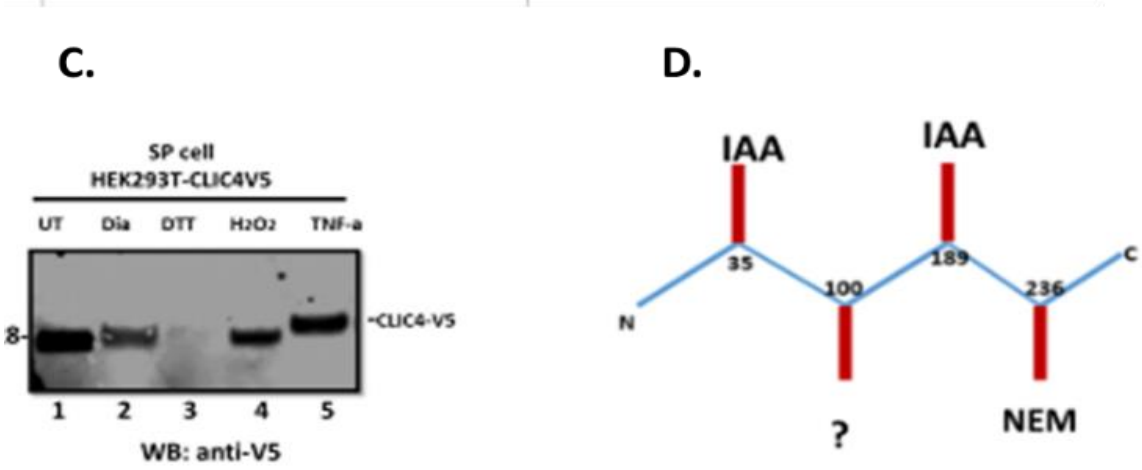


Figure 3-23 C-D, The mass spectrometry analysis of CLIC4 under oxidized condition (5mM) H_2O_2 in mammalian ER. MS/MS was carried out and identified upon the abundance of CLIC4 (human) custom sequence in "house BMS database". The research carried out with variable potential modifications of oxidation (C) oxidation (M) carbamidomethyl (C) N Ethylmaleimide (C) and other standard mods that all provided by mass spectrometry analysis. **A.** The CLIC4 was identified as being presented and obtained protein sequences coverage for human CLIC4 in oxidized sample with 67% for H_2O_2 treated sample. The percentage of peptides coverage was marked with bold red color. **B.** The MS/MS fragmentation indicated the coverage of 3 peptides, where the data is found in bms with this address BMSO 500705 shaghayegh (CLIC4/Humane) against in BMS CLIC4 (Humane). The coverage included three fragmentations (AGSDESIGN**C**PFSQR, KFLDGNEMTLAD**C**NLLPK, and DEFTNT**C**PSDK) for 3 cysteine residues (C35, C189, and C236) in three peptides, respectively. These exist as modified by IAA, and NEM suggesting two cysteine residues were placed in an oxidized position in oxidized samples that were able to be modified with IAA, there was evidence for one cysteine that was modified with NEM but with respect to score/expect, it considered as a false positive **C.**The oxidized samples under treatment with (5mM) H_2O_2 at 37˚C for 5 minutes were exposed to modification with NEM to trap the free thiols (-SH) and be modified with second alkylated reagent (AMS) to increase the mass for highlighting the electrophorese shift on the SDS-PAGE and a western blot was carried out with anti-V5 antibody **D.** The cartoon shows the schematic diagram for CLIC4 protein with 3 cysteines including (Cys35), (Cys189) and (Cys236) that were modified with IAA and NEM indicating CLIC4 might be set in an oxidized position.

(Panel e)

A.

1	MALSMPLNGL	KEEDKEPLIE	LFVKAGSDGE	SIGNCPFSQR	LFMILWLKGV
51	VFSVTTVDLK	RKPADLQNLA	PGTHPPFITF	NSEVKTDVNK	IEEFLEEVLC
101	PPKYLKLSPK	HPESNTAGMD	NSRPEANEAL	ERGLLKTLQK	IFAKFSAYIK
151	LDEYLNSPLP	DEIDENSMED	IKFSTRKFLD	GNEMTLADCN	LLPKLHIVKV
201	VAKKYRNFDI	PKEMTGIWRY	LTNAYSRDEF	TNTCPSDKEV	EIAYSDVAKR
251	LTKGKPIPNP	LLGLDST			

Figure 3-24 A Coverage peptide sequences for oxidized TNF-α

B.

Modified Cysteine (Cys35)

AGSDGESIGNCPFSQR

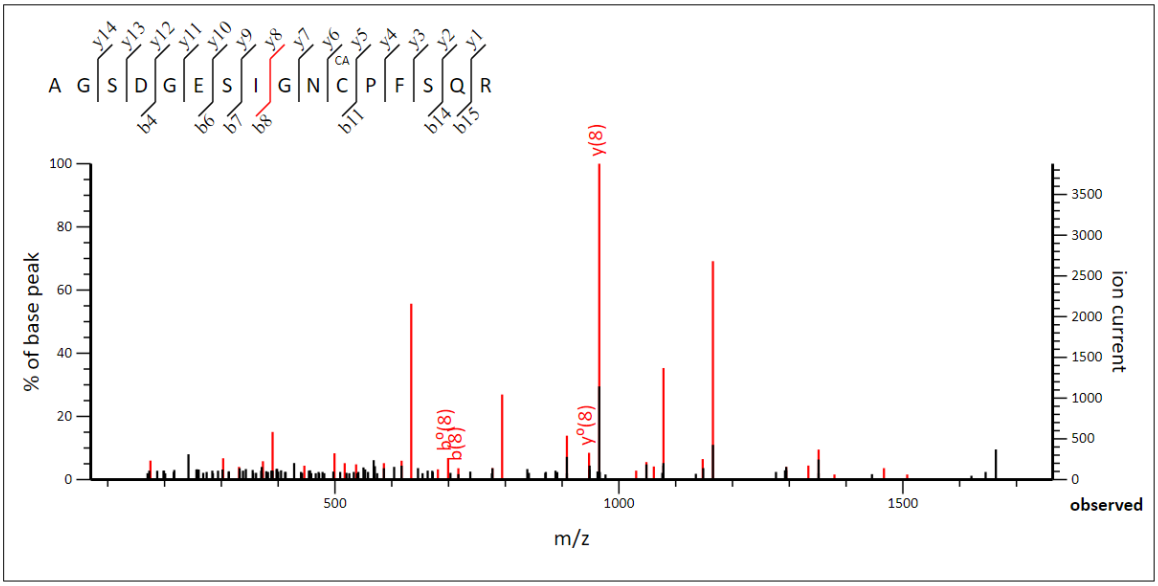


Figure 3-24-B1 Mass spectrometry analysis for TNF-α oxidized sample: indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 35 (as a catalytic cysteine) via variable modification: Carbamidomethyl (C), ions score: 117 and Expect: 3.4e-12, matches: 33/154 fragmentations and using 51 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 472

AGSDGESIGNC3PFSQR

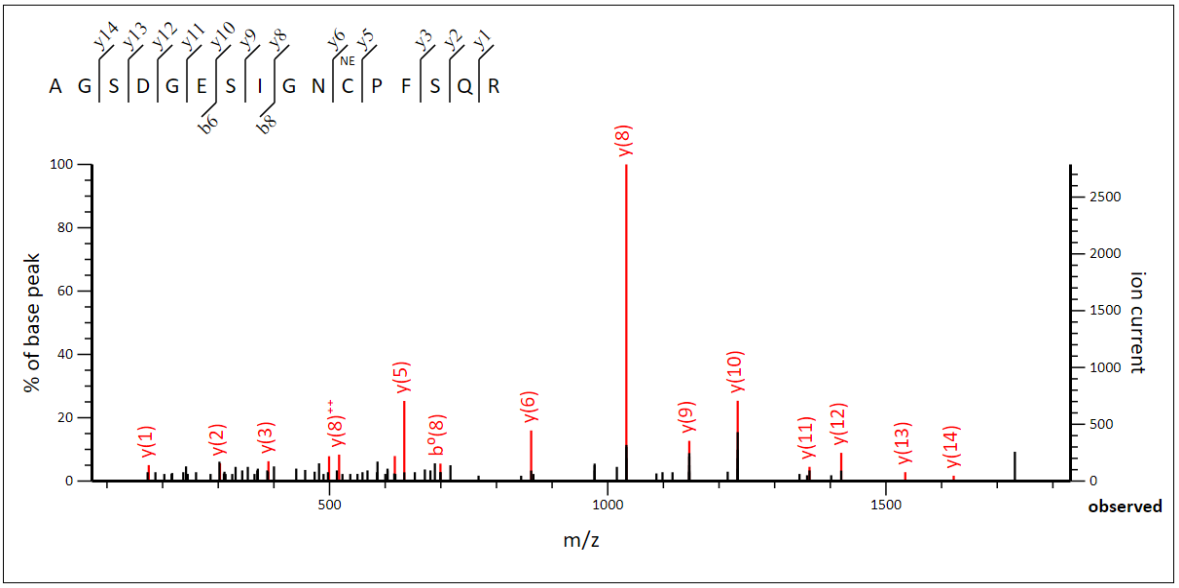


Figure 3-24-B2 Mass spectrometry analysis for TNF-α oxidized sample: indicating the MS/MS fragmentation AGSDGESIGNCPFSQR following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 35 (as a catalytic cysteine) via variable modification: N-ethylmaleimide (C), ions score: 117 and Expect: 3.4e-12, matches: 33/154 fragmentations and using 51 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 475

DEFTNTCPSDK

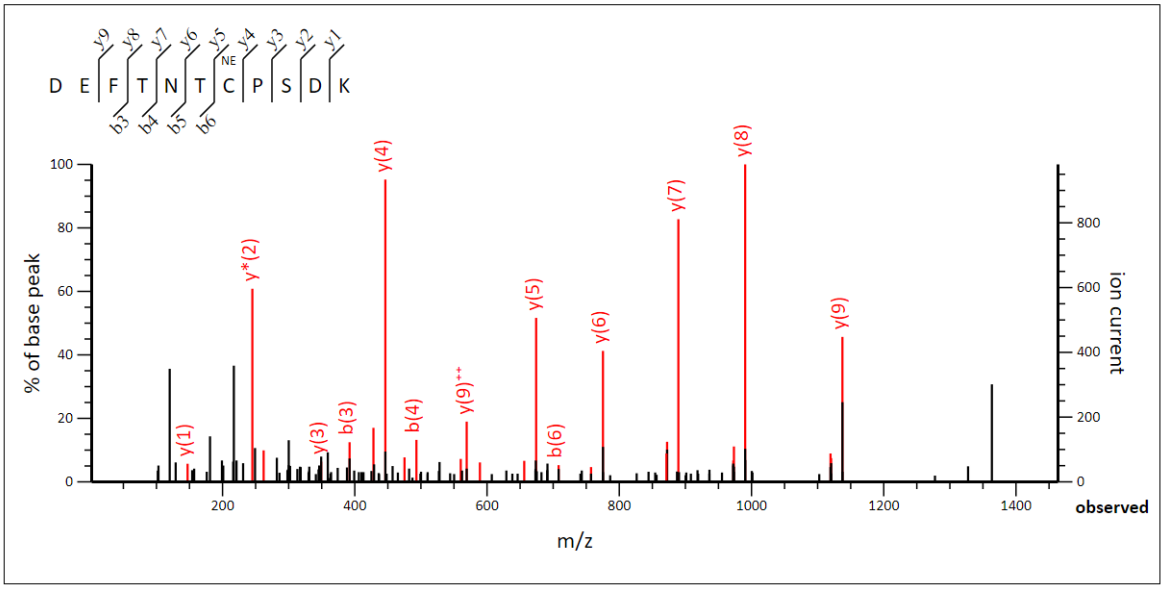


Figure 3-24-B3 Mass spectrometry analysis for TNF-α oxidized sample: indicating the MS/MS fragmentation DEFTNTCPSDK following trypsin digestion from (Lysine) K. via ion chromatogram peaks and identify the modified cysteine 35 (as a catalytic cysteine) via variable modification: N-ethylmaleimide (C), ions score: 66 and Expect: 2.3e-07, matches: 25/140 fragmentations and using 56 most intense peaks, the data can be found in bms|BMS050070|Shaghayegh CLIC4 [human] in BMS, CLIC4 [human], Match to Query 399

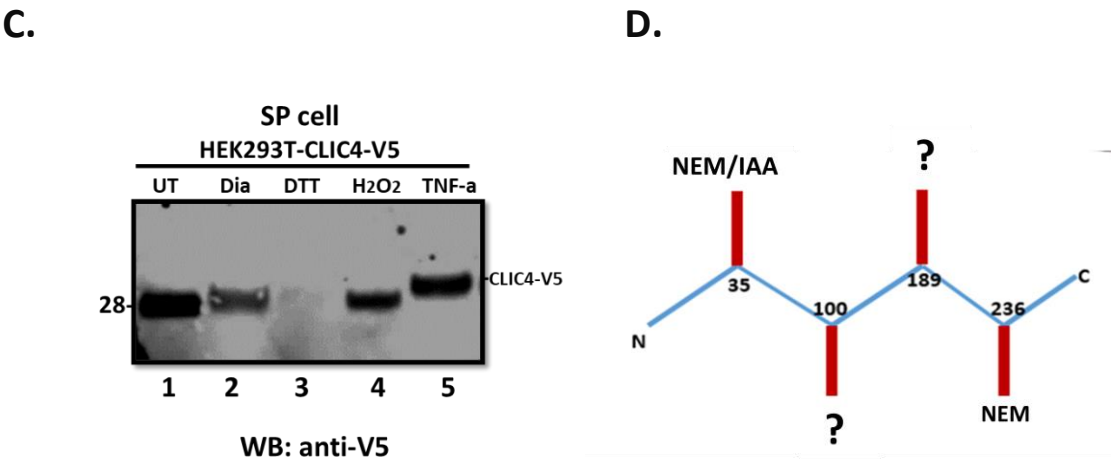


Figure 3-24 C and D the oxidized samples under treatment with (10uM) TNF-α at 37˚C for 5 minutes were exposed to modification with NEM to trap the free thiols (-SH) following isolation of SP cells and purification with anti-V5 agarose beads that was described above, the oxidized samples were identified upon the abundance of CLIC4 (human) custom sequence in "house BMS database". The modifications included NEM (C), NEM + H_2O (C), (4VP) for reduced cysteines and (IAA) for oxidized cysteines, and some other more standard mods that were all provided by mass spect analysis center. A. The search identified CLIC4 as being presented and obtained protein sequences coverage for human CLIC4 in oxidized sample with 40% for H_2O_2

treated sample. The percentage of peptides coverage was identified with bold red color. B. The MS/MS fragmentation indicated the coverage of 3 peptides, where the data is found in bms with this address BMSO 500705 shaghayegh (CLIC4/Humane) against in BMS CLIC4 (Humane). The coverage included two fragmentations (AGSDESIGNC-35-IAAPFSQR, AGSDESIGNC35-NEM PFSQR and DEFTNTCPSDK) for two Cysteine residues (C35, and C236) in two peptides, respectively. These exist as modified by IAA, and NEM, suggesting cysteine 35 as a modified cysteine in (CXXS) motif was placed in an intermediate or transient position, which means CLIC4 under TNFα treatment partially reduced and partially oxidized, where CLIC4 was modified with both NEM and IAA, and the other one cysteine was modified with NEM. **C.** The oxidized samples under treatment with (10uM) TNF-α at 37˚C for 5 minutes were exposed to modification with NEM to trap the free thiols (-SH) and modified with second alkylated reagent (AMS) to increase the mass for highlighting the electrophorese shift on the SDS-PAGE and a western blot was carried out with anti-V5 antibody. In the case of TNF-α treatment CLIC4 presented in an intermediate position between oxidized and reduced state, indicating partially oxidized and partially reduced (lane 5). **D.** the cartoon shows the schematic diagram for CLIC4 protein with two cysteines including (Cys35) and (Cys236) that were modified with IAA and NEM indicating CLIC4 might be in a transient form, although there were two unknown redox states for the rest of cysteine residues.

The mass spect result indicated that with optimization of loading peptide with 10ul of peptide in state of 6ul per well to run a MS/MS, indicating 70% protein sequence coverage for untreated sample, 80% for reduced sample, 27% for oxidized sample for Diamide, 67% for H_2O_2, and 44% for TNF-α. The mass spect analysis identified CLIC4 as being present with different sequencing coverage for cysteine residues per each species. In the previous experiment we showed that CLIC4 under treatment with several redox reagents can migrate in different mobility due to being placed in several redox states depending on each condition, as we showed that untreated CLIC4 migrated at the same level as reduced samples, suggesting CLIIC4 predominantly placed in a reduced state.

Here, MS/MS analysis confirmed that the untreated and reduced samples presented in a reduced position due to being modified their 4 cysteine residues with NEM, suggesting that CLIC4 predominantly maintained in a reduced form and placed in an activated form for its diverse functions either for oxidoreductase activity (Zanivan S, et al., 2017) or for ion channel activity for shuttling the electrons directly (Littler R.D, et al., 2010) or through an interaction with membrane binding proteins for transporting the cytosolic reductive equivalent indirectly (Duncan RR, et al., 1997 and Cao X et al., 2019). However, the oxidized samples with (H_2O_2) appeared with a reduction in migration or a low-speed electrophoresis shift in comparison with untreated and reduced samples, suggesting CLIC4 was placed in

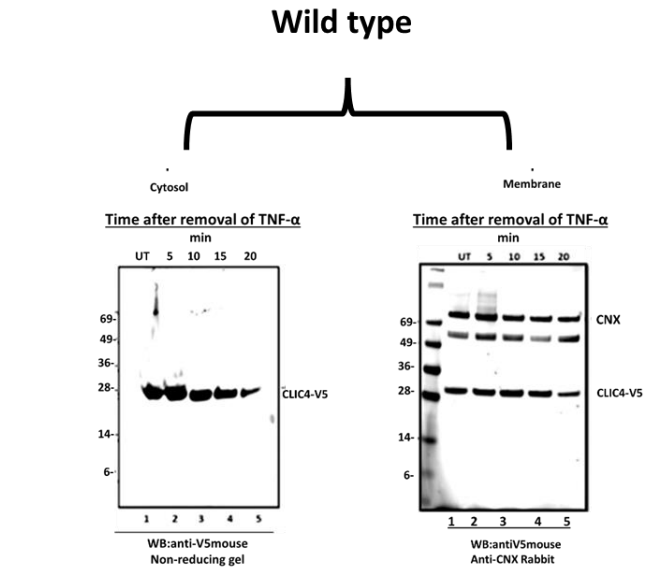
an oxidized position under H_2O_2 treatment and our mass spect result can confirm the redox state of CLIC4 owning to being modified cysteines with IAA. While TNF-α treated samples were observed with a difference speed in migration, demonstrating CLIC4 under TNF-α treatment partially reduced and partially oxidized, and it could occur because of the existence of cysteine 35 in its redox (CxxS) motif and ability to modify with both IAA and NEM; demonstrating Cysteine 35 can form a transient disulfide binding motif with other target protein or low molecular weight thiol such as GSH, Grx, and Trx (Alkhameci H, et al., 2015).

Our result indicated that the modified cysteine residues (cysteine 35) can play an important role in the redox status of CLIC4 in the mammalian ER, thus in the next experiment we designed the mutant form of cysteine 35 to alanine as a mutant trap to identify the role of (Cys35) in CLIC4 function under redox sate.

3.2.11 The catalytic cysteine 35 plays an important role in reduction and translocation of CLIC4 from the cytosol into the ER.

In the previous experiments we illustrated that after removal of the oxidative stress, CLIC4 immediately reduced and return to the active form, suggesting it might be considered for transduction of the cytosolic compartment from the cytosol into the ER (Robinson P, et al., 2020). To address the functional role of CLIC4 during the oxidative stress induced by TNF-α in the ER, we first need to examinate the reduction and re-localization of CLIC4 from the cytosol into the ER. We assume that the re-localization of CLIC4 from the cytosol into the ER in response to oxidative stress could be resulted from a modification of cysteine 35 through disulfide binding formation with low molecular weight thiol such as GSH, Grx, and Trx or other target protein (Alkhameci H, et al., 2015), if this assumption is correct, then cysteine 35 as a modified cysteine redox might can be involved with intracellular translocation in response to oxidative stress through post-translational modification. To investigate if this hypothesis can occur, we designed a mutant stable cell line by replacing cysteine 35 with alanine in the redox motif (CXXS) which could be a requirement for understanding of the redox status of CLIC4 in response to oxidative stress.

A)



B)

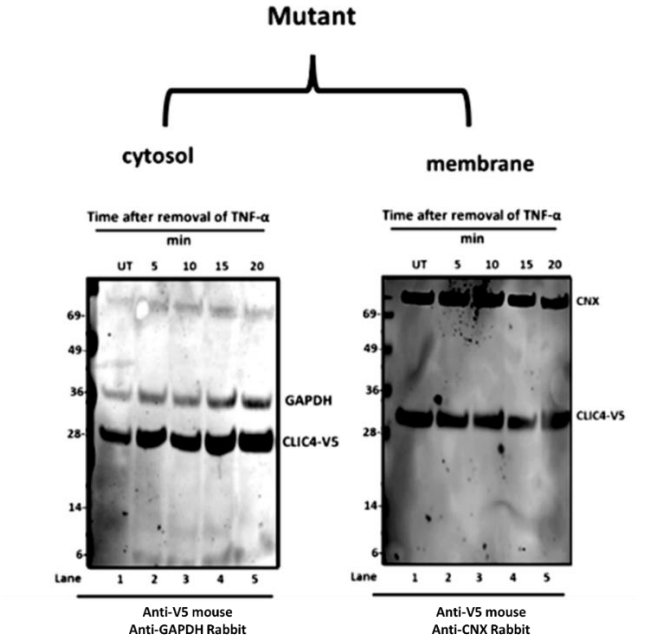


Figure 3-25 mutant CLIC4 not able to translocate from the cytosol into the ER To examine the reduction of CLIC4 in wildtype and mutant stable cell lines following oxidative stress, both wildtype and mutant stable cell lines were either left untreated (lane1) or treated with TNF-α (10uM) for 5 min (lane 2-5). TNF-α was removed with fresh medium, and then incubated at 37℃ at various time points following oxidation. The cytosolic and

membrane fractions were isolated at the same time with the same purification pathway as described previously, the redox state of CLIC4 was determined by alkylation with AMS and western blotting with an anti-V5 antibody.

To investigate whether modification of catalytice cysteine residues can play a particular role in reduction of oxidized CLIC4 after removal of TNF-α, we designed a mutant catalytice cysteine in the redox motif (CXXS) of the active site of CLIC4 and isolated the membrane and cytosol fractions at the same time with the same purification pathway from the wild type and mutant cell lines. To address the role of Cys35 in the reduction process of oxidized CLIC4 along with CLIC4 translocation from the cytosol into the membrane following oxidative stress, the wild type and mutant cells were either left untreated (Figure 3-25 A and B, lane 1) or treated with TNF-alpha (10μM) (Figure 3-25 A and B, lanes 2–5). TNF-α was removed, and the cells were resuspended in the fresh medium and incubated at 37 °C. At specified times following oxidation, the cells were subjected to the fractionation at the same time with the same purification pathway, and the redox state of CLIC4 was determined by alkylation with AMS and western blotting with an anti-V5 and specific organelles markers for cytosol and membrane, GAPDH and CNX, respectively.

Our result indicated that cytosolic wild-type CLIC4 was reduced after 20 minutes when the cells were washed with fresh medium to remove the extra TNF-α, and CLIC4 returned to the reduced position (Figure 3-25-A, left panel, lane 5), which was accompanied by a depletion of CLIC4 from the cytosol toward to the ER membrane (Figure 3-25-A, right panel, lane 5), suggesting it can be considered the presence of a reductive pathway to recover CLIC4 from the addition of TNF-α to return to the reduced state or active form due to oxidoreductase activity of CLIC4. In addition, depletion, or deplation of CLIC4 can be occurred owing to its ion channel activity for translocation of CLIC4 from the cytosol toward the ER membrane.

However, the mutant CLIC4 (CXXS replaced with AXXS) failed to be reduced 20 min after removal of TNF-α or not be recovered from extra oxidizing agents and still located in the oxidized position (Figure 3-25 B, Lane 5). Furthermore, no observation of re-localization of mutant CLIC4 from the cytosol into the ER has been seen (Figure 3-25 B, Lane 5), suggesting the catalytic cysteines (Cys35) of CLIC4 required for both functional activities of CLIC4 either

reduction of CLIC4 as an oxidoreductase enzyme or translocation as an ion channel function (Venezuela Set al., 2009). In addition, cysteine 35 can regulate CLIC4 ion channel activity (Alkhameci H, et al., 2015), and ion channel activity, in turn, can regulate oxidoreductase activity (Zanivan S, et al., 2017), thus cysteine 35 could be involved with both functions of CLIC4 either oxidoreductase activity or ion channel function, which in turn, both can involve with a reductive pathway in mammalian cells, as mutant CLIC4 was failed in reduction and translocation. Thus, in the next experiment, we examined the effect of inhibition of ion channel function on the reduction of CLIC4 via IAA-94.

3. 2. 12 CLIC4 ion channel activity can regulate the reduction of CLIC4 following oxidative stress.

The inhibition of CLIC4 enzymatic activity coincides with previous electrophysiological experiments that demonstrated that inhibition of enzymatic activity of CLIC4 by utilising IAA-94 drug with low dose concentration can block channel activity of CLIC4 (Venezuela S, et al., 2009). To determine whether IAA-94 inhibitor has a significant effect on reduction of CLIC4 following TNF-α oxidative stress-induced, HEK293T-CLIC4-V5 cell lines were treated with 40μM IAA-94 and incubated for 1 hour at 37 ̊C. The IAA-94 inhibitor was removed with fresh medium. Then cells either treated with (10μM) TNF-α or left untreated. The redox state of CLIC4 was determined at various time points (Figure 3-25 lanes 1-4).

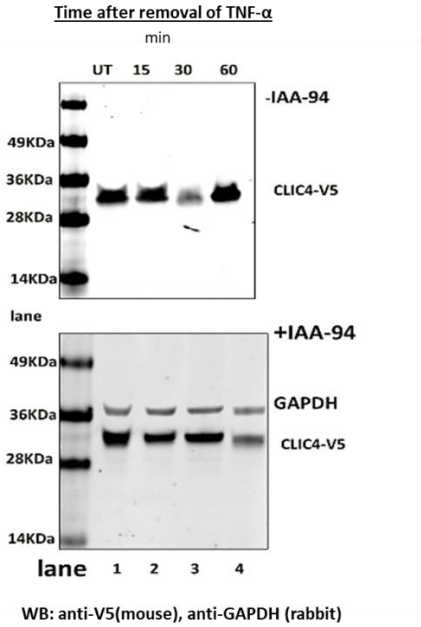


Figure 3-26 CLIC4 ion channel activity can regulate the reduction of CLIC4 following oxidative stress. To determine whether IAA-94 has a significant effect on reduction of CLIC4 following TNF-α oxidative stress-induced, we first treated HEK293T cells that stably expressed CLIC4-V5 with 40uM IAA-94 and incubated for 1 hour at 37℃. IAA-94 inhibitor was removed with fresh medium. Then cells were treated with (10μM) TNF-α or left untreated. TNF-α (oxidizing agent) was removed, and cells were washed with fresh medium and incubated at various time points following oxidation with TNF-α. The redox state of CLIC4 was determined by alkylation with AMS and western blotting with an anti-V5 antibody and anti-GAPDH. All species were fully reduced with 50mM (DTT).

Previous electrophysiological studies indicated that IAA-94 inhibitor is a membrane permeable drug, and its cellular inhibitory effects could be due to inhibition of CLICs enzymatic activity rather than directly blocking their ion channel activity (Wasson C, et al., 2021). It is also likely that this enzymatic activity regulates the CLICs ion channel function (Alkhameci H, et al., 2015). To evaluate the regulatory effect of ion channel function of CLIC4 on its oxidoreductase activity, we treated HEK293T-CLIC4-V5 with 40μM IAA-94 inhibitor with 60 min incubation at 37℃. Then the inhibitor was replaced with fresh medium and cells were either treated with 10μM TNF-α for 5 min at 37℃ (Figure 3-26, lanes 2-4) or left untreated (Figure 3-26, lane 1). TNF-α was removed with fresh medium and the redox state of CLIC4 was determined at various times with subjecting to AMS modification and western blotting with anti-V5 and anti-GAPDH.

The result indicated that in the absent of CLICs inhibitor (IAA-94) drug, in 15 min after removal of TNF-α CLIC4 was migrated at the same level as untreated samples, indicating CLIC4 be partially reduced and partially oxidized (Figure 3-26, lane 1). In 30 min following removal of TNF-α induced-oxidative stress CLIC4 was reduced, suggesting there is a reductive pathway to recover CLIC4 from extra oxidizing agent and maintained CLIC4 in a reduced state (Figure 3-26, lane 3). By contrast, in 60 min after removal of TNF-α, CLIC4 not be able to be recovered from extra oxidizing agent (Figure 3-26, lane 4), suggesting for an oxidoreductase to be functional as an isomerase or reductase is when it be placed in a reduced form to be capable of donating electrons and be quickly oxidized. After each cycle of reduction and oxidation the oxidoreductase must be returned to original one via reductive and oxidative pathways (Lyric R.M, et al., 1970). Hence, there is a reductive pathway to reduce CLIC4 after removing oxidized agent and CLIC4 can return to active state.

By contrast, in the presence of IAA-94 treatment, no reduction was observed in CLIC4 (Figure 3-26 lane 3, bottom panel), suggesting IAA-94 can inhibit the oxidoreductase activity of CLIC4 by inhibition of its ion channel activity. This result leaves two possible explanations for the inhibition of the CLIC4 ion channel by IAA-94 (K, et al., 2009) firstly, the inhibitor can block electron transferring to conduct electrons (Li S, et al., 2022), and the inhibition of the channel is mediated by the inhibition of the enzymatic activity of the soluble form of CLIC4. The more likely explanation is that the inhibitor, IAA-94, can act by binding near the active site (CXXS) of the soluble form of CLIC4 thus inhibiting its enzymatic activity and consequently its channel activity can occur (Ponalagu D, et al., 2019). Therefore, Cysteine 35 can have a significant role on reduction of CLIC4, and there is a requirement to investigate about possibilities of GSH in reduction and consequently activate of CLIC4 either enzymatic or ion channel activity. Hence, in the next chapter we need to examinate the GSH potential and its ability on CLIC4 reduction.

3-3 Discussion and Conclusion

Members of the CLIC family are soluble proteins capable of spontaneously inserting into lipid membranes and intracellular membranes to form chloride selective ion channels (Padmakumar VC, et al., 2012). To date, characterization of these proteins has focused on their membrane insertion and their ion channel activity, with no distinct function assigned to their soluble form. The glutaredoxin-like activity of CLIC4 was further supported by its lack of activity in the common Trx disulfide reductase (Littler R.R. et al., 2010). Grx2 and Grx3 dithiol glutaredoxin have been found to being reduced by thioredoxin reductase (TrxR) as well as GSH and glutathione reductase (GrxR) (Alkhameci H, et al, 2015). However, CLIC4 were not reduced by the selenonzyme, thioredoxin reductase (TrxR) (Littler R.R et al., 2010). Given the lack of activity by CLIC4 protein, could speculate that CLIC4 have GSH-dependent enzymatic activity. The functional role of CLIC4 as a glutaredoxin protein which act as enzymes by deglutathionylating the mixed disulphide between glutathione (GSH) and HEDS reagent suggesting soluble form of CLIC4 with Grx-like activity may act to catalyse the reduction of disulfide and thus function as oxidoreductases in mammalian cells (Zanivan S, et al., 2017).

In this project to characterise the functional role of CLIC4 either as an oxidoreductase or ion channel during oxidative stress, we first identified the expression and localization of

CLIC4 in several subcellular compartments under oxidative stress. Hence, we designed a tagged protein with a 9-peptide epitope tag (V5) in the C-terminal of CLIC4 protein to generate a stable cell line. The subcellular fractionation was prepared from a HEK293T cell line which was transiently and stably transfected with CLIC4-V5 tagged. It was proven that the V5 epitope tag is a good tool to monitor the localization of protein in intracellular compartments. Moreover, to optimize which oxidative stress inducers are capable to induce CLIC4 translocation between subcellular compartments, we examined two oxidative stress agents including TNF-α and H2O2. TNF-α is a good oxidative stress indicator to investigate the impact of the reduction and translocation of CLIC4 on redox status inside intracellular organelles notably ER.

Inside the ER, we assumed that CLIC4 as one member of CLICs family with oxidoreductase activity was involved in the reductive pathway in the lumen. The function of CLIC4 would be driven by redox state. In the reduced state, it can bind and reduce substrate proteins while in the oxidised state, the substrate can be oxidised and released (Alkhameci H, et al., 2015). Hence, after disulfide exchange with client protein, it is necessary to reduce CLIC4 so it can function as a reductase. Note it is important to know CLIC4 due to (CXXS) redox motif in its active site to complate the oxidoreductase activity is dependent to GSH (Alkhameci H, et al., 2015). Hence, CLIC4 was chosen as an indicator of redox status to determine the impact of the reductive pathway on the redox state in the ER lumen. Determination of redox state of CLIC4 in HEK293T WT which was stably overexpresses CLIC4-V5 was optimised based on a previously described approach (Oka O, et al 2013). To confirm the redox state of CLIC4 in the ER compartment, we have applied MS/MS analysis under several redox condition to provide us with site, abundance, and post translational modification of cysteine residues in CLIC4. Finally, to understand of reason for relocalization of CLIC4 from the cytosol into the ER in response to oxidative stress, we isolate cytosolic and membrane fractionation from wildtype and mutant HEK293Tcell line, which stably overexpresses mutant CLIC4-V5 (Cysteine-35 to Alanine). In addition, we applied IAA-94 drug as a CLICs inhibitor to investigate the effect of ion channel activity on reduction of CLIC4 and consequently on inhibition of CLIC4 translocation (Wasson C, et al., 2021).

Here we demonstrated that CLIC4 expressed in the presence and absence of oxidizing agents and localized to the several intracellular organelles and in response to TNF-α with

respect to concentration CLIC4 can relocalize from the cytosol into the nucleus and ER. CLIC4 nuclear translocation has been the focus of much research, but ER CLIC4 translocation had not been clearly defined until now. Furthermore, previous studies indicated that knockdown CLIC4 that was produced by subjecting to a specific CLIC4 small interfering RNA significantly increased the expression of CHOP as an ER apoptotic indicator and can proceed the cell death, suggesting CLIC4 has a vital role in the ER (Xue H, et al., 2016, and Zhong J, et al., 2012). Hence, in this study, to evaluate the functional role of CLIC4 in the ER, we generated the SP cells as a source of the ER that included both ER membrane and ER lumen (Wilson R, et al., 1995), and we examined endogenous and exogenous of CLIC4 in the ER compartment. Moreover, in this project, as described above we induced the oxidative stress by TNF-α for its ability to induce relocalization of CLIC4 into the ER from the cytosol and increase the formation of disulfide bonds in the ER through ROS accumulation, which could be responsible for reduction of cellular GSH level (Xue x, et al., 2005). The consequences of the oxidative stress in the ER can be accumulation of non-native disulfide bonds in the ER (Bulleid N, et al., 2006).

ER is known as a host of disulfide formation in folding protein for entry to the mammalian secretory pathway and indicated for ER oxidoreductase for reduction and isomerization of non-native disulfide bonds. But an oxidoreductase must be placed in a reduced state to be activated for reduction of non-native disulfide bond and eliminate the oxidative stress in the cells. Here we evaluate the redox state of CLIC4 as an oxidoreductase via subjecting to modification with several alkylation reagents in the absence or presence of TNF-α, our result demonstrated that CLIC4 was placed in a reduced state in normal physiological conditions, suggesting there is a reductive pathway in mammalian cells to maintain CLIC4 as an oxidoreductase in a reduced position or activated form. In addition, we show during oxidative stress CLIC4 first be oxidized and after removal of the oxidizing agent, be reduced but this reduction occurred gradually as CLIC4 after 5 min of removal of TNF-α is partially reduced and after 20 min is completely, suggesting the presence of a reductive pathway should enable CLIC4 to recover from the addition of oxidizing agent. The reduced state for an oxidoreductase enzyme is requirement to capable it to donate the electron to its substrate. This process is dependent on the catalytic cysteines of CLIC4. We showed CLIC4 mutant was not able to be reduced and even not able to be translocate from the cytosol to the ER. This suggested that the reducing power generated outside of ER membrane was transferred into ER lumen depending on the disulfide bond exchange reaction between Trx

(Cao X et al., 2019) and a potential membrane protein with oxidoreductase activity (Duncan R.R, et al., 1997).

The potential membrane protein characterized by ability to membrane insertion and functional redox motif in its active site, which is highlighted with CXXC or CXXS because of sharing the structural homology with the small redox thioredoxin (Bindoli A, et al., 2009). CLIC4 due to ion channel activity with membrane binding ability and oxidoreductase activity could be a good candidate for one of the mis components for reductive pathway. To evaluate the significant role of redox motif in reduction and translocation of CLIC4 from the cytosol into the ER, we generated the mutant stable cell line through replacing (cysteine to Alanine) from CXXS to AXXS as a mutant trap approach to examine the redox activity of modified cysteine. The result is suggested the catalytic cysteines (Cys35) of CLIC4 are required for both functional activities of CLIC4, while the mutant form of CLIC4 either failed in reduction of CLIC4 as an oxidoreductase enzyme or failed in translocation of CLIC4 from the cytosol to the ER as an ion channel function (Venezuela S, et al., 2009), demonstrating that the catalytic cysteine (Cys35) is required for ion channel activity and reduction of CLIC4 can be parallel to translocation from the cytosol into the ER. By comparing mutant CLIC4, not only translocation of CLIC4 from the cytosol toward to the ER failed but also no gradual reduction appeared, suggesting that re-location of CLIC4 can be regulated via its oxidoreductase activity

In addition, we emphasize that although the ER lumen is optimized for the formation of disulfide bonds in secretory proteins (Chakravarti S, et al., 2006), the active site of CLIC4 as an oxidoreductase enzyme needs to be either partially or completely reduced. Such a reduced state would be required if these proteins are involved in disulfide bond reduction or isomerization. To determine the redox status of CLIC4 in the ER, we generate SP cells as a source of the ER, we applied the MS/MS analysis under several redox conditions through purification of CLIC4 by immunoprecipitation. Mass spect data indicated that CLIC4 in untreated and reduced samples appeared in a reduced position because of all 4 cysteine residues were modified with NEM, suggesting CLIC4 is located in an active conformation and act as an oxidoreductase enzyme instead of ion channel and probably localized in the ER lumen, rather than ER membrane.

Finally, we indicated that after incubation of cells with CLICs inhibitor (IAA-94) drug, no reduction of CLIC4 was observed even in 30 min after removing of TNF-α, suggesting CLIC4

in addition to its ion channel activity can include a kinetic advantage for its soluble form and IAA-94 could be inhibited the oxidoreductases activity of CLIC4 by making an overlapping insertion near active site of CLIC4 (Ponnalagu D, et al., 2019). This result leaves two possible explanations for the inhibition of the CLIC4 ion channel by IAA-94 (K, et al., 2009), in which the inhibitor can block electron transferring to conduct electrons (Li S, et al., 2022) the inhibition of the channel is mediated by the inhibition of the enzymatic activity of the soluble form of CLIC4. The more likely explanation is that the inhibitor, IAA-94, can act by binding near the active site of the soluble form of CLIC4 thus inhibiting its enzymatic activity and consequently its channel activity can occur. Therefore, there is a requirement to investigate the possibilities of GSH and shuttling electrons through CLIC4 ion channel activity.

Chapter 4 GSH reduces CLIC4 in response to oxidative stress in the Endoplasmic Reticulum.

4.1 Introduction

The globular soluble form of CLIC4 in addition to ion channel activity appeared as an oxidoreductase with the ability to catalyze conjugation of glutathione (GSH) and is involved in oxidative stress response in the cells (Hayes JD, et al., 2005). The oxidoreductase activity of CLIC4 belonged to glutathione dependent thiol transferase activity to catalyse the glutathione-dependent reduction of dehydroascorbate (DHA) (Board PG, et al., 2000). However, the enzymatic activity of CLIC4 be similar to that of the glutaredoxins (Pastore A, et al., 2012), which are structurally related to the thioredoxins and involved in couple reaction to GSH, NADPH and glutathione reductase (GR) for reduction of intracellular disulfides. CLIC4 contains a GSH binding site (G-site) within its conserved thioredoxin domain structurally belongs to the glutaredoxin family with monothiol (Cys-Gly-Phe-Ser) motif. By forming with GSH and target proteins and even low molecular weight thiols (Trx or Grx) (Pastore A, et al., 2012) can act as an oxidoreductase (Littler R.R, et al., 2010). For CLIC4 to act as an oxidoreductase for performing isomerisation or reduction reactions, it must be preserved in a reduced redox state to remain active.

Reduced and oxidized redox states of a protein were indicated with Cysteine redox state that markedly identified with post translational modifications of protein (Bulleid N, et al., 2005). Consequently, there is a requirement for a specific labelling followed by mass spectrometry (MS) to identify the abundance of cysteine residues and a (MS/LC) to detect a relative comparison of protein expression levels in redox states (Zhao S, et al., 2021). Hence, in chapter 3, to determine the redox state of CLIC4 through mass spect analysis, CLIC4 was modified with several alkylation reagents to compare oxidation and reduction states upon modification with NEM to trap free thiol and make to increase the mass in the +125.7 and for oxidation state via modification through IAA with +57 increase the mass, in which in both of case increase the ion distribution in chromatogram but the difference could be due to the average of charge for each cysteine residue that mass spectrometer could be difference and detected with an short ion mass spectra peak for NEM, and with

an intense ion spectra peak for IAA, detecting reduced and oxidised cysteine, respectively (Cao X et al., 2019).

In addition, we indicated that CLIC4 can be reduced following removal of oxidative stress and there is a reductive pathway for reduction of CLIC4. A candidate for the reduction of the CLIC4 is reduced Glutathione (GSH) (Haynes W, et al., 2016). Glutathione is a ubiquitous molecule found in all parts of the cell where it includes a range of functions from detoxification to protection from oxidative stress. It provides the main redox buffer for cells and as such has been implicated in the formation of native disulfide bonds (Van Lit M, et al 2011). However, the discovery of the enzyme Ero1 raises question the exact role of glutathione in this process. In this Chapter, we determine a significant role for reduction of CLIC4 and consider its role for protecting the cell from the oxidative stress, which might be resulting tofformation of disulfide bond in the ER (Chakavarthi S, et al, 2003). GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is synthesized in the cytosol from the precursor amino acids glutamate, cysteine, and glycine (Lu SC, et al., 2013). The cell contains concentrations of GSH (up to 10 mM), which are maintained in this reduced form via a cytosolic NADPH-dependent reaction catalysed by glutathione reductase (GR) (Chakavarthi S, et al., 2003).

The formation disulfide bonds rarely form in the cytosol because of the high concentrations of GSH (Bulleid N, et al., 2004). GSH provides a redox buffer against ER-generated oxidative stress in addition to a role in disulfide-bond formation (Cao X et al., 2019). There are two major pathways including Trx1/ TrxR1 or the GSH/GR/Grx pathway that might prevent disulfide formation in proteins synthesized in the cytosol. NADPH involved with both pathways for the ultimate electron donor so lack of NADPH could be inhibited disulfide formation in the reticulocyte lysate (Poet GJ, et al., 2017). Thus, two main essential cytosolic factors are NADPH and GSH for reductive pathways to decrease the oxidative stress effect in the ER by reduction of disulfide formation (Poet GJ, et al., 2017), although transport of these of components into the ER is still not understood. Therefore, there is a requirement to identify the membrane protein involved in electron and cytosolic components transfer from the cytosol into the ER. Hence, CLIC4, due to either its ion channel activity or oxidoreductase activity, could be a good candidate to participate in

reductive pathway either for reduction non-native disulfide formation or for transporting cytosolic reductive equivalent from the cytosol to the ER (figure 4-1).

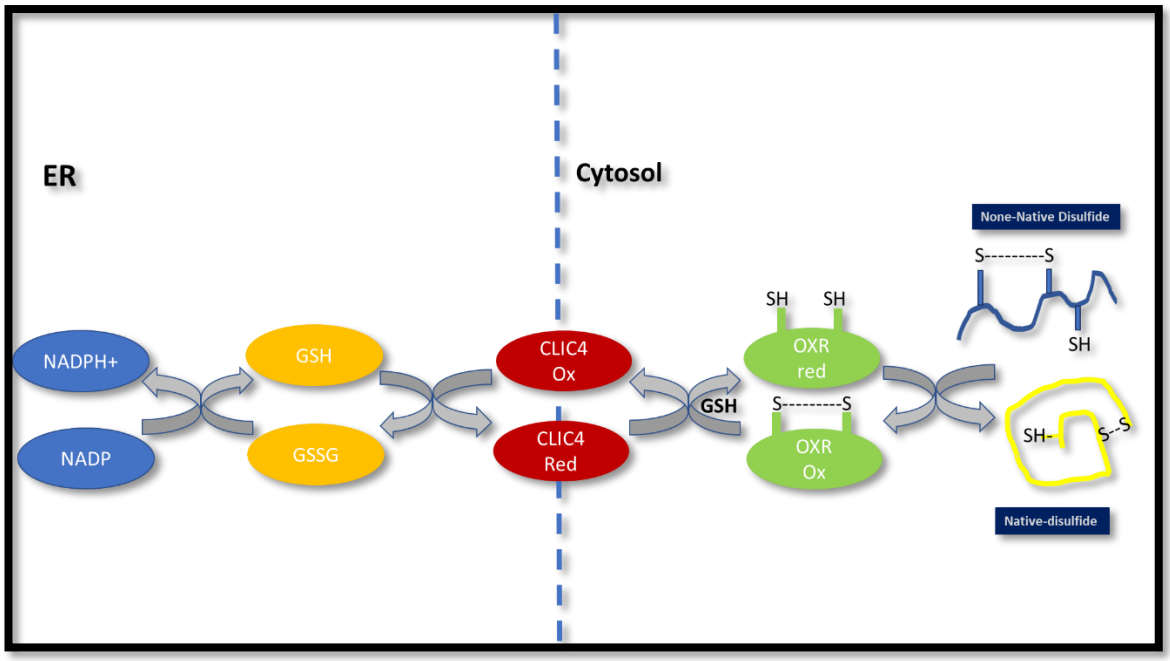


Figure 4-1 Hypotetical ER redactive pathway The cartoon shows a hypothetical reductive pathway that implicated the reduction of non-native disulfides through GSH, NADPH and a unknown binding membrane protein. CLIC4 might be considered as a good candidate for membrane binding protein because of either its ion channel activity or ezyme catalytic activity.

To uncover the potential ability of CLIC4 in participating in reductive pathway due to its oxidoreductase and ion channel activity in eukaryotic cells, in chapter 3, I examined the redox state of two family members of CLICs in mammalian cells by modification of free cysteines with the alkylating agent AMS. I showed that both CLIC1 and CLIC4 exist in a predominantly reduced form in intact cells, suggesting that they might be mostly presented in a soluble form with enzyme catalytic activity. Following oxidative stress, I demonstrated that CLIC4 is immediately reduced and recovered, demonstrating that a reductive pathway is in place to maintain CLIC4 in a reduced state. However, in this chapter I show that following oxidation, CLIC4 is not reduced in microsomes unless GSH is present. Also, here, we show that the only cytosolic component that can reduce CLIC4 could be GSH and in turn, it is reduced by cytosolic glutathione reductase. Inhibition of either glutathione reductase or glutathione synthesis prevents the reduction of CLIC4 following oxidation. In addition, following oxidative stress induced by TNF-α, CLIC4 is reduced and recovered by glutathione. I also demonstrated that glutathione rapidly reduces CLIC4 at physiological concentrations

in Bacterial cells. Furthermore, we evaluated the impact of the GSH on the redox state of CLIC4 to turn it into an activated form as a reduced state. Subsequently, to examine the specific potential of cytosolic components for facilitating reduction, we subjected the cytosolic fraction to PD10 column due to depleting low molecular mass components. To indicate which of the cytosolic reductive equivalents could be responsible for a reduction of CLIC4, I prepared the microsome fraction from HEK293T humane cell line with overexpressing CLIC4-V5 and examined several reducing components including NADPH, GSH, cysteine, and cystamine at physiological concentrations. Moreover, we indicated only GSH can reduce CLIC4 and activate the oxidoreductase activity. In the next chapter, we will evaluate the potential reduction of GSH on CLIC4 and overall protein through an advanced technology using TMT labelling with respect to protein expression. These findings define a clear role for CLIC4 as a GSH dependent oxidoreductase or a membrane binding protein in the reductive pathway to reduce non-native disulfide bonds during ER oxidative stress and the key question in the future is how to identify the membrane protein involved in the reductive pathway which be able to transfer GSH from the cytosol into the ER.

4.2 Results

4.2.1 Glutathione is the only cytosolic component that required for reduction of CLIC4-V5 following oxidative stress

Glutathione is an essential component to reduction of oxidoreductase. To identify the functional role of CLIC4 in reductive pathway and further characterize the reductive pathway, I fractionated cells to evaluate whether the reductive pathway could function in isolated cytosol, ER, and microsomal membrane. Our results indicated that the expression of CLIC4 in isolated cytosolic fraction following removal of TNF-α totally changed and appeared a translocation from the cytosol into the ER membrane (Figure 4-2, A, right panel lane 2-5), demonstrating CLIC4 could be oxidized by the addition of TNF-α and then following removal of oxidizing agent in 15 min it was recovered, suggesting cytosolic components are involved in reductive pathway and make a reduction of CLIC4 faster than membrane fraction (Figure 4-2, A, left panel lane 1-6). However, when the membrane fractions were isolated and resuspended in membrane buffer in the absence of TNF-α, no reduction of CLIC4 was observed (Figure 4-2, A, left panel, lane1). Consequently, all the factors required for the reductive pathway do not exist in the isolated membrane fraction, but I can see some recovery of CLIC4 expression in 30 minutes following removal of TNF-α that could be due to some contamination with cytosolic fraction. I repeated this experiment with microsomal fraction, and we indicated that no recovery for oxidized CLIC4 occurred in isolated microsome, demonstrating cytosolic components can be considered as a significant factor for reduction of CLIC4 and are important components for activating a reductive pathway. In addition, to investigate whether we could consider a reductive pathway in microsomal membrane to understand the functional role of CLIC4, I repeated the experiment, but this time resuspended the oxidized microsomes in some isolated cytosol components. Based on previous studies, under these conditions, the recovery of CLIC4 occurred but only under Glutathione incubation. The results indicated that reductive cytosolic equivalents are required for efficient reduction or recovery of CLIC4 following TNF-α induced oxidative stress.

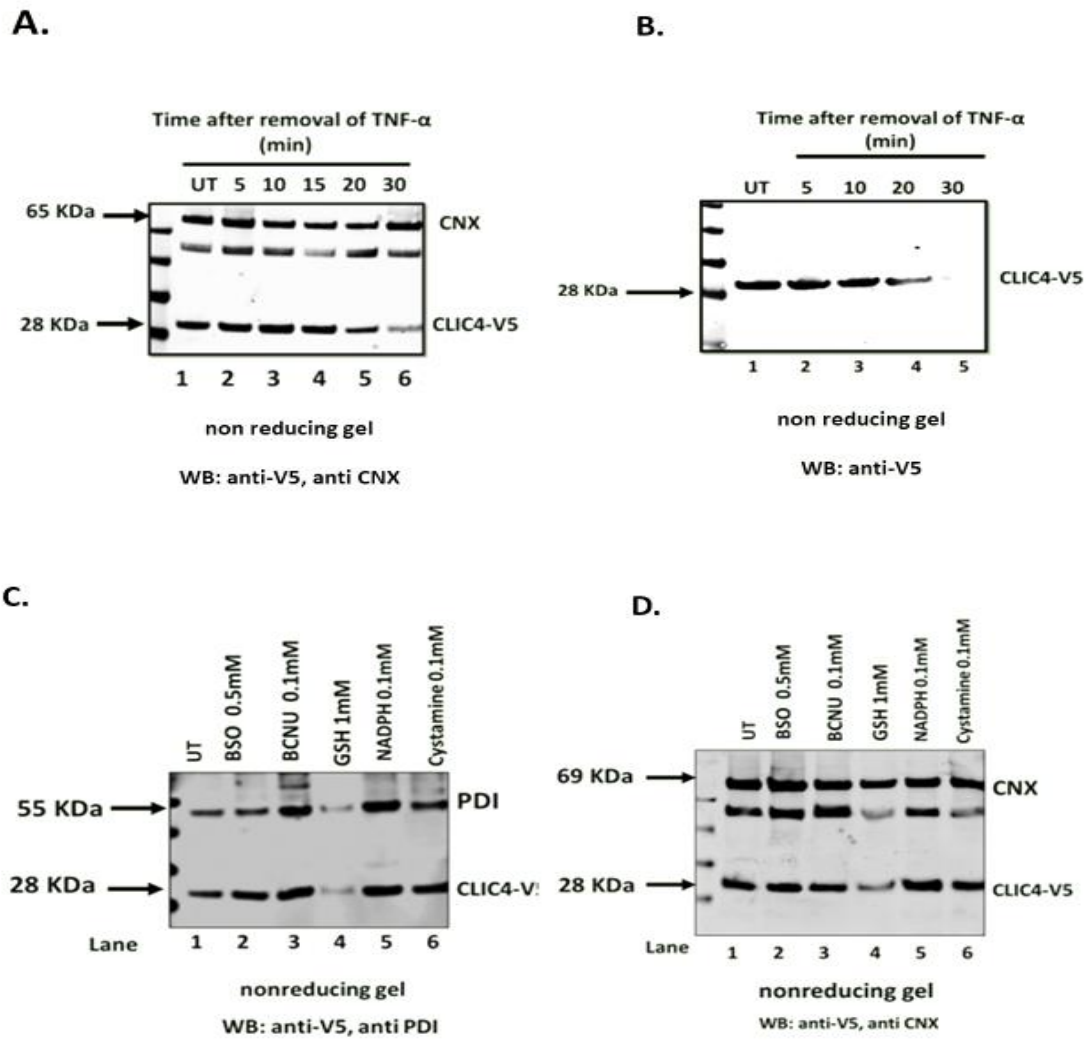


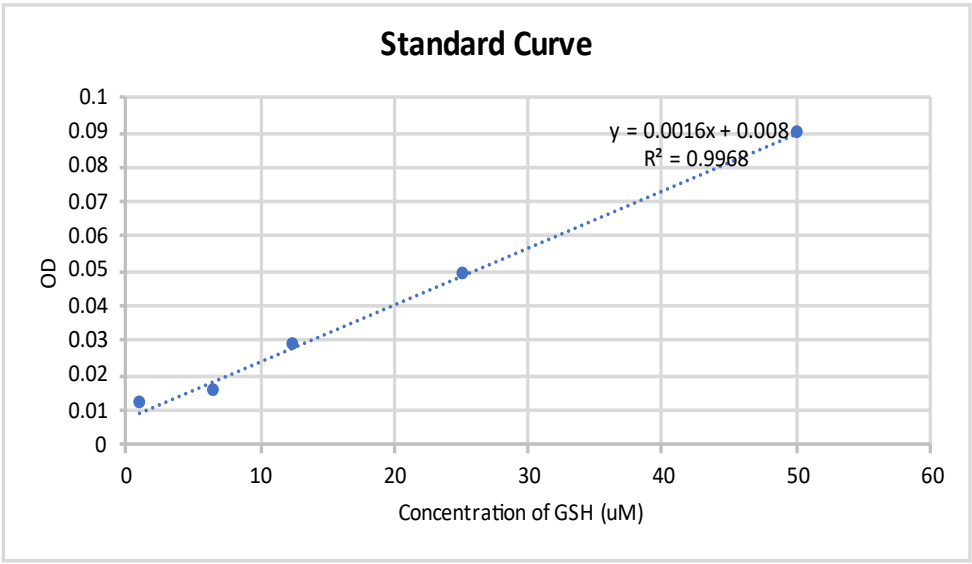
Figure. 4-2 Glutathione is the cytosolic component and essential for reduction of CLIC4 following oxidative stress induced by TNF-α. A. The redox state of CLIC4 was determined by alkylation with AMS, SDS-PAG, and western blotting with an antibody for V5 to recognise the epitope tag of CLIC4-V5. A. The membrane fractions prepared from HEK293T cells were either left untreated (lane 1) or oxidized with TNF-α (10uM) for 5 minutes (Lanes 2-6). TNF-α was removed, the redox state of oxidized membrane fractions was determined at various times. **B.** Cytosolic fractions that expressed stably recombinant CLIC4-V5 were oxidized with TNF-α. The redox state of CLIC4 was determined after 1 hour as described above**. C.** Microsomes were either left untreated (lane 1) or oxidized with 10uM TNF-α for 5 min and incubated at 37˚C for 1 hour in buffer A containing depleted cytosol fraction that dialysed with a PD10 column that previously equilibrated with buffer A and eluted in the same buffer. NADPH (1mM) as indicated, Carmustine (BCNU) (0.1mM) or BSO (0.1 mM) and cysteamine (0.1mM) were also included in addition to depleted cytosol. The samples were assayed for redox state as above **D.** Microsome vesicles were isolated from HEK293T that stably expressed the recombinant CLIC4-V5 were oxidized with TNF-α as in A described and incubated at 37˚C in buffer A (microsomal buffer) containing various low molecular mass compounds at the concentrations indicated. The redox state of CLIC4 was determined after 1 h. The species were resolved by non-reducing SDS-PAGE and transferred to nitrocellulose filters for western blotting (WB), which were hybridized with anti-CNX (top) as an ER membrane marker or anti-V5 (bottom) antibodies.

To examine the specific potential of cytosolic components for facilitating reduction, we subjected the cytosolic fraction to PD10 column due to depletion of low molecular mass components. Previous studies (Fertelli M, et al., 2002) indicated that the low molecular weight components such as GSH, NADH, NADPH, cysteine and cysteamine or proteins such as thioredoxin and glutaredoxin could contribute to the reduction of ER oxidoreductase. To identify which of those components could be responsible for CLIC4 reduction, we first examined several reducing components that are present within cytosol for their ability to reduce CLIC4 at physiological concentrations. All the compounds tested, only GSH was able to reduce oxidized CLIC4 (Figure 4-2, C and D line 4). GSH is maintained in a reduced form within the cytosol by the enzyme glutathione reductase with reducing equivalent coming by NADPH. Therefore, I examined NADPH in indicated concentration (Figure 4-2 C and D lane5), only GSH was able to efficiently reduce or recover CLIC4 following oxidative stress induced by TNF-α (Figure 4-2 C and D lane 4), suggesting that is the active component in the cytosol required for reduction of microsomal CLIC4. The recovery of reduced CLIC4 was stopped when an inhibitor of glutathione reductase (carmustine) and (BSO) glutathione synthetases were added to the cytosol (Figure 4-2 C and D, lane 3 and 4), suggesting that the reduction of GSSG to GSH is required for renovation of the reductive pathway. Moreover, no prevention of CLIC4 recovery was observed when cysteamine was added to the cytosol (Figure 4-2 Cand D, lane 6), suggesting that it is not involved in ER reductive pathway and reduction of CLIC4. The results indicated that CLIC4 can be reduced by GSH to be activated as an oxidoreductase enzyme to reduce the non-native disulfide bonds to maintain the redox balance in the ER. Please note that in the Figure 4-2-C the ER microsomal marker is PDI that has same oxidoreductase activity same as CLIC4 so I can see in both proteins affected by GSH reduction and realized with a reduction.

4.2.2 Glutathione can reduce CLIC4 in intact cells

In the previous result we demonstrated that glutathione could reduce CLIC4 using microsomal membrane assay. In this experiment, to make sure whether GSH was also involved in the reductive pathway and can be a main factor for reduction or recovery of CLIC4 following oxidative stress induced by TNF-α within intact cells. To examine this hypothesis, I either removed the intracellular GSH by inhibiting the initial enzyme in GSH synthesis (γ-glutamyl cysteine synthetase) with 0.5 mM BSO or inhibition of glutathione reductase with Carmustine one of the main inhibitors of GR (glutathione reductase) as a significant enzyme in Grx-1 pathway to produce the total cellular glutathione (Fertelli M, et al., 2002). By this approach I can prevent the reduction of GSSG following oxidative stress induced by TNF-α. The normal intracellular concentration of GSH is 10 mM and known as physiological buffer (Tavender T.J, et al., 2010); this was reduced to less than 2 mM due to the incubation with BSO; although it is enough for ER oxidoreductase, it could be insufficient for reduction or recovery of CLIC4 following oxidative stress (Panel A), (Figure 4-3).

A.



B.

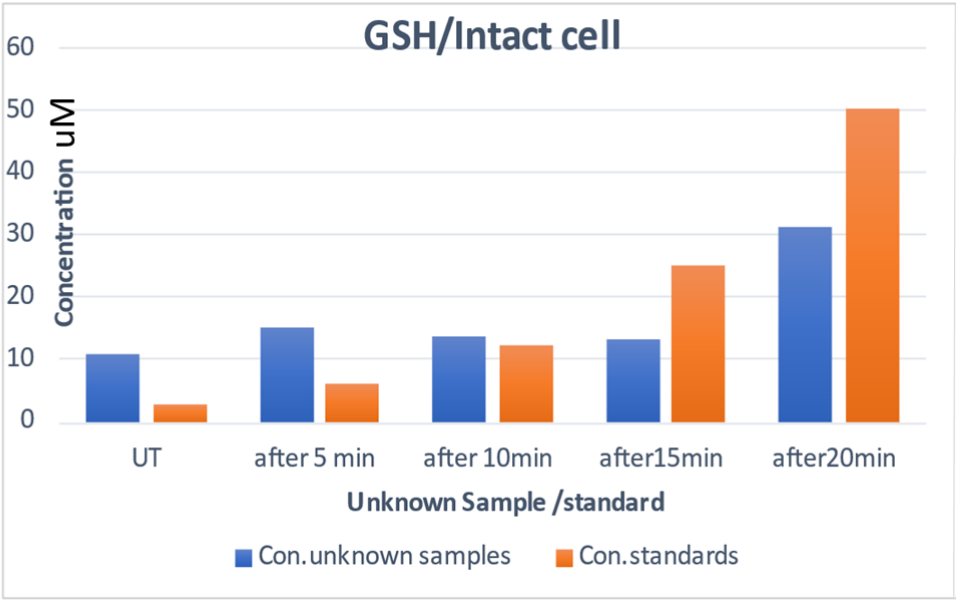
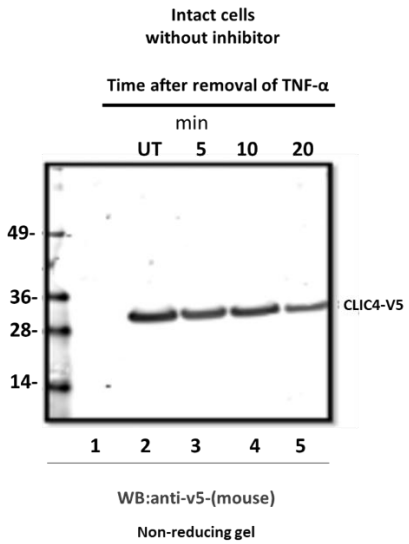


Figure 4-3(Panel A) Reduced glutathione (GSH) can reduce and recover of CLIC4 following oxidative stress induced through TNF-α. A. Standard Curve to measure the amount of GSH in intact cells**. B.** The stable HEK293T-CLIC4-V5 cell lines were either left untreated or treated for oxidation with (TNF-α 10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were allowed to recover in the fresh medium. The total glutathione concentration was measured essentially through a Glutathione kit to detect a range of 0.01-3µM GSH to 10nM GSH equivalents using Ellman's Reagent (DTNB) and glutathione reductase (GR). The cells were lysed in 8mM HCl, 1.3% (w/v) 5-sulfosalicylic acid on ice for one hour. The proteins were removed by centrifugation. The acidified sample was added to 0.2 mM NADPH, 0.6 mM Ellman's reagent, glutathione reductase (1–2 units/ ml), 150 mM phosphate buffer, pH 7.4. The rate of

absorption at 412 nm was measured over 1 min with respect to GSH standards. GSH concentrations were calculated by a formula provided by manufacturer instruction.



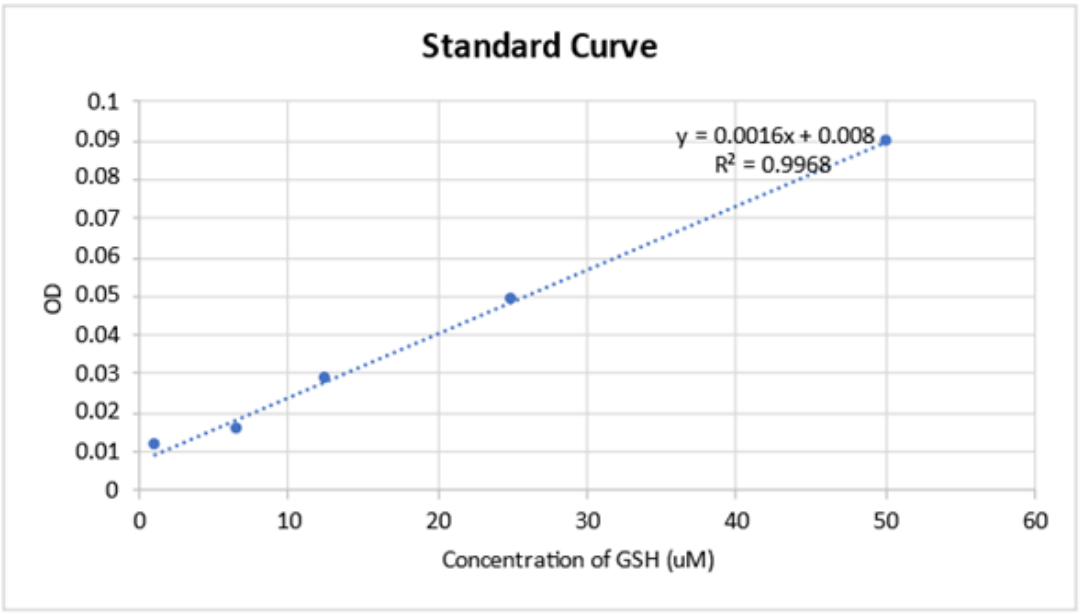
C. In addition, for understanding of translocation of CLIC4 in response to Glutathione level alteration, we first examined the intact cells in the absence of Glutathione inhibitors that were left untreated or treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were allowed to recover in fresh medium cells were subjected to alkylation with AMS, SDS-PAGE, and western blotting with anti-V5 antibody.

In the previous experiment, we indicated that GHS was the only cytosolic equivalent component that was able to reduce CLIC4 under oxidative conditions. In this experiment, we examined the amount of GSH before and after the removal of oxidative stress induced by TNF-α at various time points to understand the consumption of GSH for recovering CLC4. The result indicated that CLIC4 was recovered from an additional oxidizing agent after 20 minutes of removing TNF-α (Figure 4-3, C), conversely, the amount of GSH was dramatically increased, suggesting the consumption of GSH for reduction of GSH in 20 min after removal of TNF-α was stopped, demonstrating the total amount of CLIC4 was reduced and no need for extra GSH for reduction. However, the process of reduction was observed with depletion of CLIC4 during time point of oxidative stress, suggesting CLIC4 can re-localize between subcellular organelle for its membrane binding potential, notably from the cytosol into ER.

4.2.2.1 BSO inhibits reduction of CLIC4 and consequently inhibit translocation of CLIC4 from the Cytosol into ER.

To determinate the role of GSH in re-localization of CLIC4 from the cytosol in response to oxidative stress, before isolating membrane and cytosolic fractions, HEK293T cell line which stably overexpresses CLIC4-V5, was treated with (0.5mM BSO) for inhibiting of Glutathione synthetase and incubated at 37'C for overnight. On the next day, the Glutathione inhibitor was removed, and cells were allowed to recover in a fresh medium, then cells were left untreated and treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were washed in the fresh medium. After fractionation of cells, samples were subjected to AMS modification and western blot analysis. Our result indicated that BSO, not only inhibit of reduction of clic4 but also can inhibit of re-localization of CLIC4 from the ER into cytosol, suggesting GSH can involve in reduction and translocation of CLIC4 between subcellular organelles (Panel B), (Figure 4-3, panel B)

(Panel B)



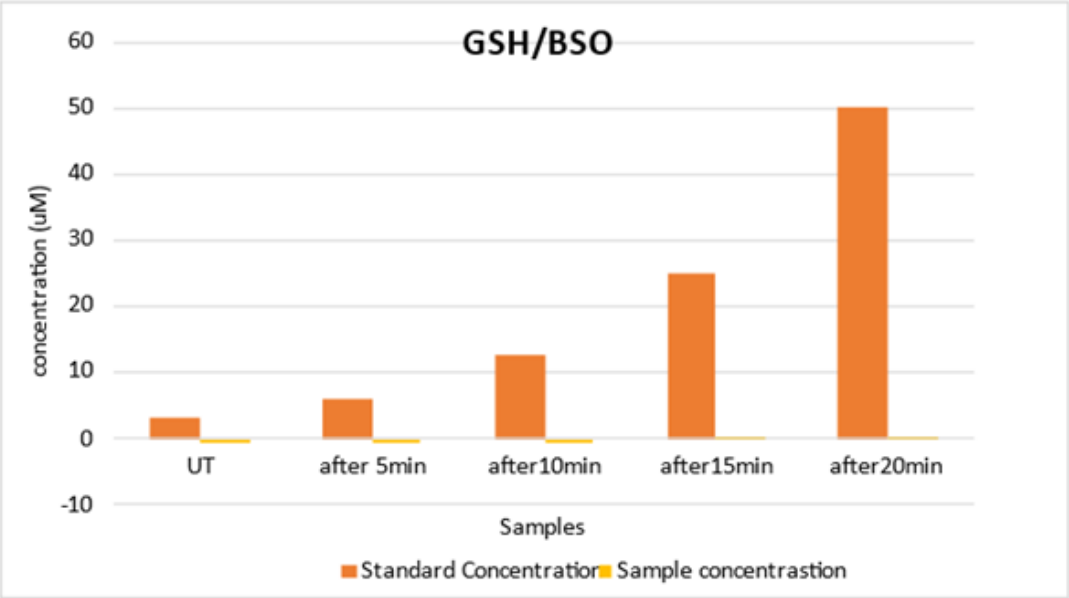
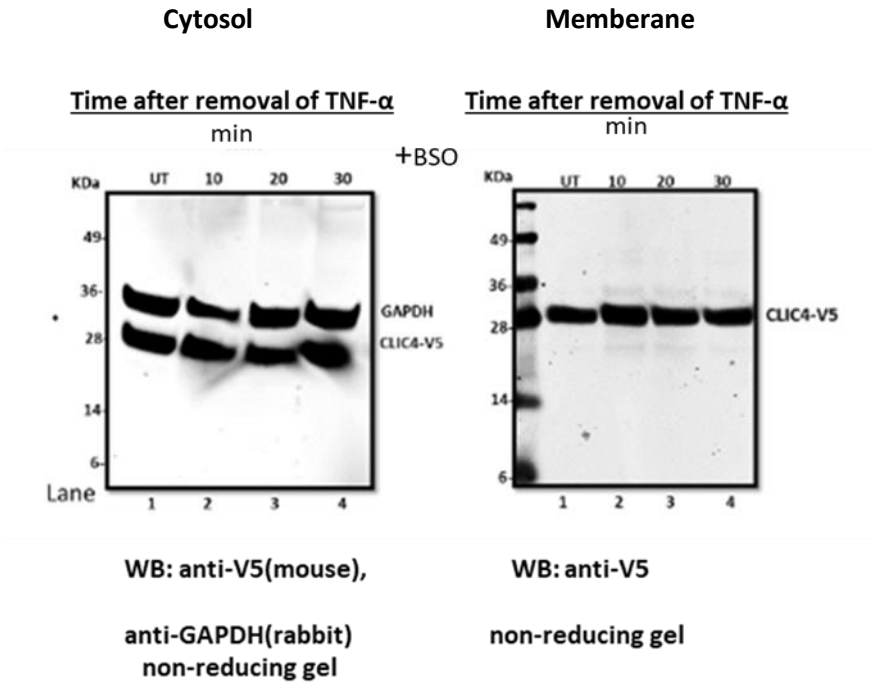


Figure 4-3 (Panel B) The reduction and recovery of CLIC4 following oxidative stress induced through TNF-α can avert by the inhibition of GSH in mammalian cells , A. Standard Curve to measure the GSH under (0.5mM BSO) treatment. **B.** The stable HEK293T-CLIC4-V5 cell line was first treated with (0.5mM for BSO) and incubated at 37'C overnight. Then cells were washed with fresh medium and were either left untreated or treated with (TNF-α 10uM) for 5 minutes. The oxidizing agent was removed at various time points and the cells were allowed to recover in a fresh medium. The total glutathione concentration was measured essentially through a Glutathione kit to detect a range of 0.01-3µM GSH to 10nM GSH equivalents using Ellman's Reagent (DTNB) and glutathione reductase (GR). The cells were lysed in 8mM HCl, 1.3% (w/v) 5-sulfosalicylic acid on ice for 1 h. The proteins were removed by centrifugation. The acidified sample was added to 0.2mM NADPH, 0.6mM Ellman's reagent, glutathione reductase (1–2 units/ml), 150 mM phosphate buffer, pH 7.4. The rate of absorption at 412nm was measured over 1 min with respect to GSH standards. GSH concentrations were calculated by the formula that was provided by manufacturer instruction. At various intervals, the level of GSH was determined using Ellman's reagent that was provided in the glutathione kit.



C. In addition, for understanding the translocation of CLIC4 in response to Glutathione level alteration, we isolated membrane and cytosolic fractions. The stable HEK293T-V5 cell line was first treated with (0.5mM BSO) and incubated at 37'C overnight .

On the next day, the Glutathione inhibitor was removed, and cells were allowed to recover in a fresh medium, then cells were left untreated and treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were washed in the fresh medium. Before the lysis of the cells, cells were subjected to the same purification pathway for membrane and cytosolic fractionation. Following treatment, cells were washed with PBS supplemented with an EDTA-free protease inhibitor tablet and centrifuged for 3 min at 300 rpm, then cells were resuspended in 1ml ice-cold buffer A for 10 min. Cells were lysed by passing through a 1ml syringe, 30 times. The remaining sample was spun at 1000g, for 7 min at 4 °C. Pellet was a nuclear fraction; the supernatant contains cytosol and membrane. The supernatant was spun at 100,000g for 30 min at 4°C. The supernatant was removed to a fresh 1.5ml tube that was the cytosol and precipitated with ice-cold acetone (5x volume) and the cytosolic fraction was resuspended in 100ul specific cytosolic buffer in the presence of protease inhibitor and analyzed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and GAPDH (C. Left panel). The pellet from the last ultracentrifuges was the membrane fraction and resuspended in 100ul specific membrane buffer in the presence of protease inhibitor and analyzed by reducing SDS-PAGE and western blotting (WB) with anti-V5 antibody.

4.2.2.2 Carmustin inhibits reduction of CLIC4 and consequently inhibit translocation of CLIC4 from the Cytosol into ER.

To determinate the role of GSH in re-localization of CLIC4 from the cytosol in response to oxidative stress, before isolating membrane and cytosolic fractions, HEK293T cell line which stably overexpresses CLIC4-V5 treated with (0.1mM BCUN) for 3 hours and incubated at 37'C before treating the cells, the Glutathione inhibitor(inhibitor of glutathione reductase) was removed, and cells were allowed to recover in a fresh medium, then cells were left untreated and treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were washed with the fresh medium. Before fractionation to cytosol and membrane. samples were subjected to AMS modification and western blot analysis. Our result indicated that BUCN (Carmustin), not only inhibits of reduction of CLIC4 but also can inhibit of re-localization of CLIC4 from the ER into cytosol, suggesting GSH can involve in reduction and translocation of CLIC4 from cytosol into the ER (Figure 4-3, panel C)

A.

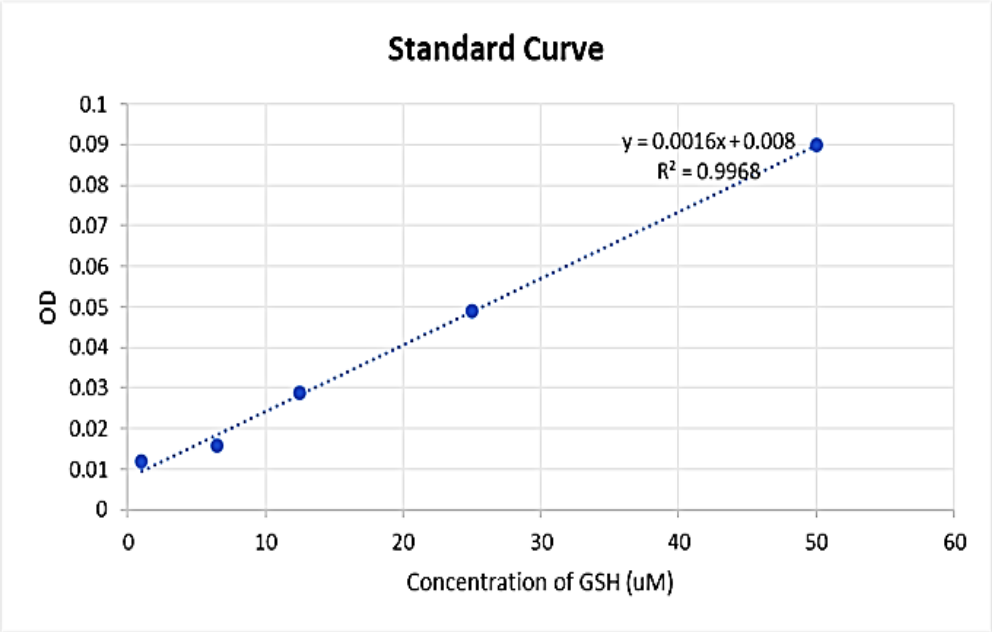
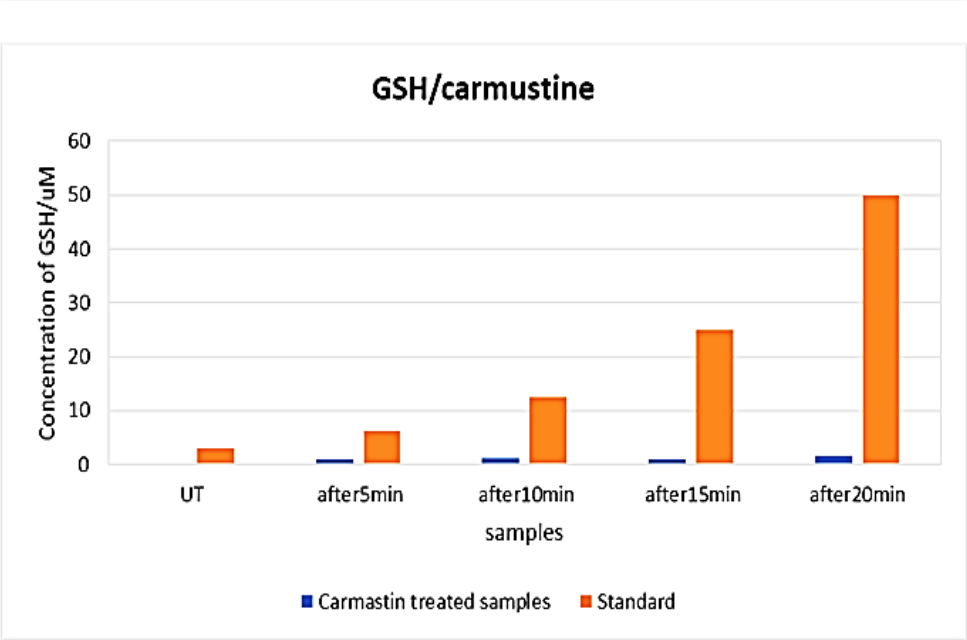


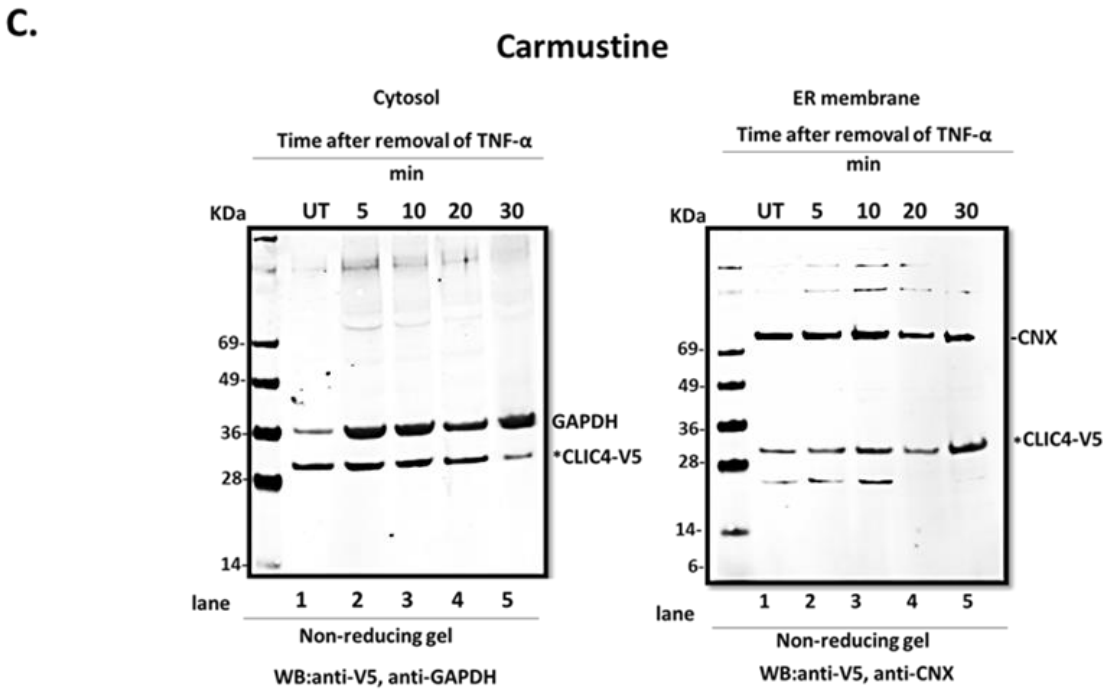
Figure 4-3 (Panel C), -inhibition of reduction of glutathione in vivo condition can avert the reduction and recovery of CLIC4 following oxidative stress induced through TNF-α. A. standard Curve to measure the GSH under (0.5mM BSO) treatment.

B.



B. The stable HEK293T-CLIC4-V5 cell line, was first treated with (0.5mM for BSO) and incubated at 37'C overnight. Then cells were washed with fresh medium and were either left untreated or treated with (TNF-α 10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were allowed to recover in a fresh medium. The total glutathione concentration was measured essentially through a Glutathione kit to detect a range of 0.01-3µM GSH to 10nM GSH equivalents using Ellman's Reagent (DTNB) and glutathione reductase (GR). The cells were lysed in 8mM HCl, 1.3% (w/v) 5-sulfosalicylic acid on ice for 1

h. The proteins were removed by centrifugation. The acidified sample was added to 0.2mM NADPH, 0.6mM Ellman's reagent, glutathione reductase (1–2 units/ml), 150 mM phosphate buffer, pH 7.4. The rate of absorption at 412nm was measured over 1 min with respect to GSH standards. GSH concentrations were calculated by a formula that was provided by manufacturer instruction. At various intervals, the level of GSH was determined using Ellman's reagent that was provided in the glutathione kit.



C. In addition, for understanding the translocation of CLIC4 in response to Glutathione level alteration, we isolated membrane and cytosolic fractions. The stable HEK293T-V5 cell line was first treated with (0.1mM BCUN) for 3 hours and incubated at 37'C before treating the cells, the Glutathione inhibitor was removed, and cells were allowed to recover in a fresh medium, then cells were left untreated and treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed at several time points and the cells were washed with the fresh medium. Before the lysis of the cells, cells were subjected to the same purification pathway for membrane and cytosolic fractionation. Following treatment, cells were washed with PBS supplemented with an EDTA-free protease inhibitor tablet and centrifuged for 3 min at 300 rpm, then cells were resuspended in 1ml ice-cold buffer A for 10 min. Cells were lysed by passing through a 1ml syringe, 30 times. The remaining sample was spun at 1000g, for 7 min at 4 °C. Pellet was a nuclear fraction; the supernatant contains cytosol and membrane. The supernatant was spun at 100,000g for 30 min at 4°C. The supernatant was removed to a fresh 1.5ml tube that was the cytosol and precipitated with ice-cold acetone (5x volume) and the cytosolic fraction was resuspended in 100ul specific cytosolic buffer in the presence of protease inhibitor and analyzed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and GAPDH (c. Left panel). The pellet from the last ultracentrifuges was the membrane fraction and resuspended in 100ul specific membrane buffer in the presence of protease inhibitor and analyzed by reducing SDS-PAGE and western blotting (WB) with anti-V5 and CNX antibody.

In the last experiment, I identified that GSH can reduce CLIC4 using the microsomal assay, now we want to determine whether GSH was also involved in the reductive pathway within intact cells. To do this, we depleted the intracellular pool of GSH by inhibiting the initial enzyme in GSH synthesis (β-glutamyl cysteine synthetase). Although previous studies indicated that this level of GSH could be appropriate for the reduction of the oxidoreductase enzyme and keeping them in a reduced form at the steady state in the redox condition (Fratelli M, et al., 2002), our result illustrated that the impact of decreased GSH on the recovery of CLIC4 from oxidation after 20 minutes (Figure 4-2-2, (Panel B), B, C). The addition of Carmustine to the cells following the removal of TNF-α inhibited the recovery of GSH from GSSG, hence, the level of GSH still was at a decreased level (Figure 4-2-2 (Panel C), B). The inhibition of GSH recovery also prevented any reduction of CLIC4 following removal of TNF- α (Figure 4-2-2 (Panel C), C), obviously demonstrating that for CLIC4 with its oxidoreductase activity to persist in a reduced position, the main requirement is the presence of intracellular GSH. Moreover, the time point recovery of the intracellular pool of GSH following treatment with TNF-α (Figure 4-2-2, (Panel A), B) is related to the reduction of CLIC4 (Figure 4-2-2, (Panel A), C). Therefore, these results demonstrate that the levels of intracellular GSH have a significant effect on recovery of CLIC4 following oxidative stress and this can occur through the reductive pathway within the SP or intact cells.

On the other hand, the result indicated that the inhibition of GSH recovery in the mammalian cells through (BCUN) and (BSO) at two inhibitors of Glutathione reductase (GR) and Glutathione synthetase (GS) shows a significant effect on the translocation of CLIC4 from the cytosol into the ER membrane and no cytosolic mis localization was absorbed, suggesting depletion of GSH not only has an effect on the reduction of CLIC4 after removal of an oxidizing agent but also can prevent translocation of CLIC4 into ER membrane. In conclusion, GSH can play a significant role in the reduction of CLIC4 either for its oxidoreductase activity or its ion channel activity.

4.2.3 NADPH does not show any reduction of CLIC4 in intact cells

In the previous result, we demonstrated that glutathione could reduce CLIC4 using a microsomal membrane assay. In this experiment, to make sure whether only GSH as a cytosolic equivalent can be considered for reducing CLIC4, we examined the level of NADPH, which was also involved in the reductive pathway and can be the main factor for the reduction or recovery of CLIC4 following oxidative stress induced by TNF-α within intact cells. To examine this hypothesis, we measure the amount of NADPH at several points after the removal of TNF-α. With this approach, we can evaluate the effect of NADPH on the reduction of CLIC4. The normal intracellular concentration of the total NADPH concentration in the cytosol is 3.1-0.3, and after treatment with TNF-α, it can increase but doesn't show any reduction on CLIC4 after removal of TNF-α or recovery of CLIC4 following oxidative stress.

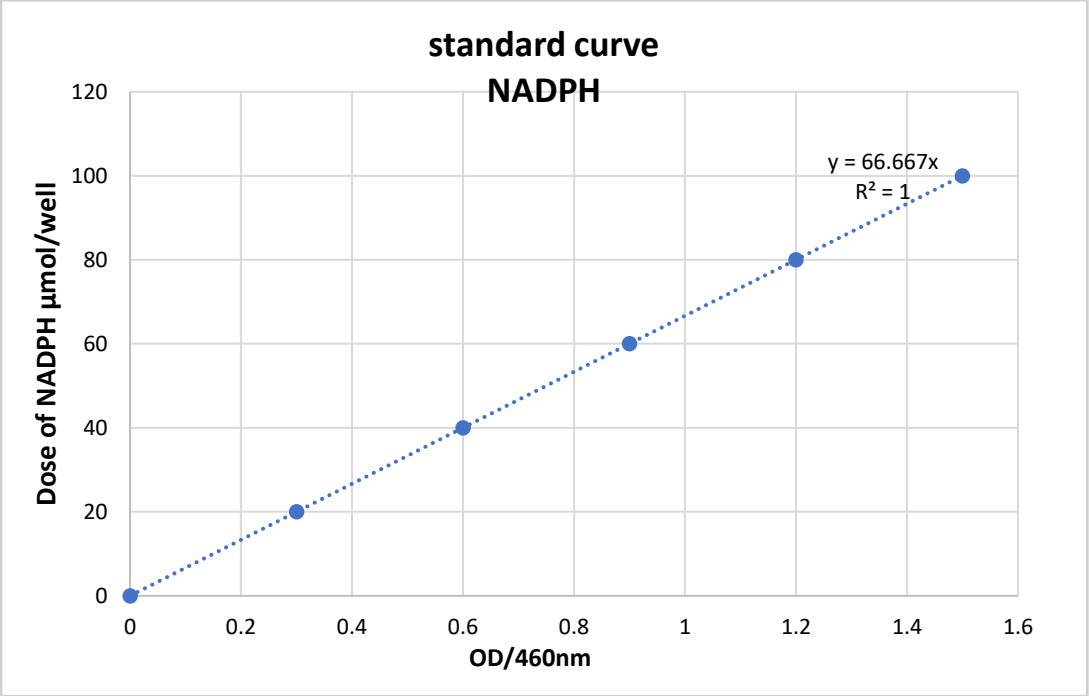


Figure 4-4 NADPH standard curve NADPH was measured with the Colorimetric NADPH Assay Kit in a 96-well white/clear bottom plate using a microplate reader. As low as 3 µM of NADPH can be detected with 30 minutes of incubation with absorbance measurement at 460nm.

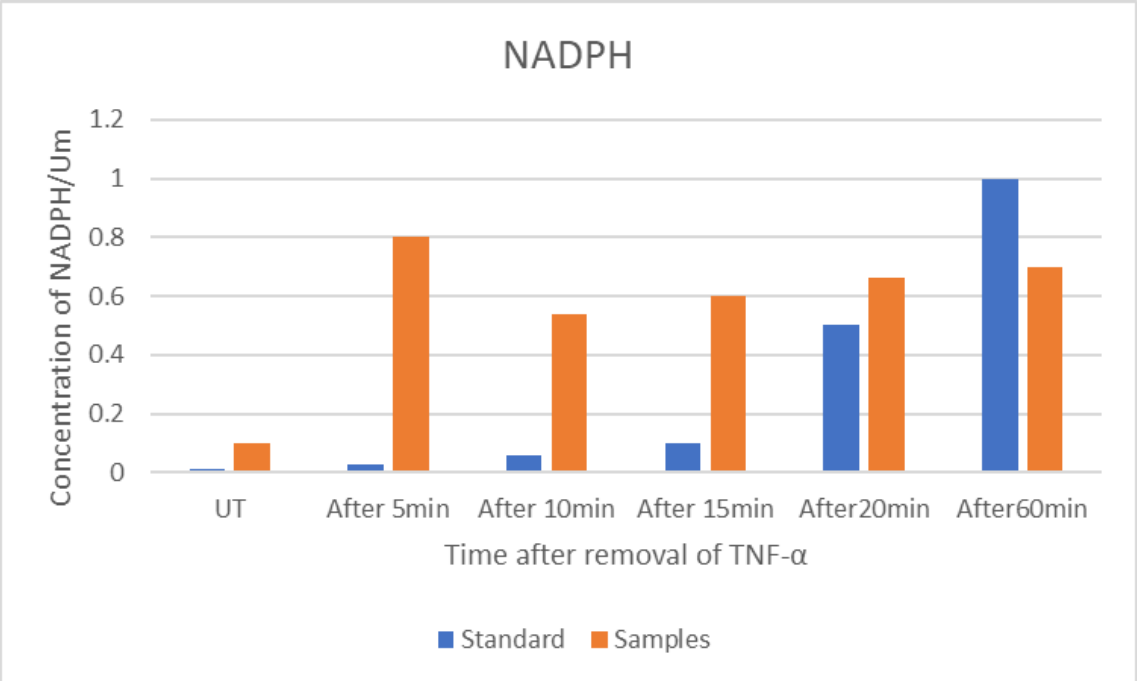


Figure 4-4-1 NADPH not able to reduce CLIC4 in mammalian cells The stable HEK293T-CLIC4-V5 cell lines were either left untreated or treated with (TNF-α 10uM) for 5 minutes. The oxidizing agent was removed in several time points and the cells were allowed to recover in fresh medium. The amount of NADPH was measured using colorimetric NADPH Assay Kit. The NADPH probe is a chromogenic sensor that has its maximum absorbance at 460 nm upon NADPH reduction. The absorption of the NADPH probe is directly

proportional to the concentration of NADPH in the solution and provides a sensitivity to detect as little as 3µM NADPH in a 100µL assay volume. In this assay we first prepared NADPH reaction mixture and then added to NADPH standards or test samples. The plate reader incubated at room temperature for 15-120 minutes before evaluating and monitoring the absorbance at 460 nm. Our result indicated that after removal of TNF-α, no change appeared in the NADPH concentration and the amount of NADPH increased after oxidation the cells and no alteration of the NADPH concentration was observed after removal of TNF-alpha, suggesting compared to GSH, NADPH as a cytosolic reductive pathway does not have a significant effect on reduction of CLIC4.

4.2.4 Glutathione can directly reduce CLIC4-V5 in mammalian cells

To determine whether GSH can directly reduce CLIC4 in vivo (within cells), we first examined whether the reduction of CLIC4 in microsomes was depended on the concentration of combined GSH. Increasing the concentration of GSH from 1 mM (that we previously examined) to 10 mM increased the rate of reduction of CLIC4 (Figure 4-5, A and B), consistent with a direct role for GSH in reducing CLIC4 and activating CLIC4 for its oxidoreductase function in the ER to cope with oxidative stress by reduction of non-native disulfide bond following the TNF-α treatment (Xeu X, et al., 2005).

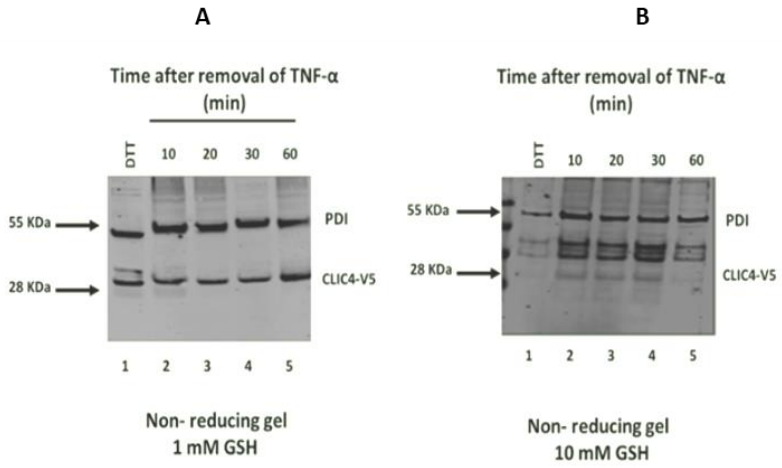


Figure 4-5 Glutathione directly reduces CLIC4-V5 A. Microsomes were reduced with 5mM DTT (lane 1) or oxidized with 10uM TNF-α and the oxidized agents were removed with resuspension in microsomal buffer and quick spin to remove supernatant and resuspended the pellet in microsomal buffer A and then incubated in (1mM) GSH at 37'C for 1h (lanes 2-5). The redox state of CLIC4-V5 was determined at various times with subjection to AMS modification, SDS-PAGE, and western blotting with anti-V5 and anti-CNX. **B.** The microsomes were prepared from the stable HEK293T-CLIC4-V5 cell lines and reduced with 5mM DTT (lane 1) or oxidized with TNF-α (lane 2-4) and then incubated for 5 minutes. The oxidizing agent was removed in the

several point time with resuspension of the microsomes in the microsomal buffer and quick spin to remove supernatant and resuspended the pellet in microsomal buffer A and incubated with (10mM) GSH at 37'C for 1 h. The redox state of CLIC4-V5 was determined at various times by subjection to AMS modification, SDS-PAGE, and western blotting with anti-V5 and anti-PDI.

To determine whether GSH can directly reduce CLIC4, we need to evaluate whether the reduction of CLIC4 in microsomes occurred when GSH was added according to concentration. With the increase in the amount of added GSH from 1 to 10 mM the rate of reduction of CLIC4 increased (Figure 4-5, A and B), according to a particular role for GSH for reduction of CLIC4 as an oxidoreductase to convert to active form for isomerization or reduction of non-native disulfide bonds in the ER. On the other hand, CLIC4 as an ion channel with oxidoreductase activity to be capable of transferring cytosolic components or other reductive equivalents to the ER, it needs to be reduced as well, hence the reduction of CLIC4 via GSH was important for the potential function of CLIC4 in the ER.

4.2.5 GSH can reduce purified recombinant CLIC4 in Bacterial cells

The His-tagged CLIC4 was constructed on PET 23a (+) that was modified with TEV-His tag (cysteine protease cleavage) for purification with His tag gravity column and was provided in the Woolhead lab at University of Glasgow. To evaluate whether GSH could reduce purified recombinant CLIC4, we first oxidized the protein with TNF-α and then removed the excess TNF-α (oxidizing agent) by gel filtration and followed the reduction of oxidized protein either in the absence or presence of 10 mM GSH (Figure 4-6). Disulfide status was determined at individual time points by first quenching disulfide exchange by acidification followed by treatment with AMS. Hence using this approach, a decrease in electrophoretic mobility indicates reduction of disulfide bonds. In the absence of GSH no reduction of purified protein was observed. However, in the presence of GSH the reduction of CLIC4 can occur faster than in the absence of GSH (Figure 4-6, panel E). Note: these experiments included 5 steps: Purification of CLIC4-His (Panel A), SEC of CLIC4-His (Panel B), SEC of CLIC4-His and TNF-alpha to remove TNF-alpha (Panel C), check fractionation and MALS examination to confirm remove of TNF-alpha and check the size of CLIC4-His (Panel D), then examination of reduction of CLIC4-His by GSH in the bacterial cells (panel E).

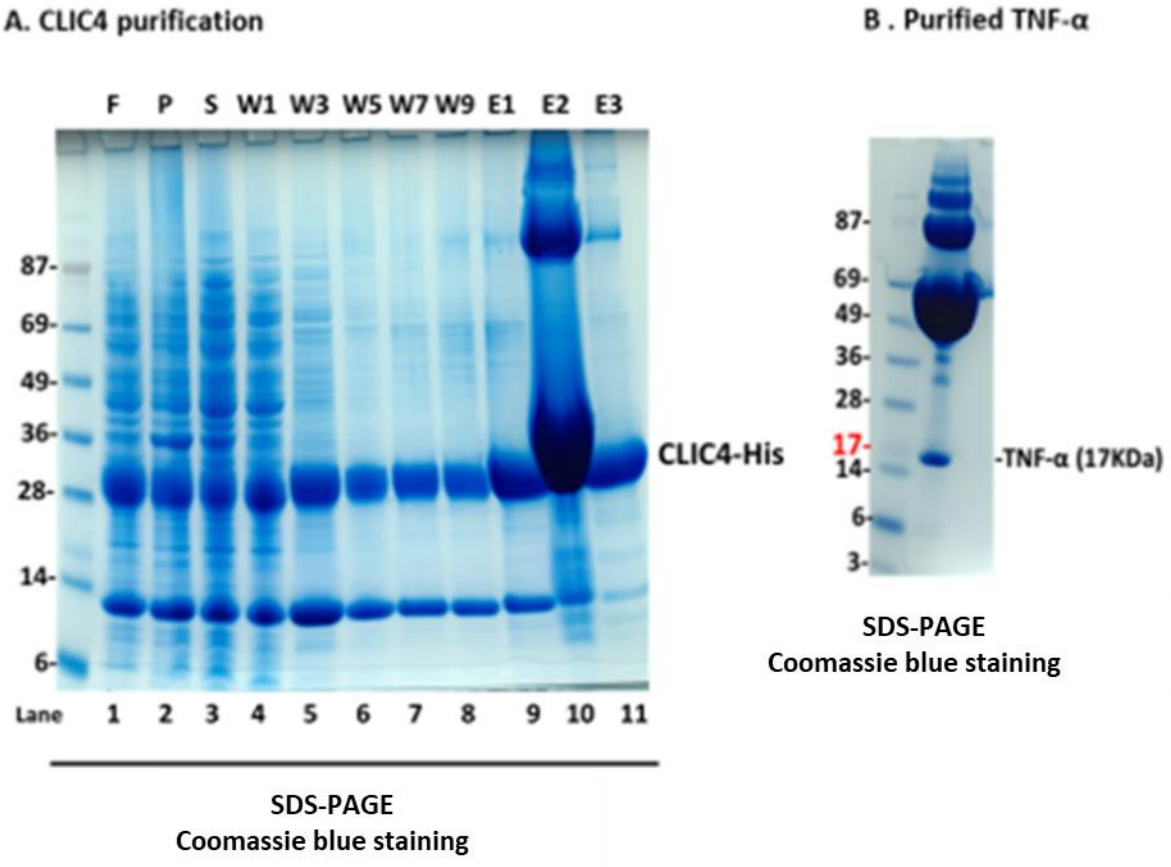
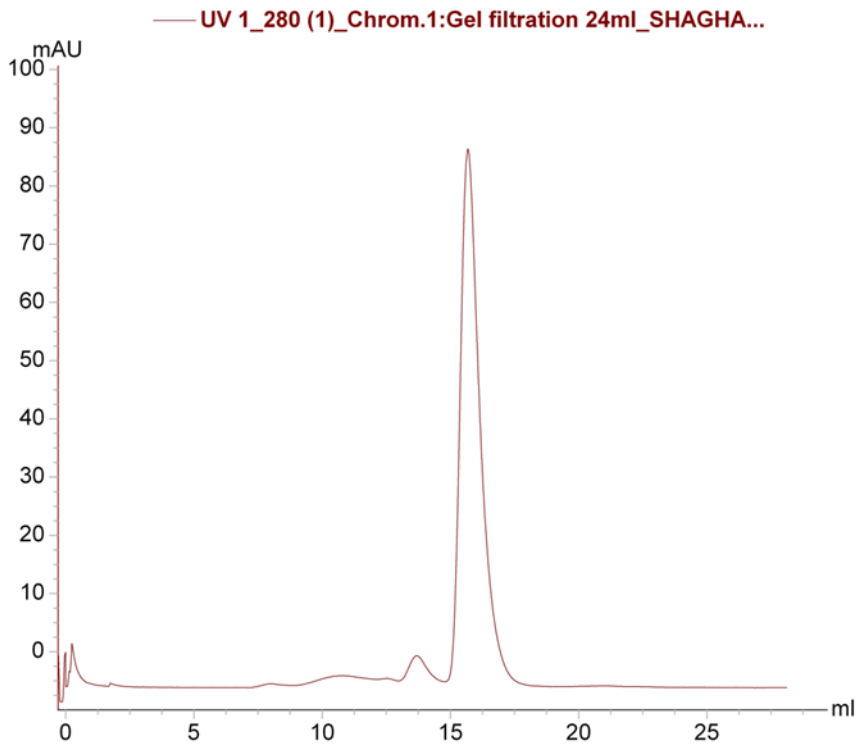


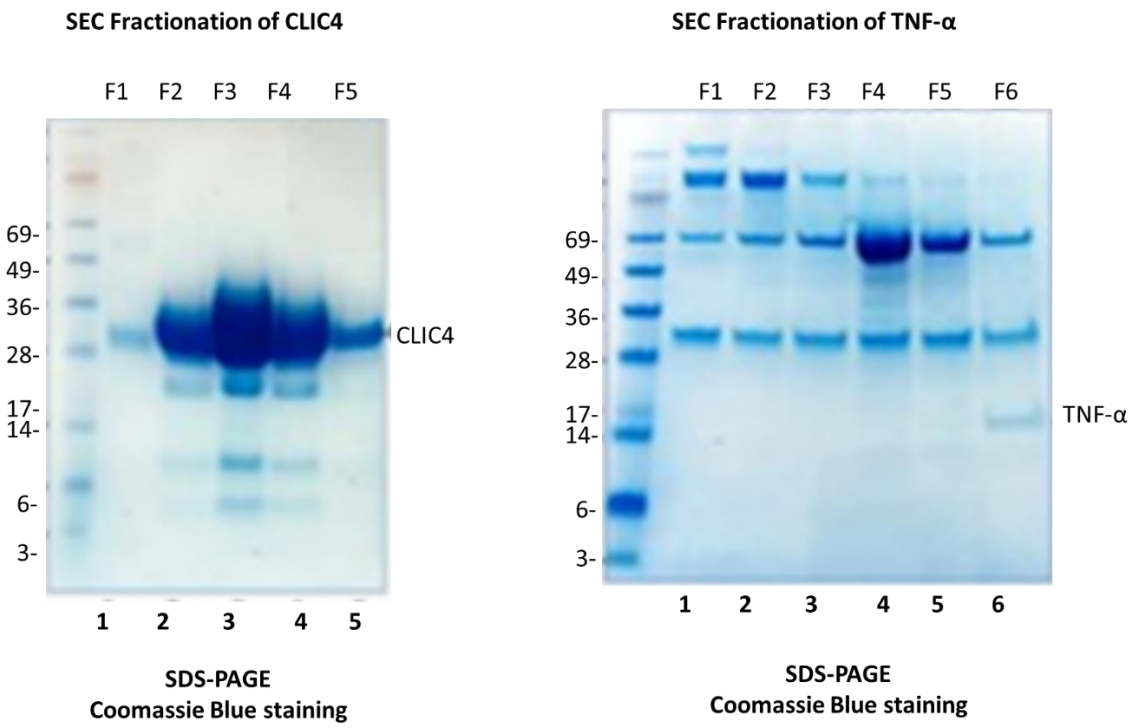
Figure 4-6-A GSH can reduce purified recombinant CLIC4 in Bacterial cells the Escherichia coli strain BL21(DE3) PLysS containing a modified version of PET32 a (+), expressing His-tagged CLIC4 that modified with TEV (cysteine cleavage protease) + His tag and purified by His60NiGravity Columns through standard protocol in the Woolhead lab at Glasgow University. To purify CLIC4, we first inoculate 5ml containing 5ul of AMP (from stock 100mg/ml) with a glycerol stock scrapping per 500ml of LB and then incubate at 37'C for overnight. On the next day, we inoculated 500ml of LB, which contained 500ul of AMP with 5ml of overnight culture and incubated at 37 in 200 rpm and checked the OD600 to reach about 0.6 at this OD we can induce the IPTG (1mM from 1Mstock), then we need to reduce the temperature to 30'C to allow for 4h. Then pellet the cells by centrifugation at 10000 rpm for 10 min at 4℃. Decant LB from the centrifuge tube and resuspend cells in 20ml 1XTBS buffer containing 1EDTA-freeprotease inhibitor tablet and 20mg of lysozyme from stock 100mg/ml. At this stage, we need to incubate the cells in ice for 30 min to allow the cell wall of bacterial cells to be easily broken via French press with 2 passes at 8000psi for lysing cells and centrifuge the cells to pellet out debris at 17000rpm for 30 min at 4C. To purify the CLIC4-His, we used His-binding nickel column, and equilibrate a His-binding nickel column with 10 bed-volume of binding buffer (40mMTris, 300mM NaCl, PH8). To wash the column, we applied the supernatant to the Ni-column and wash Ni-column with 10 bed-volumes of Wash buffer (40mMTris,300mMNaCl, adding 20mM imidazole). To elute the protein, we need to add 3 bed-volume of Elution buffer and collecting separately at a fresh Eppendorf tube. Also, for quantity the protein concentration using the Nano-Drop, also, we applied extinction coefficient of CLIC4 that at 0.1% is 0.7. This will give us a more accurate concentration. Note: before subjecting His-tagged purified CLIC4 to SEC, protein needs to be dialyzed from extra salt for this prepose used Slide-A-Lyzer dialysis Cassette (10,000MW)

in 3x1.5 L in (a commercial PBS, PH7.4 at 4'C with stirring). Also, the commercial TNF-α was subjected to SDS-PAGE Coomassie blue staining to determine the expression and the size of protein (Figure 4-6-B).



a.

Figure 4-6-B GSH can reduce purified recombinant CLIC4 in bacterial cells (a) To purified CLIC4 from oxidizing agent, we applied the (Size exclusion chromatography (SEC) or gel filtration) to remove TNF-α from CLIC4 upon its size by passing through a resin packed column. SEC works upon kind of resins as consist of a matrix of beads with lacking reactivity. Following entering of the sample into the column, the larger molecules than the pores are unable to diffuse into the beads, so they elute first. Molecules that located within this range of size between the very big and very small can penetrate the pores upon their size and able to be isostatically via (mobile phase) be eluted and no need to use different buffer. For performing the size exclusion chromatography, we need to purify and dialyze the protein with Slide-A-Lyzer dialysis Cassette (10,000MW) in 3x1.5 L in (a commercial PBS, PH7.4 at 4'C with stirring). Collecting CLIC4 from dialysis cassette and using a VIVASPIN 20ML CONCENTRATOR (Vs20389) and again centrifuged proteins at 300xg for 3min before subjected to a gel filtration with the same buffer (commercial PBS) that caried out with superposed 12 (HR10/30) column in this SEC experiment. (SEC was performed in the Protein lab by permission Prof. Blatt andby Dr. Waghmare).



b. SDS-PAGE Coomassie blue staining of Fractionation SEC purified CLIC4-His (left panel), and TNF -alpha in right panel.

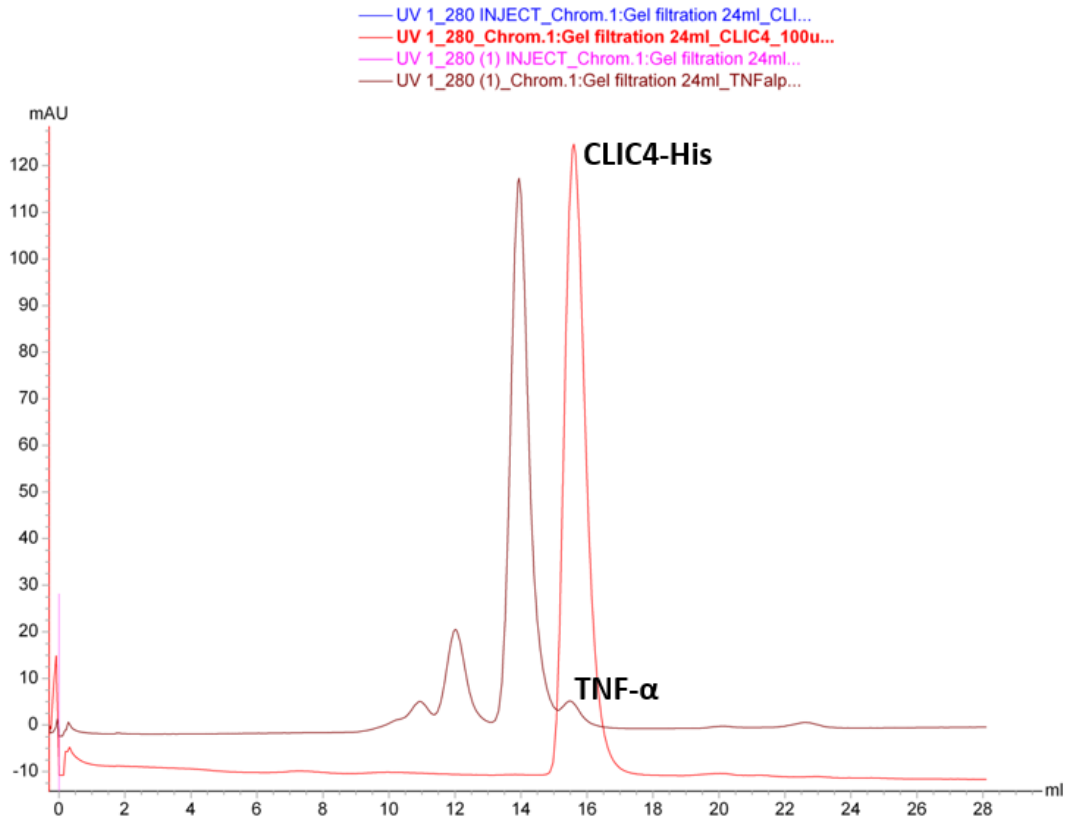


Figure 4-6-C GSH can reduce purified recombinant CLIC4 in Bacterial cells. The purified His-tagged CLIC4 was either treated with 10mM DTT or oxidized with (10uM) TNF-α, oxidizing agent was removed in several

time points by size exclusion chromatography to remove of TNF-α from CLIC4. Sample volumes of 0.5% to 4% of the total column volume are applied at low flow rates using long columns, often 60 cm or longer. As the separation takes place in only 1 CV, it is essential to have a well packed column for good results in SEC. The result indicated that no aggregation was absorbed with CLIC4 and TNF-alpha, when samples were observed at a Coomassie-stained SDS-PAGE gel, indicating TNF-α was removed and only CLIC4 was eluted at a correct size and no evidence for interaction between two proteins was absorbed.

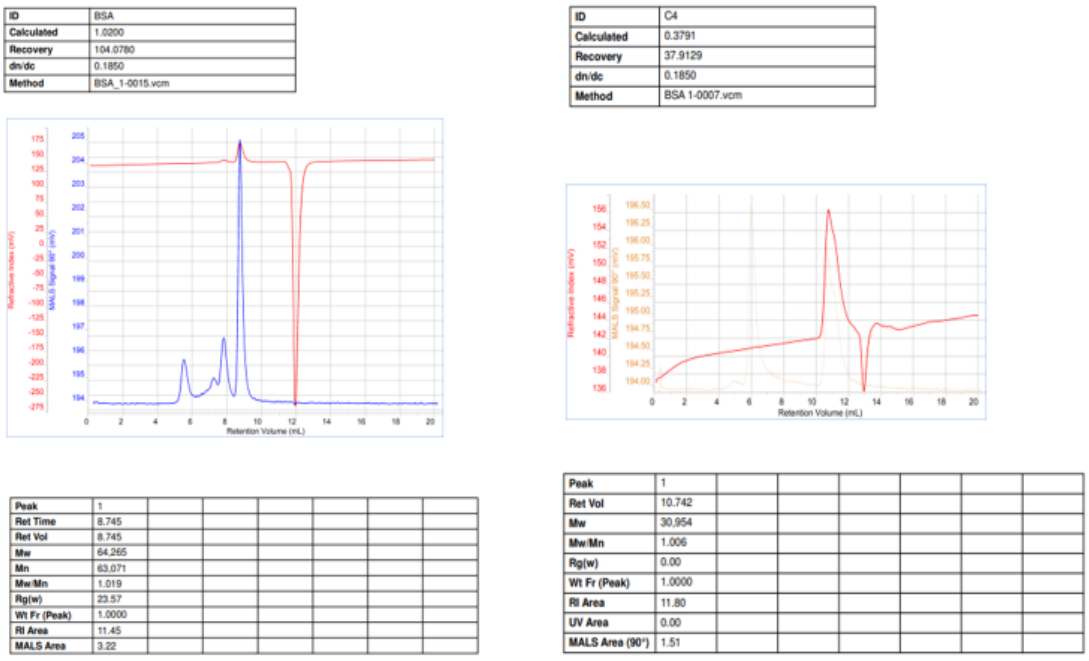


Figure 4-6-D GSH can reduce purified recombinant CLIC4 in Bacterial cells. The MALS was caried out for purified sample to monitor and analyse the size of protein with determining the molecular size upon anisotropic light scattering produced by larger molecules and is associated with their size upon tertiary protein structure. The right panel shows the control sample BSA with 64.265MW, and the left panel indicated CLIC4 protein with 30.954 MW. The indicated molecular weight was appeared a bit more than the actual of molecular weight of CLIC4 (28KD) and can be confirmed the only CLIC4 was purified. The MW is 30,954 Daltons, one peak and the MW/MN value is 1.006 which indicates a monodisperse polymer where all the chain lengths are equal (The MALS study was take place on Glasgow Structural study).

E.

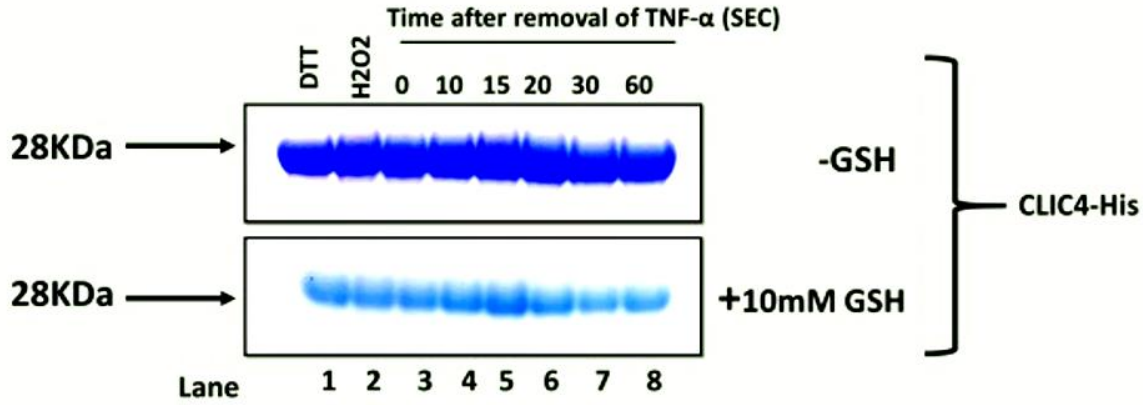
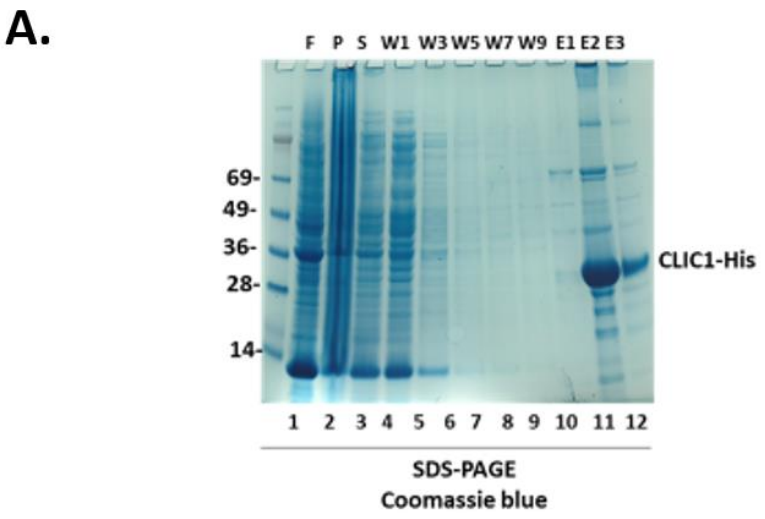


Figure 4-6-E GSH can reduce purified recombinant CLIC4 in Bacterial cells. After removal of TNF-α by SEC, Purified CLIC4-His was incubated in either 50mM phosphate buffer, pH 7.4 minus GSH (upper panel) or plus GSH (10 mM) (lower panel) for various times (lanes 3-8). A sample treated with H_2O_2 (2mM) was included as a control for oxidized sample (lane 2). The redox state of CLIC4-His was trapped by the addition of 10% (v/v) trichloroacetic acid and the redox state determined by alkylation of free thiols with AMS, separation by SDS-PAGE, and staining with Coomassie Blue. The migration of proteins is totally different with vivo condition. The reduced protein migrated as a smear rather than a tight band even when protein is reduced with DTT, suggesting GSH has a significant effect on reduction of CLIC4 in Bacterial cells.

Purified CLIC4-His tagged was either reduced with DTT (10mM) (lane 1) or oxidized with TNF-α (10uM) (lane 3-8). TNF-α was removed by size exclusion chromatography, and then CLIC4 was incubated in either 50mM phosphate buffer, pH 7.4, minus GSH (upper panel) or plus GSH (10 mM) (lower panel) for various times (lanes 3–7). The redox state of CLIC4 was trapped by the addition of 10% (v/v) trichloroacetic acid and the redox state determined by alkylation of free thiols with AMS, separation by SDS-PAGE, and staining with Coomassie Blue. The reduced protein migrated as a smear rather than a tight band even when the protein was reduced by DTT (lane 1), a likely consequence of the acidification process. The reduced protein migrated as a smear rather than a tight band even when the protein was reduced by DTT, suggesting that it can be a consequence of the acidification of proteins (Figure 4-5, panel E, upper and lower panel lane 3-8). On the other hand, this could normally happen very irregularly, these results indicated that CLIC4 can be directly reduced by GSH, and maybe the reduction of CLIC4 was presented at the same time or faster than in mammalian cells. The fact that GSH can reduce CLIC4 in vitro. In the next

experiment, CLIC1 included a disulphide band between cysteine 24 and Cysteine 59 and was considered as a control for disulfide exchange intermediate structure.



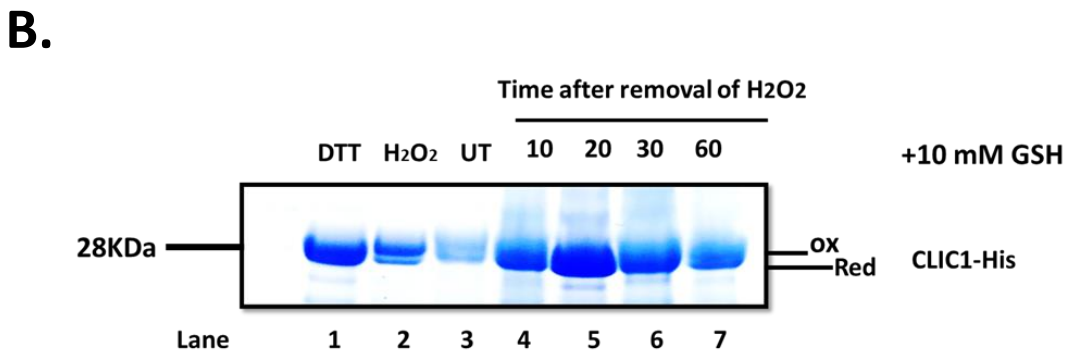
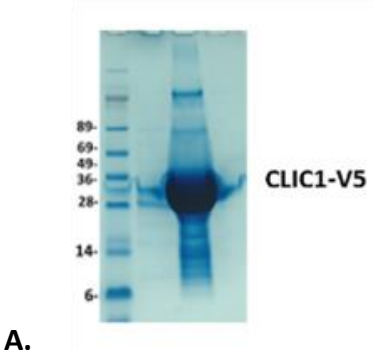
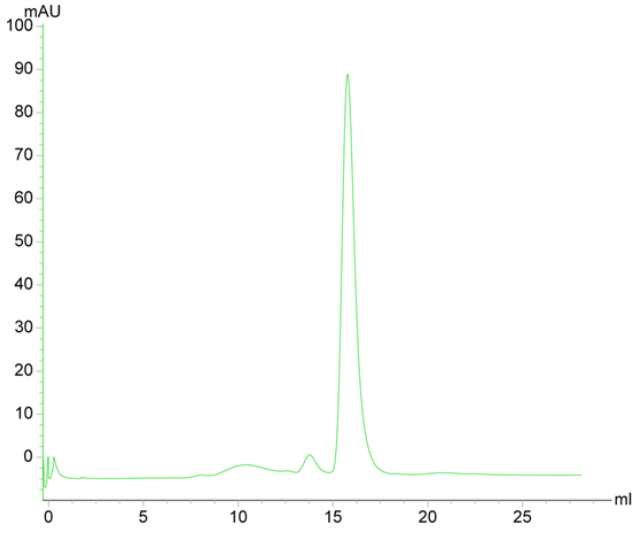


Figure 4-7 (panel a) GSH can reduce purified recombinant CLIC1 in bacterial cells: the Escherichia coli strain BL21(DE3) PLysS containing a modified version of PET-28a (+), expressing His-tagged CLIC1 and purified through standard protocol in the Woolhead lab at Glasgow University through a His60NiGravity Columns. To purify CLIC1, we first inoculate 5ml containing 10ul of Kan (from stock 500mg/ml) with a glycerol stock Scrapping per 500ml of LB and then incubate at 37℃ for overnight. On the next day, we inoculate 500ml of LB, which contained 1ml of Kan with 5ml of overnight culture and incubated at 37℃ in 200rpm and check the OD600 to reach about 0.6 at this OD we can induce the IPTG (1mM from 1Mstock), then we need to reduce the temperature to 30℃ to allow for 4 h. Then pellet the cells by centrifugation at 10,000 rpm for 10 min at 4℃. Decant LB from the centrifuge tube and resuspend cells in 20ml 1XTBS buffer containing 1EDTA-freeprotease inhibitor tablet and 20mg of lysozyme from stock 100mg/ml. In this stage, we need to incubate the cells in ice for 30min to allow the cell wall of bacterial cells to be easily broken via French press with 2 passes at 8000psi for lysing cells and centrifuge the cells to pellet out debris at 17000rpm for 30 min at 4℃. To purify the CLIC1-His, we used His-binding nickel column, and equilibrate a His-binding nickel column with 10

bed-volume of binding buffer (40mMTris, 300mM NaCl, PH8). Then to wash the column, we applied the supernatant to the Ni-column and washed Ni-column with 10 bed-volumes of wash buffer (40mMTris,300mMNaCl, adding 20mM imidazole). To elute the protein, we need to add 3 bed-volume of Elution buffer and collect separately at a fresh Eppendorf tube. To quantity the protein concentration using the Nano-Drop (Panel E, A and B). CLIC1 included a disulphide band between cysteine 24 and Cysteine 59 and consider as a control for disulfide exchange reaction. Purified CLIC1-His was either reduced with 10mM DTT (lane 1) or oxidized with 2mM (H_2O_2), and the extra oxidizing agent was removed via Slide-A-Lyzer dialysis cassette (10,000MW), in3X1.5L PBS PH7.4 for 3 hours. Then samples were incubated in 50mM phosphate buffer, pH 7.4 with GSH (10 mM) for various times (lanes 4-7). A sample treated with H_2O_2 (2mM) was included as a control for oxidized sample (lane 2). The redox state of CLIC4-His was trapped by the addition of 10% (v/v) trichloroacetic acid and the redox state determined by alkylation of free thiols with AMS, separation by SDS-PAGE, and staining with Coomassie Blue. The migration of proteins is totally different with intact cells. The reduced protein migrated as a smear rather than a tight band even when protein was reduced with DTT.



A.



B.

Figure 4-7 (panel b) GSH can reduce purified recombinant CLIC1 in Bacterial cells: the Escherichia coli strain BL21(DE3) PLysS containing a modified version of PET-28 a (+), expressing His-tagged CLIC1 and purified

through standard protocol in the Woolhead lab at Glasgow University through a His60NiGravity Columns. To purify CLIC1, we first inoculated 5ml containing 10ul of kan (from stock 50mg/ml) with a glycerol stock Scrapping per 500ml of LB and then incubated at 37'C for overnight. On the next day, we inoculate 500ml of LB, which contained 1ml of kan with 5ml of overnight culture and incubated at 37 in 200rpm and check the OD600 to reach about 0.6 at this OD we can induce the IPTG (1mM from 1Mstock), then we need to reduce the temperature to 30'C to allow for 4 h. Then pellet the cells by centrifugation at 10,000 rpm for 10 min at 4'C. Decant LB from the centrifuge tube and resuspend cells in 20ml 1XTBS buffer containing 1EDTA-free protease inhibitor tablet and 20mg of lysozyme from stock 100mg/ml. At this stage, we need to incubate the cells in ice for 30 min to allow the cell wall of bacterial cells to be easily broken via French press with 2 passes at 8000psi for lysing cells and centrifuging the cells to pellet out debris at 17000rpm for 30 min at 4C. To purify the CLIC1-His, we used His-binding nickel column, and equilibrate a His-binding nickel column with 10 bed-volume of binding buffer (40mMTris, 300mM NaCl, PH8). Then, we applied the supernatant to the Ni-column and washed Ni-column with 10 bed-volumes of wash buffer (40mMTris,300mMNaCl, adding 20mM imidazole). To elute the protein, we need to add 3 bed-volume of Elution buffer and collect separately at a fresh Eppendorf tube.

To quantity the protein concentration using the Nano-Drop, (Panel F, A and B). CLIC1 was subject to size exclusion chromatography for elute purified CLIC1 with respect to the size (Panel F, C). Purified CLIC1-His was either reduced with 10mM DTT (lane 1) or oxidized with 2mM (H_2O_2), and the extra oxidizing agent was removed via Slide-A-Lyzer dialysis cassette (10,000MW), in3X1.5L PBS PH7.4 for 3hours. Then samples were incubated in 50mM phosphate buffer, pH 7.4 with GSH (10 mM) for various times (lanes 4-7). A sample treated with H_2O_2 (2mM) was included as a control for oxidized sample (lane 2). The redox state of CLIC1-His was trapped by the addition of 10% (v/v) trichloroacetic acid and the redox state determined by alkylation of free thiols with AMS, separation by SDS-PAGE, and staining with Coomassie Blue. The migration of proteins is totally different with vivo condition. The reduced protein migrated as a smear rather than a tight band even when protein reduced with DTT.

The result indicated that GSH reduces CLIC4/1 in vitro condition and could be considered as a good candidate for reduction of oxidoreductase enzyme in vitro. However, CLIC1 as an oxidoreductase only in the presence of GSH can be activated for reduction of disulfide band in the insulin; in the fact that, the catalytic activity of CLICs protein is dependent on GSH and in the absence of GSH CLIC4 and CLIC1 not able to reduction (Alkhameci H, et al., 2015). Hence, CLICs protein with oxidoreductase activity including CLIC4, CLIC1, CLIC2 (Alkhameci H, et al., 2015, and CLIC3 (Zanivan S, et al., 2013) needs to form an intermediate structure

for an exchange reaction with GSH and CLIC4 may be reduced via GSH when present in the ER as an oxidoreductase and should be formed the mixed disulfide as an intermediate structure with GSH for reduction or even isomerization reaction (Venezuela S, et al., 2016). In addition to CLIC4, CLIC1 as one of the CLICs family members with oxidoreductase activity with (CXXS) redox motif with ability to form disulfide bonds between catalytic cysteine24 and non- catalytic cysteine 59 can be considered as a control for direct reduction of GSH and ability to form disulfide intermediate structure with 2xGSH (Alkhameci H et al., 2015).

4.2.6 Glutathione can form a mixed disulfide with CLIC4

In the last experiment we indicated that GSH can directly reduce CLIC4/CLIC1. Here, to determine whether this transitional action can be formed within the ER, we treated microsomes with TNF-α to oxidize CLIC4, 5 min after removal of oxidizing agent added a BioGEE to microsomes during the recovery phase. The Biotinylated glutathione ethyl ester (BioGEE) is a cell-permeant and used for the detection of GSH because under conditions of oxidative stress, cells may transiently integrate glutathione into proteins. Stressed cells incubated in BioGEE will also incorporate this biotinylated glutathione incorporate into proteins, facilitating the identification of oxidation-sensitive proteins. To determine if a mixed disulfide was formed, microsomes were first oxidized and then incubated in the absence and presence of BioGEE for 30 min to allow recovery to occur and then treated with NEM to trap any mixed disulfide formed and to supress extra Bio GEE. Biotinylated proteins were isolated on streptavidin-agarose beads eluted with 50mM DTT, and protein separated by SDS-PAGE. CLIC4 was then detected by western blotting. We expected in the absence of BioGEE no CLIC4 was isolated with the streptavidin beads (Figure 4-7) but our result indicated that a small amount of CLIC4 was isolated, suggesting CLIC4 partially makes interaction with GSH. However, when recovery took place in the presence of BioGEE, CLIC4 was isolated (Figure 4-7, lane 2) from cell lysates. No CLIC4 was isolated when the streptavidin beads were blocked with biotin prior to the isolation, demonstrating the specificity of the interaction (Figure 4-7, lane 4). These results demonstrate that the biotinylated reagent acted upon oxidized CLIC4 bringing about the formation of an intermediate motif between GSH and CLIC4 suggesting that GSH can directly reduce CLIC4. Note that we applied PDI specific marker for indicating microsome fraction. Since both

CLIC4 and PDI are categorised with oxidoreductase activity, the marker appeared a similar response to biotinylating. Therefore, we cannot see a tight band as a specific marker.

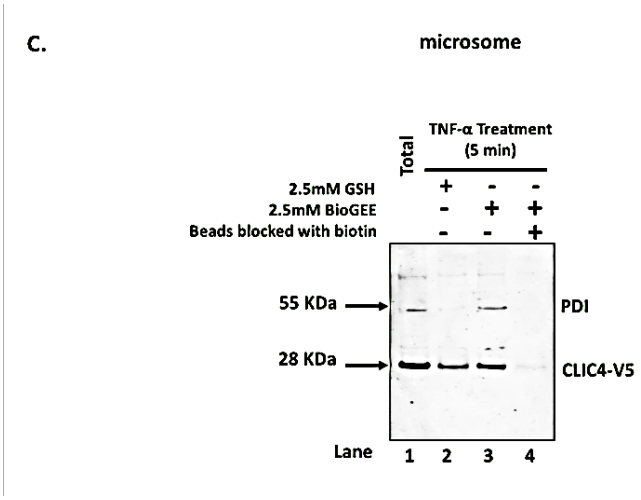


Figure 4-8 Glutathione can form a mixed disulfide with CLIC4 and can recover CLIC4-V5 following oxidative stress. Microsomes were oxidized with TNF-α (10uM) and then incubated with either GSH (2.5 mM) (lane 2) or BioGEE (2.5 mM) (lanes 3 and 4) for 30 min. Disulfide exchange was inhibited with NEM (25 mM) at 4 ℃. Biotinylated proteins were isolated with streptavidin-agarose (lanes 2 and 3) or streptavidin-agarose that was already blocked with excess biotin (40mM) for 30 minutes at 4℃ (lane 4). The proteins were eluted with DTT (50 mM) and analysed by SDS-PAGE and Western blotting with an anti-CLIC4 antibody.

Our previous results indicated that the expression of CLIC4 under redox condition totally depends on GSH in 1 mM concentartion and CLIC4 appeared in a reduced position. Now, to determine the direct effect of GSH on reduction of CLIC4, we increase the GSH concentration to 10 mM. Our result indicated that increasing the added GSH could make a change to the expression level of CLIC4 in microsomal membrane and make the reduction of CLIC4 in 30 min follwing TNF-α treatment (Figure 4-5, B) suggesting CLIC4 after incubation in the 10 mM of GSH, recoverd and returned to a reduced position. In addition, we examined the expression of CLIC4 under redox state in-vitro condition to evaluate whether GSH could have a direct effect on purified recombinant CLIC4-His. For this experiment we first oxidized the CLIC4-His with TNF-α and then removed the excess TNF-α by gel filtration and followed the reduction of oxidized CLIC4 either in the absence or presence of 10mM GSH (Figure 4-6, E). The redox state of purified CLIC4-His was trapped by 10%(v/v) trichloroacetic acid (TAC) for 1 hour on ice and the proteins were precipitated by the addition of 25%(v/v) acetone. The redox state of CLIC4 was determined by alkylation

of free thiol with AMS. In the absence of GSH no reduction of purified protein was observed (Figure 4-6 panel D, upper panel). However, in the presence of GSH rapid reduction can occur in a few minutes (Figure 4-5, panel D, lower panel). The reduced proteins migrated as a smear rather than a tight band even when the protein was reduced by DTT (Figure 4-6, panel D, lane 1), a likely consequence of the acidification process. Therefore, we can conclude migration of CLIC4 under redox condition in vitro is totally different with in vivo. Nevertheless, it is clear from these results that CLIC4 can be directly reduced by GSH, and the rate of reduction was equivalent to or even faster than the reduction of CLIC4 in cells or in intact cells.

Moreover, we used purified CLIC1-His as a control. I found that CLIC1-His appeared a reduction in migration as well and clearly indicated a rapid reduction under 10mM GSH incubation (lane 4-6, Panel E,D), demonstrating, GSH can reduce CLIC1/4 in an indicated concentration and both of them appeared a similar response to GSH because of its oxidoreductase activity. Although we illustrate that GSH can reduce CLIC4 in vitro condition, still we need to find more evidence to confirm that GSH can directly reduce CLIC4 when present in the ER. If GSH does indeed directly reduce oxidized CLIC4, then a mixed disulfide intermediate between CLIC4 and GSH should be formed as a member of the reduction process. This particular property of CLIC4 make it as an enigma of the CLIC proteins by Ion channels, redox, enzymes activity, which all functional properties is depended to its redox motif and interaction with GSH (Littler R.R, et al., 2010).

To determine whether this transitional action can be formed within CLIC4 and GSH in the ER, we treated microsomes with TNF-α to oxidize CLIC4, 5 min after removal of oxidizing agent added a BioGEE to microsomes during the recovery phase. The Biotinylated glutathione ethyl ester (BioGEE) is a cell-permeant and used for the detection of glutathiolation because under conditions of oxidative stress, cells may transiently incorporate glutathione into proteins. Stressed cells incubated in BioGEE will also incorporate this biotinylated glutathione derivative into proteins, facilitating the identification of oxidation-sensitive proteins.

To determine if a mixed disulfide was formed, microsomes were first oxidized and then incubated in the absence and presence of BioGEE for 30 min to allow recovery to occur and

then treated with NEM to trap any mixed disulfide formed and to supress extra Bio GEE. Biotinylated proteins were isolated on streptavidin-agarose beads eluted with 50 mM DTT, and protein separated by SDS-PAGE. CLIC4 was then detected by western blotting. In the absence of BioGEE no CLIC4 was isolated with the streptavidin beads (Figure 4-8, C, lane 3). However, when recovery took place in the presence of BioGEE, CLIC4 was isolated (Figure 4-7, C, lane 2) from cell lysates. No CLIC4 was isolated when the streptavidin beads were blocked with biotin prior to the isolation, demonstrating the specificity of the interaction (Fig 4-7, C, lane 4). These results demonstrate that the biotinylated reagent acted upon oxidized CLIC4 bringing about the formation of a mixed disulfide and suggest that GSH can directly reduce CLIC4 in microsomal mammalian cells. Note that we applied PDI as a specific marker to identify microsome fraction. Since both CLIC4 and PDI are oxidoreductase enzyme, the marker showed a similar response to biotinylating. Therefore, we cannot see a tight band as a specific marker. Reduction CLIC4 by GSH can be performed by glutathionylation and can be activated by oxidative stress.

4.2.7 GSH cannot reduce the mutant form of CLIC4

In the previous experiment we showed GSH can be considered as a good candidate for reduction of CLIC4 in the mammalian cells. Furthermore, we showed that in vitro and in vivo conditions, GSH can reduce CLIC4 through formation of the intermediate structure between CLIC4 and GSH. Here, we assume that the formation of the transient or intermediate structure between GSH and CLIC4 can be because of the existence of the redox motif (CXXS) in the N-terminal of thioredoxin-like domain in the CLIC4 (Littler D.R, et al., 2005). Thus, if this assumption could be correct, GSH cannot form a transient intermediate structure with a mutant form of CLIC4 redox motif. Hence, to investigate whether reduction of CLIC4 via GSH depended on modified cysteine 35, we designed a mutant trap approach through replacing cysteine 35 to Alanine. The reason to use the alanine can be due to disability of Alanine for formation of intra and inter disulfide bands with other non-catalytic cysteines.

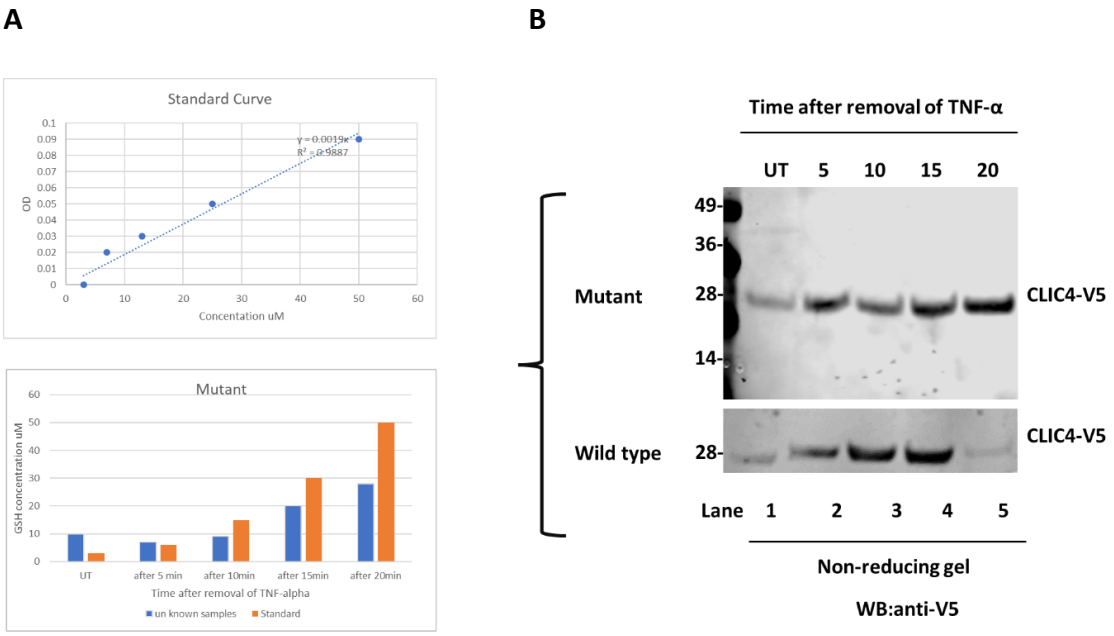


Figure 4-9 Reduction of CLIC4 via GSH can be dependent on modified cysteine 35. A, the upper panel shows the standard curve for measuring the level of GSH in the mutant cells. Linear detection range 0.01-3µM GSH equivalents considered with an enzymatic method via Ellman's Reagent (DTNB) and glutathione reductase (GR). DTNB reacts with GSH to form a yellow product. The rate of change in the density, measured at A_{412} nm, which is directly proportional to the glutathione concentration in the sample. The mutant HEK293T-CLIC4-V5 cell lines were either left untreated or treated with TNF-α for 5 minutes. The oxidizing reagents were removed after several time points. Then, the stable mutant cells were lysed in 8mM HCl, 1.3% (w/v) 5-sulfosalicylic acid on ice for 1 h. The proteins were removed by centrifugation. The acidified sample was added to 0.2mM NADPH, 0.6mM Ellman's reagent, glutathione reductase (1–2units/ml), 150 mM phosphate buffer, pH 7.4. The rate of absorption at 412nm was measured over 1 min with respect to GSH standards. GSH concentrations were calculated by the formula that was provided by manufacturer instruction. At various intervals the level of GSH was determined using Ellman's reagent that provided in glutathione kit. **B.** In addition, to determine the reduction of CLIC4 in response to Glutathione level alteration in mutant cells, cells were left untreated or treated with TNF-α (10uM) for 5 minutes. The oxidizing agent was removed, at several time points and the cells were washed with the fresh medium. The lysed cells for redox analysis were subjected for AMS modification and analysed by reducing SDS-PAGE and western blotting (WB) with anti-V5 in non-reducing gel.

The results above indicated that after oxidizing mutant cells with TNF-α, a decrease at cellular GSH level was absorbed due to recovery of cellular oxidoreductase enzyme by GSH. But this diminution at cellular GSH level in the mutant cells was less than wildtype cells, suggesting the catalytic cysteines in the redox motif of oxidoreductase active site enzyme has a significant effect on establishing intermediate structure between GSH and CLIC4 as a stage of reduction process. The level of GSH before oxidizing was more than after oxidizing with TNF-α, suggesting GSH was used for the recovery of enzymatic protein. On the other

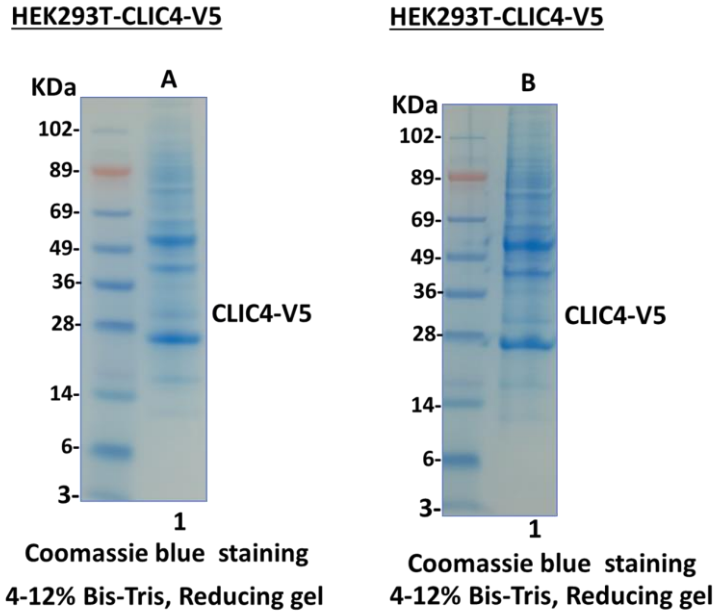
hand, after 20 min of removal of TNF-α, the level of GSH dramatically increased in mutant, suggesting oxidoreductase totally recovered and the consumption GSH stopped (4-9, A, lower panel after).

To determine the effect of GSH on reduction of CLIC4 in mutant cells, we examined the redox state of CLIC4 in the wild type and mutant cells. We designed two groups of cells treated and oxidized cell lines. Cells were either left untreated or treated with (10uM) TNF-α for 5 minutes at 37'C. The oxidizing agent was removed in several time points, and cells were resuspended in the fresh medium to recover from the extra oxidizing agent. then to determine the redox state, cells were subjected to redox state with AMS modification, SDS-PAGE, western blotting with anti-V5 (Figure 4-9, B, upper and lower panels, lanes 1-5). Our result indicated that untreated samples in wild type cells demonstrated a reduction state compared to oxidized samples (Figure 4-9, B lower panel, lane 1 and 2) and in 20 minutes after removal of oxidizing agents CLIC4 was recovered and returned to reduced state (Figure 4-9, B, lower panel, lane 5), suggesting GSH was utilized for reduction of CLIC4 during oxidation process and after full reduction of CLIC4, GSH was dramatically increased, that we showed that in the last experiment (Figure 4-3-1, panel A, C). To compare mutant cells, no reduction of CLIC4 was absorbed either untreated or in several time points after removal of TNF-α (Figure 4-9, B, upper panel, lanes 1-5), suggesting the mutant form of CLIC4 cannot form an intermediate structure with GSH, hence, the reduction reaction with exchange thiol-GSH cannot occur and no reduction was indicated in CLIC4 even after removal of TNF-α). In the next experiment, I designed a workflow through TMT labelling to investigate the significant effect of GSH reduction on CLIC4 or overall protein with respect to protein expression.

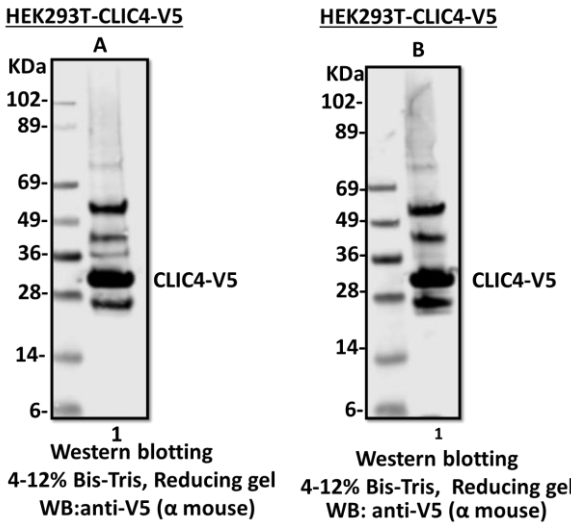
4.2.8 TMT labelling aides us to understand the redox state of cysteine residues

To determine the abundance of CLIC4 cysteine residues concerning protein expression in a humane HEK293T cell line, there is a requirement to check the coverage of cysteine residues of the stable cell line with overexpressing of CLIC4-V5 to achieve sufficient results. Hence, I first examined two colonies of the HEK293T cell line that stably expressed CLIC4-V5 to pick up a good coverage of CLIC4-V5 (Figure 4-10 A, B, C, and D). Following making the microsomal membrane as a source of the ER membrane, the redox state in mammalian cell was trapped by alkylation of free thiols with 25mM *N*-ethylmaleimide (NEM) and species exposed with IP-buffer. Isolated microsomal membranes were either purified through agarose beads for (Mass spectrometry analysis) before Coomassie blue staining or subjected to several antibodies to identify the right band for cutting by western blotting with anti-V5 (mouse), anti-V5 (rabbit), and anti-CLIC4 (rabbit) antibodies (Figure 4-10 A, B, and C), respectively.

A)



B)


HEK293T-CLIC4-V5
A
KDa
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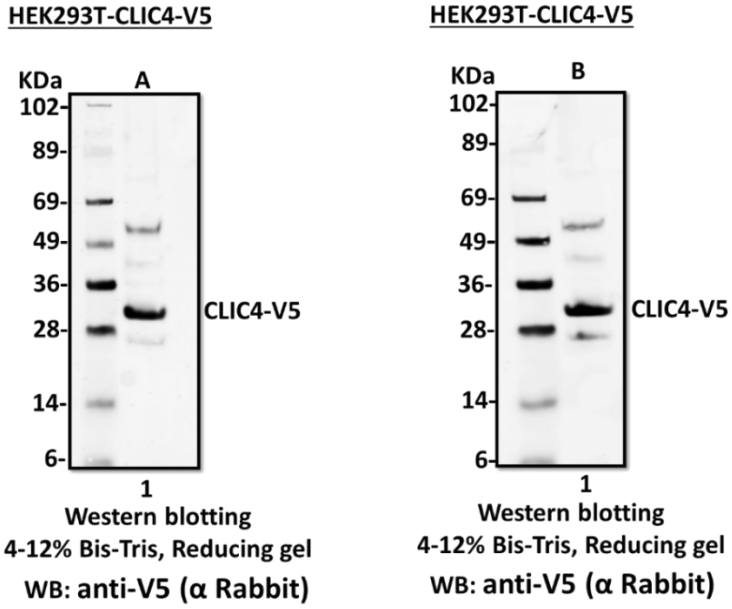
89-
69-
49-
36-
28-
14-
6-
CLIC4-V5
1
Western blotting
4-12% Bis-Tris, Reducing gel
WB:anti-V5 (α mouse)
HEK293T-CLIC4-V5
B
KDa
102-
89-
69-
49-
36-
28-
14-
6-
CLIC4-V5
1
Western blotting
4-12% Bis-Tris, Reducing gel
WB: anti-V5 (α mouse)

C)


HEK293T-CLIC4-V5
KDa
A
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69-
49-
36-
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14-
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CLIC4-V5
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Western blotting
4-12% Bis-Tris, Reducing gel
WB: anti-V5 (α Rabbit)
HEK293T-CLIC4-V5
KDa
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102-
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49-
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Western blotting
4-12% Bis-Tris, Reducing gel
WB: anti-V5 (α Rabbit)

D)

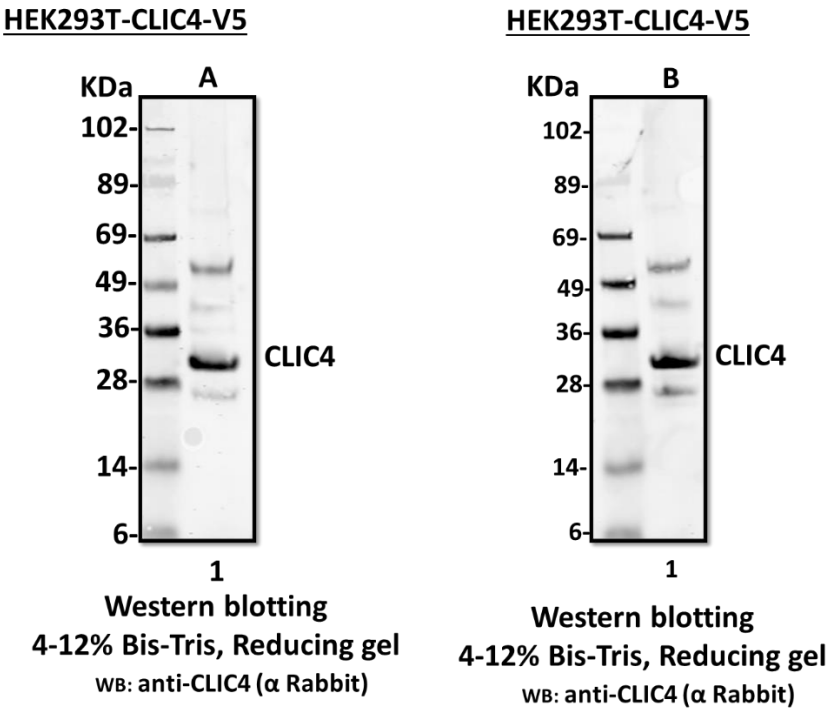


Figure 4-10 Purification of the mammalian microsomal membrane to evaluate the abundance of CLIC4 cysteine residues to examine the redox state of CLIC4 concerning protein expression, the purification of CLIC4-V5 is a requirement. The HEK293T cells stably transfected with recombinant CLIC4-V5 construct were precleared by incubation with protein A-Sepharose (PAS) for 30 min at 4 °C, before incubation with anti-V5-conjugated agarose beads (Sigma) for 16 h at 4 °C. The beads were washed three times with lysis buffer supplemented with 0.5% SDS and then incubated with 10 mM DTT for 5 min to elute cross-linked complexes. Then samples were boiled for 5 min to dissociate the immunocomplex from the beads and centrifuged at 13,000 × g for 1 min at 4 °C to recover the sample from beads and run on the gel for Coomassie blue staining for two samples (Figure A) and western blotting with both anti-V5 either for a mouse (Figure B) or rabbit antibody (Figure C) and anti-CLIC4 antibodies (Figure D) to find a right place of protein to gel extraction. Following subjecting the gel bands to a standard mass protocol, to determine the abundance of cysteine sites and coverage of the protein sequencing, CLIC4 was subjected to mass spectrometry to analyse the sample on the ESI instrument and search the data through BMS internal database and NCBI.

After purification of CLIC4-V5 from the human cell line, samples subjected to MS/LC in Glasgow proteomics (Figure 4-11). The mascot results indicated with 68% and 67% coverage for cysteine residue for A and B, respectively.

http://fun gen1.ibls.gla.ac.uk/mascot/cgi/master_results_2.pl?file=..%2Fdata%2F20210929%2FF142596.dat

http://fungen1.ibls.gla.ac.uk/mascot/cgi/master_results_2.pl?file=..%2Fdata%2F20210929%2FF142597.dat,

The mass spectrometry results show that the coverage of 4 cysteines is good for CLIC4 in both samples (68% for A and 67% for B). All 4 cysteine-containing peptides (C35, C100, C189, C234) are detected, and all cysteines are modified. Mostly the modification is carbamidomethylating, but there is also evidence for N-ethylmaleimide modification of some cysteines. There is a hint that the latter modification is more abundant in sample B. Hence, for this study sample B was selected for examination. This result was used as a control to identify cysteine sites of CLIC4 and coverage of purified CLIC4 from the microsomal membrane fraction of the HEK293T cell line which stably overexpresses CLIC4-V5. In the next experiment I design next step of workfellow to analysis the effect of GSH on CLIC4 or overall protein expression.

A)

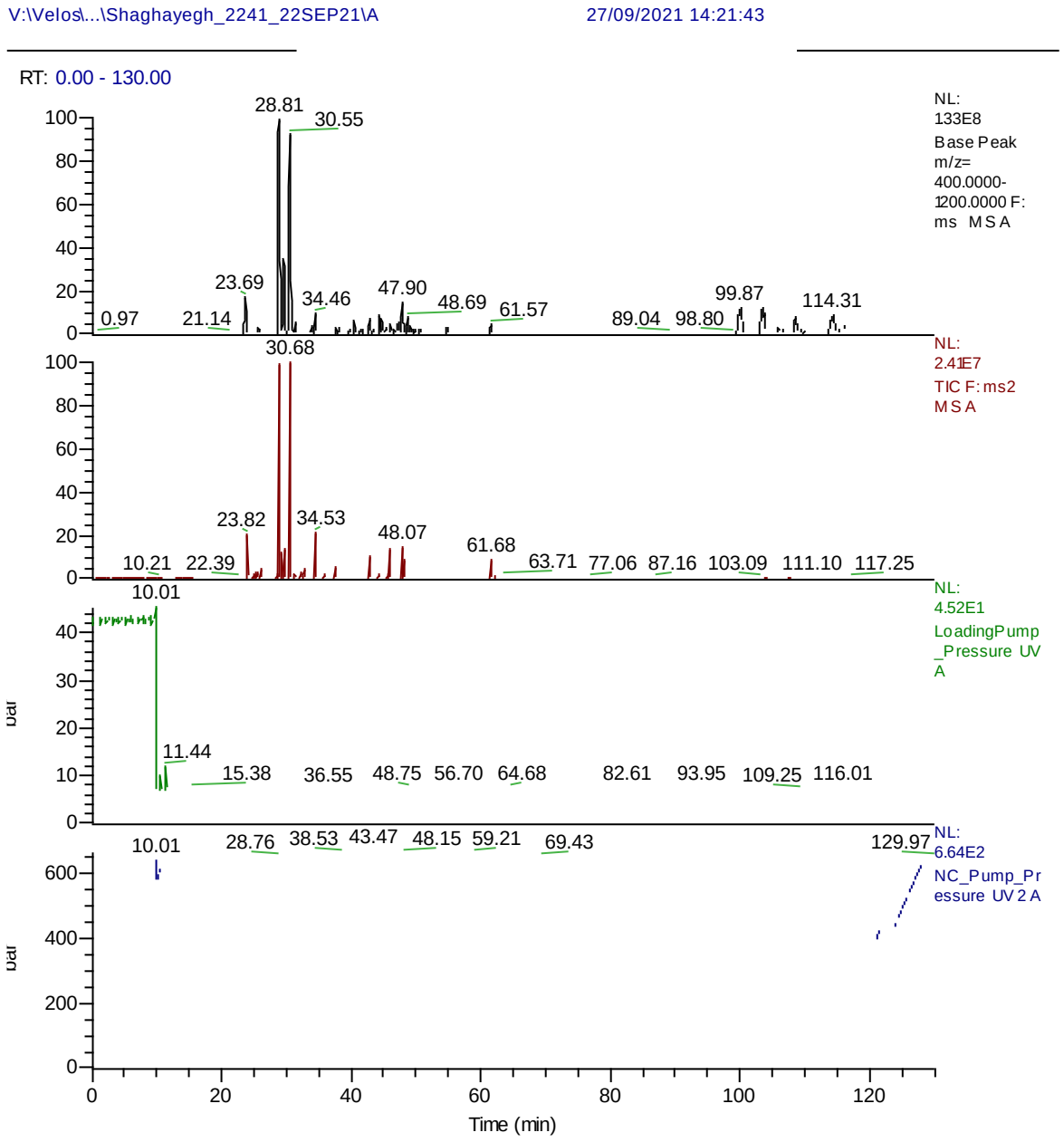


Figure 4-11 A MS/LC indicates a good coverage of Cysteine residues for microsomal membrane CLIC4 before TMT labelling for sample A (E), the Raw data view shows that the top trace in black is the full mass spec (MS1) signal where the peptides will be detected. As peptides have been digested with trypsin, the peptide terminal amino acids should either be lysine or arginine. These are basic amino acids that like to accept a proton; therefore, we expect a minimum of 2+ charge on a peptide. Other basic amino acids in the peptide can also pick up protons so peptides being 3+ and 4+ are also possible depending on the length of the peptide and the amino acid sequence. It is assumed that any singly charged ions detected by the mass spec are contaminants, such as detergents or polymers, and are excluded from fragmentation. Any multiply charged ions are selected for fragmentation; these are used for peptide identification.

B)

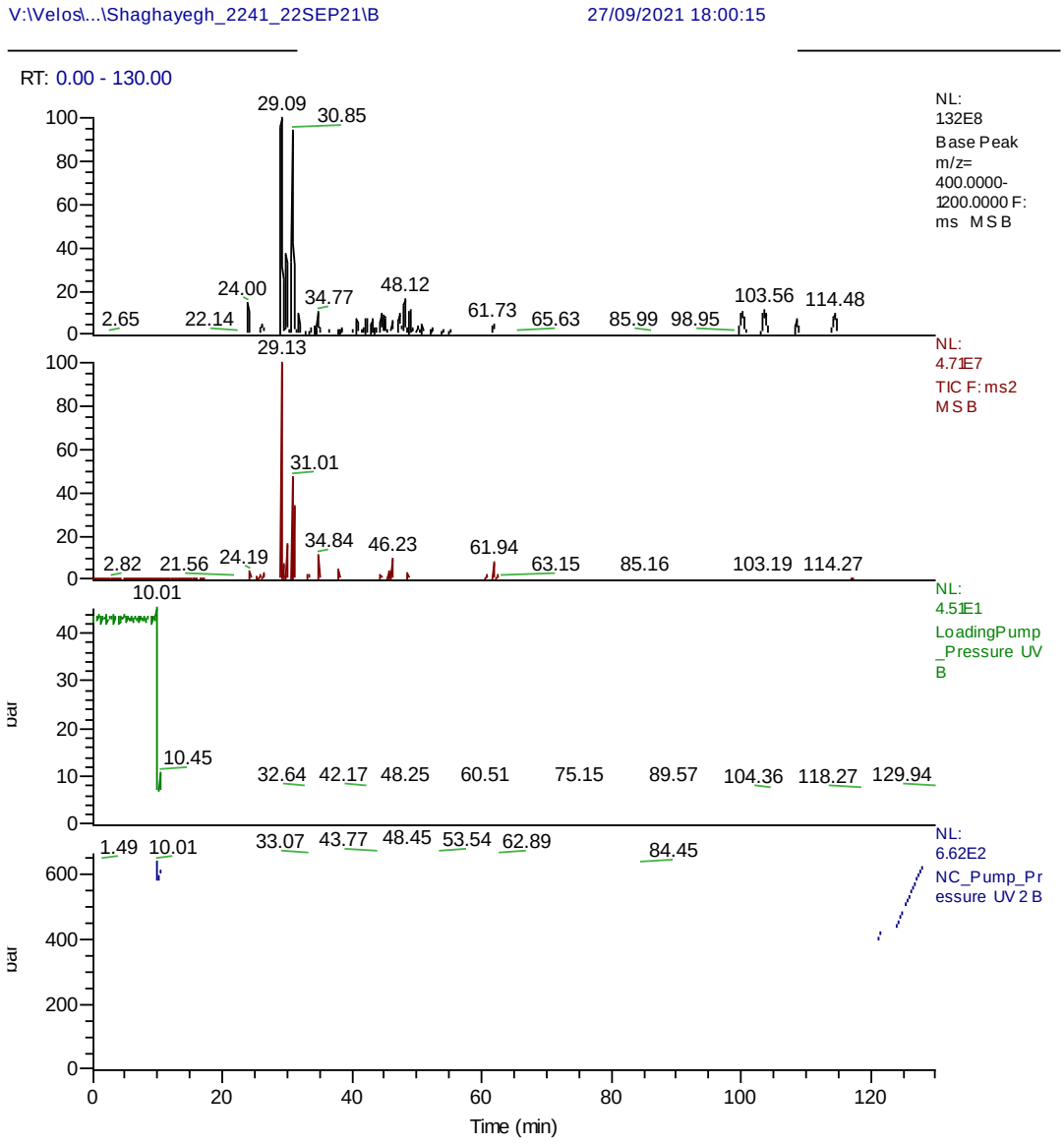


Figure 4-11 B MS/LC indicates a good coverage of Cysteine residues for microsomal membrane CLIC4 before TMT labelling for sample B (F), The second trace in burgundy shows the signals from the fragmentation spectrum (MS2). There should be lots of signals shown here for successful protein identification. If there are missing signals here this could indicate that there is an insufficient sample or are interfering ions such as detergents or polymers present in the sample. The third trace in olive/green shows the pressure of the sample loading. A dip at the start of this trace indicates a failed sample injection. The final trace in blue shows the analytical column pump pressure. The separation shows a reproducible trace based on the LC gradient. Any unexpected deviation or jumps in pressure could indicate a problem with the sample separation.

4.2.9 GSH has a significant effect on the overall protein expression in the microsomal membrane of mammalian cells

To measure the protein expression with respect to oxidative changes between two +/- GSH conditions and to normalize these changes concerning the alteration in protein expression, three biological replicates of each condition, minus Glutathione (-GSH) and plus Glutathione (+GSH), were designed, and each group was divided and treated separately to create two different sample sets (Figures 4-11). Half of each sample was incubated with N-ethylmaleimide during cell lysis to irreversibly block any free cysteines (SH) and prevent any further redox reactions. Any reversibly modified cysteines were then reduced with TCEP. Utilizing TCEP due to permeabilizing the microsomal membrane could be useful (Cao X, et al., 2020) and is a requirement for Iodo-TMT labelling (Figure 4-12).

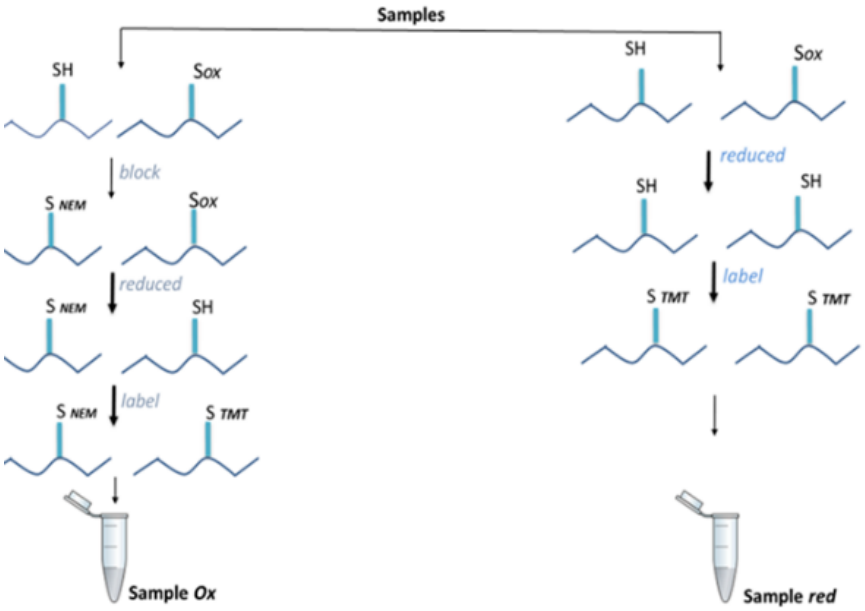


Figure 4-12 Schematic representation of sample workfellow (**A**). to designe the workfellow, Half of each sample have to be fully reduced, and all fully reduced cysteine residues are able to labeled with Iodo-TMT at PH=8, while the other half is treated with NEM to block all free cysteines (SH), and subsequently, any modified cysteine is reduced with Iodo-TMT, the image update from (Prakash A.S, et al 2018).

To quantify protein expression levels for cysteine-containing peptide, the other half of each sample was fully reduced, and all cysteine residues were labelled with Iodo-TMT. Affinity purification with an anti-TMT antibody was carried out after trypsin digestion, allowing un-labelled peptides to be depleted while IodoTMT-labeled peptides were enriched before MS analysis (Figure 4-13).

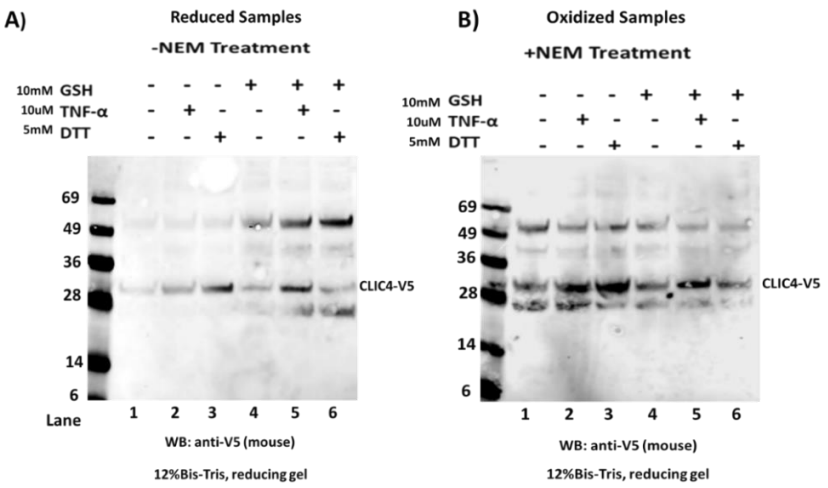
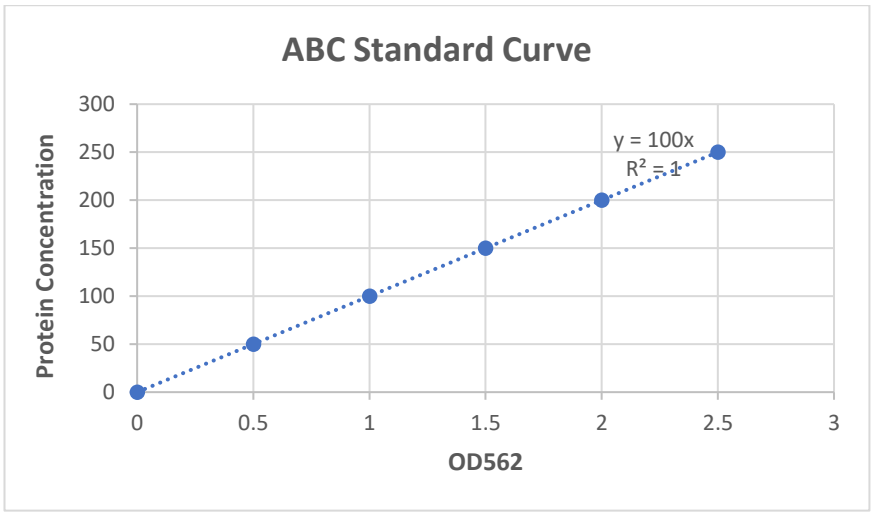
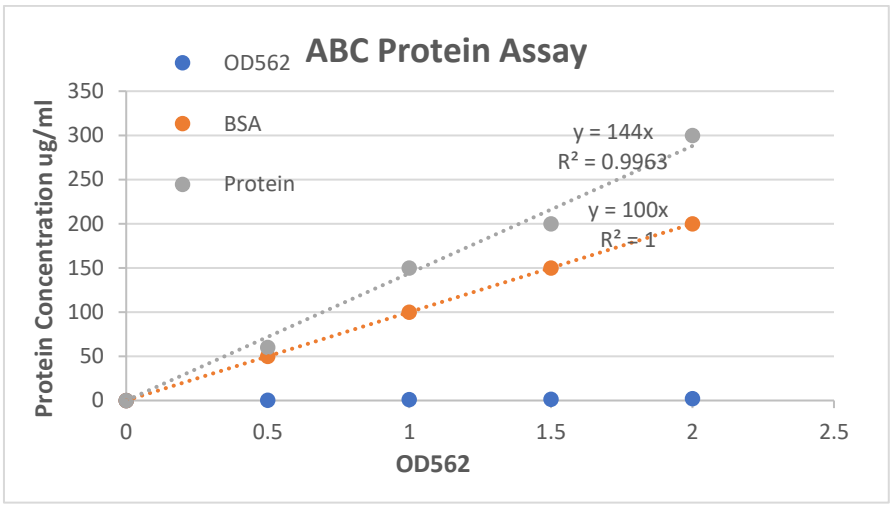


Figure 4-13 Optimization of expression of CLIC4 in two oxidized and reduced sample sets (A and B). HEK293T cells (a human embryonic kidney cell line) in suspension were either treated with DTT (10 mM) and TNF-α (10 uM) or left untreated as a control before cells were subjected to microsome isolation either in the presence or in the absent of (10mM) GSH incubation. Three of each +/-GSH microsomal membrane redox replicates, following dividing and incubating with N-ethylmaleimide separately to create two different samples sets. (A) One sample from each culture (6 samples) was resuspended in 200 µL of SDT lysis buffer to create "Reduced samples". (B) the remaining six samples were resuspended in 200 µL of (100 mM) NEM lysis buffer (Guo, J et al., 2008) to create "Oxidized samples. The samples were exposed to reduction with (100 mM) DTT, SDS-PAGE, for western blots probed with antibody to anti-V5.

The result indicates that CLIC4-V5 was expressed in both reduced (A, lanes 1-6) and oxidized (B, lanes 1-6) samples either in the presence or absence of GSH under redox conditions (this stage as a second check point to make sure CLIC4 expressed in each condition). For TMT-Labelling need to measure protein concentration with Bradford assay (Figure 4-13).



(a)



(b)

Figure 4-14 Protein concentration has a significant effect on sufficient Iodo-TMT labelling. (a) Standard curve of ABC using Bradford assays to measure the protein concentration for each condition. Each sample was split into two 30µg aliquots (24 samples in total) and made up of equal volumes with HEPES buffer. Samples were centrifuged for 10 min at 13000 × g at 4 °C and the supernatant was carefully removed. The protein pellet was washed twice with (80%) ice-cold acetone, then centrifuged to remove the supernatant. Protein pellets were resuspended in HEPES buffer, and the protein concentration was measured by a Bradford assay. (b) Typical color (orange) response curves for BSA and total protein concentration using the standard curve and the standard test tube protocol at 37 ̊C for 30-minute incubation.

The samples were divided into 24 sample with 30ug protein concentration (see appendix 4 and 5). After measurment the peptide with peptide assay kit as described in the material and methods then the samples were send to proteomics analysis at the glasgow polyomics center. The result indicated the overal microsomal protein that labelled with TMT and CLIC4 was not detected by TMT due to lack of coverage .The existance proteins upon accession number in 4 samples (see table 4-1).

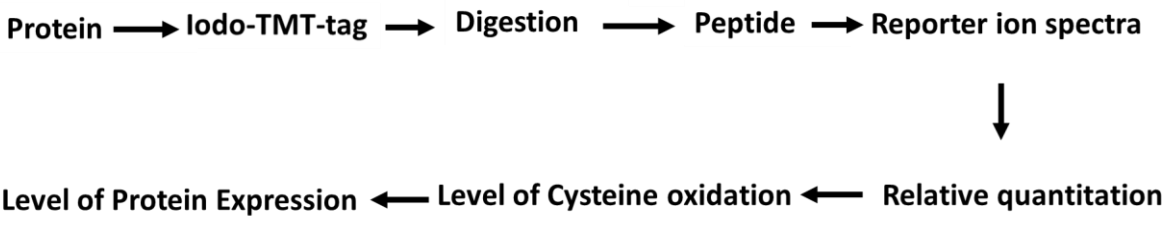
Table 4-1 Identification of the existance protein upon accession number in 4 samples

+GSH	Ox	Ox	Ox	-GSH	-GSH	-GSH	-GSH	+GSH	red	red	red	red	red
Q92945	P62805	P35613	Q9H5K3	P62805	P52926	P31930	Q9Y230	P63244	P62805	P60174	P06576	P4350	Q96QV6
Q92945	P10412	Q9UHK6	P24534	P16402	Q8IWX8	P62937	P04406	P30041	Q6FI13	P62807	Q6P0N0	P68371	P16104
Q92945	P02768	Q99623	P09429	P60174	P78527	Q96AE4	Q02543	P38646	Q16777	O60814	Q9H4B7	P04350	Q8IUE6
P62805	Q92945	P52926	P0C5Z0	Q7L7L0	P31930	P17987	Q00325	Q96D46	Q7L7L0	P68032	Q13509	A6NNZ2	P0C0S5
P16403	P11021	P35527	O15031	P08708	P62937	P06576	Q86VH4	Q8WUG5	P0C0S8	A5A3E0	Q9BUF5	P07437	P63244
Q7L7L0	Q7L7L0	Q14697	P63244	P40926	Q96AE4	P84243	P18124	Q2TAM9	P20671	Q6S8J3	Q13885	Q3ZCM7	P04264
P10809	P25705	Q9HC10	P62861	P68104	P17987	Q00839	Q8NDG6	P22626	Q9BTM1	P62736	Q04637	Q9BVA1	P10599
P68104	P10809	P23528	Q6PF15	P60709	P06576	P02768	Q13185	P09874	Q96KK5	P63267	Q01130	Q13509	P24534
P60174	Q05639	P30405	Q9UJM3	P10809	P84243	P33240	Q07955	O95714	Q99878	P68133	P61978	Q9BUF5	P09874
P84243	P35637	P26583	P62736	P00441	Q92945	P23528	Q9NPH2	Q13435	Q93077	P63261	P31948	Q13885	
P09429	Q16778	Q15637		P11142	P35637	P23396	P09429	O95470	P04908	P02768	P61604	Q04637	
P19338	Q9Y5L4	P13645		P14174	Q04760	P54577	P46778	P26583	Q92945	P08708	Q04760	Q01130	
Q9H4B7	P37802	O43390		Q9H4B7	Q00839	P10768	Q13151		P62979	P20674	P18124	P61978	
Q92945	P61604	Q04760		P00338	P02768	Q92945	P62979		P0CG48	P02768	P48552	P31948	
P61978	P08670	P62277		Q16778	P33240	P35637	P83731		P0CG47	Q99877	P25705	P61604	
P62736	P00441	Q9NPH2		P25705	P23528	Q04760	Q02543		P62987	Q5QNW6	P38646	Q04760	
P61604	P38646	P07195		P15531	P23396	O43439	Q00325		P16402	Q99879	Q71UI9	P18124	
P26583	Q8IWX8	Q9NTJ3		P04080	P54577	P04406	Q86VH4		P16403	P58876	Q96QV6	P48552	
P02768	P62979	P04264		P51659	P10768	P62913	P18124		P10412	P33778	P16104	P06576	
	P06576	Q96EH8		P35613	P12236	Q13151	Q8NDG6		P60709	B2RPK0	Q8IUE6	P25705	
	P19338	Q8NH74		P61604	P50995	P62979	Q13185		P23527	P09429	P0C0S5	P38646	
	Q9H4B7	P21941		P63104	P20674	P83731	Q07955		Q16778	Q8IWX8	P68371	Q71UI9	

4 samples with the accession number of each protein all together showing 294 proteins but only 11 proteins are the same in each sample (Table 4-2).

Table 4-2 only 11 proteins are the same in each sample

No.	+GSH	Red	-GSH	OX
1	Q92945	Q92945	Q92945	Q92945
2	P62805	P62805	P62805	P62805
3	Q7L7L0	Q7L7L0	Q7L7L0	Q7L7L0
4	P10809	P10809	P10809	P10809
5	P09429	P09429	P09429	P09429
6	Q92945	Q92945	Q92945	Q92945
7	P62805	P62805	P62805	P62805
8	P62736	P62736	P62736	P62736
9	P16104	P16104	P16104	P16104
10	P26583	P26583	P26583	P26583
11	P02768	P02768	P02768	P02768



To investigate the abundance of protein in +GSH and -GSH conditions (Red and Ox) or quantify oxidized cysteines as a percentage of total cysteine content (-GSH and +GSH), we need to measure the relative quantitation for each sample, which could be calculated from the average reporter ion abundances from each group of three replicates. Hence, resulting ratios can be used to quantify changes in protein expression (Red), changes in oxidation levels (Ox), and the percentage of oxidation level for proteins in each condition (+GSH and -GSH), as explained in formula Appendix 6).

The calculation result indicated that by considering a ratio of 1 that calculated for a protein in sample Red or Ox show no alteration in cysteine oxidation or protein expression between 2 groups of +GSH and -GSH, while a ratio of 0.5 would indicate a 50% decrease in the +GSH sample, indicating GSH has significant effect on reduction of cysteine residue and decrease in protein expression.

Our results indicate that the -GSH samples showed a significant variation in the number of proteins observed between the four samples, with the fully reduced sample identifying over 251 proteins compared with the oxidized samples, which identified less than 50. This observation could be explained by the low percentage of oxidized cysteines noted for many of the identified proteins in the -GSH and +GSH samples. Thus, as a conclusion for IodoTMT-labelling few amounts of cysteine residues are available in the NEM-blocked sample, that means the cysteine contained peptides can identified very few amounts of other peptide. And oxidation of cysteine directly can be corresponded to protein expression. Any differences in protein oxidation and expression between the two +/-GSH conditions will also alter the number of cysteine residues available for iodoTMT-labelling and therefore may affect the proteins observed in each sample.

4.2.10 Determination the effect of GSH on reduction of CLIC4

Due to lack of coverage for CLIC4 protein in proteomics experiment and to make sure of reduction of CLIC4 via GSH, we designed a purification experiment through microsomal fraction and incubation with and without GSH under redox condition. The purified proteins were subjected for a Coomassie blue staining and gel band was analysed for MS/MS in Glasgow polyomics centre.

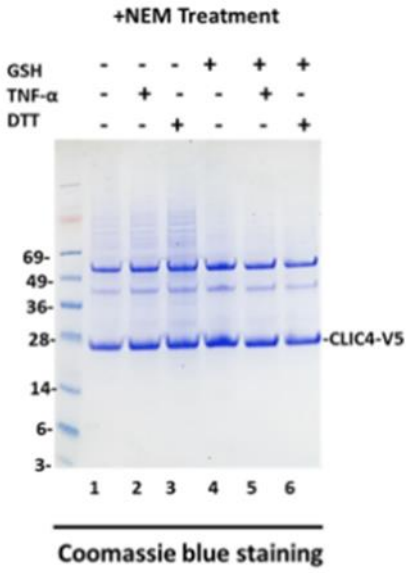


Figure 4-15 Purification of the mammalian microsomal membrane in the absent and in the present of GSH under redox condition. To evaluate effect of GSH on reduction of CLIC4, we designed 6 sample with +/-GSH. The HEK293T cells stably transfected with recombinant CLIC4-V5 construct were first either treated (5mMDTT) and (10uM TNF-α) or left untreated, then cells were subject for a microsomal fractionation, and in the final step microsomal were incubated in the absence and present of GSH. Following removal of extra GSH, the microsomal pellet was precleared by incubation with protein A-Sepharose (PAS) before incubation with anti-V5-conjugated agarose beads (Sigma). The beads were washed three times with lysis buffer and then boiled for 5 min to dissociate the immunocomplex from the beads and run on the gel for Coomassie blue staining for 6 samples and the cut the gel band and subjected for a standard digestion mass spect protocol in polyomics of Glasgow university.

The result indicated that, for 5 out of 6 the submitted samples, CLIC4 was identified upon CLIC4 protein sequence in (NCBI). And some peptides matching to CLIC4 have been fragmented. In the case of sample 2, the fragmentation of CLIC4 peptides sequences have a good coverage at SS2 samples (CLIC4/GSH). The CLIC4 sequence is found in a small database of proteins that are of interest to various collaborators. That database is called "Custom lab", and searching instead of genomic data sets makes for a much faster search and avoids all the matches to keratin and other proteins (Table 4-3) and below links:

Table 4-3 A summary of the Mascot results is informative

Sample	CLIC4 rank	Number CLIC4 peptides matched	emPAI
SS1	130	2	0.32
SS2	41	3	0.52
SS3	62	2	0.32
SS4	156	2	0.32
SS5	101	1	0.15
SS6	nd	0	0

See this link:

http://fun-gen1.ibls.gla.ac.uk/mascot/cgi/master_results.pl?file=..%2Fdata%2F20211210%2FF142835.dat;sessionID=guest_guestsession

This search matches 4 peptides, because the statistical aspect of the matching in positively affected by searching a much smaller database. The "new" peptide, FLDGNEMTLADCNLLPKLHIVK, does contain a C, that is predicted to be modified by NEM – but the score is very weak. The search was run again, adding carbamidomethylating as an additional variable C modification, and this turned up another C-containing peptide AGSDGESIGNCPFSQR , with a pretty good score:

http://fun-gen1.ibls.gla.ac.uk/mascot/cgi/master_results.pl?file=..%2Fdata%2F20211210%2FF142838.dat;sessionID=guest_guestsession

Finally, the data was run in an error-tolerant search. This type of search will test amino acid modifications iteratively to look for peptides with unanticipated modifications. It is slow and the results are often difficult to interpret:

http://fun-gen1.ibls.gla.ac.uk/mascot/cgi/protein_view.pl?file=..%2Fdata%2F20211210%2FF142847.dat&hit=SS01&db_idx=1&px=1&ave_thresh=13&_ignoreionsscorebelow=-1&report=0&_sigthreshold=0.05&_msresflags=1345&_msresflags2=258&percolate=-1&percolate_rt=0&_minpeplen=5&sessionID=guest_guestsession

This turned up 9 peptides, with various possible modifications, that match to CLIC4. 3 of the C-containing peptides are on the list but only one, AGSDGESIGNCPFSQR, includes a predicted C modification, +57 Da, carbamidomethylating.

This result indicated that 3 out of 4 cysteine residues of CLIC4 was modified with NEM and only one cysteine modified with IAA, hence, CLIC4 under GSH incubation was reduced and all reduced cysteine residues were trapped with NEM, suggesting GSH has a significant effect on CLIC4 reduction (data was released by Glasgow polyomics).

4.2.11 Potential membrane proteins in microsomes are involved in ER reductive pathway

The above LC-MS/MS analysis and proteomics experiments illustrated that GSH as a reductive cytosolic component of the reductive pathway was able to partially reduced CLIC4 and cause to decrease in the microsomal protein expression levels concerning cysteine oxidation. We considered if other membrane proteins and ER luminal proteins could be directly or indirectly reduced by Glutathione. To investigate proteins influenced by reduced Glutathione (GSH), redox proteomics as described on (Figure 4-12) was carried out. The samples were prepared for liquid chromatography with tandem mass spectrometry (LC/MS/MS) by trypsin digestion followed by tandem mass tagging to enable quantification of the changes to the ratios of tagged peptides for each sample. The proteomics results showed that the redox state of some ER proteins became more reduced in the sample which was treated with +GSH compared with in the absence of GSH. These results can be considered as evidence for reduction effect of GSH on ER proteins. we analysed the location of proteins whose redox state changed because of the treatment with GSH based on the information from UniProt (Table 4-4). They are localized to the nucleus, cytoplasm, mitochondria, lysosome, intermediate compartment, secreted pathway, and ER. The proteins localized to the nucleus, cytosol, and mitochondria that have been identified their redox status change because of the treatment with the GSH were possibly co-purified with the microsomes. The proteins localized to the ER were reduced due to the incubation of the GSH suggesting that the reducing power can be passed into the ER lumen by some unknown membrane protein through the cytosolic reductive pathway (Figure 4-16). In addition, the secreted proteins which need to form their disulfide bonds in the ER are also identified, indicating that their disulfide formation is also influenced by treatment with the reduced GSH. The change of redox status change of several microsomal membrane proteins was also recognized, which suggested that they might be involve in the electron shuttling (Appenzeller-Herzog et al., 2008).

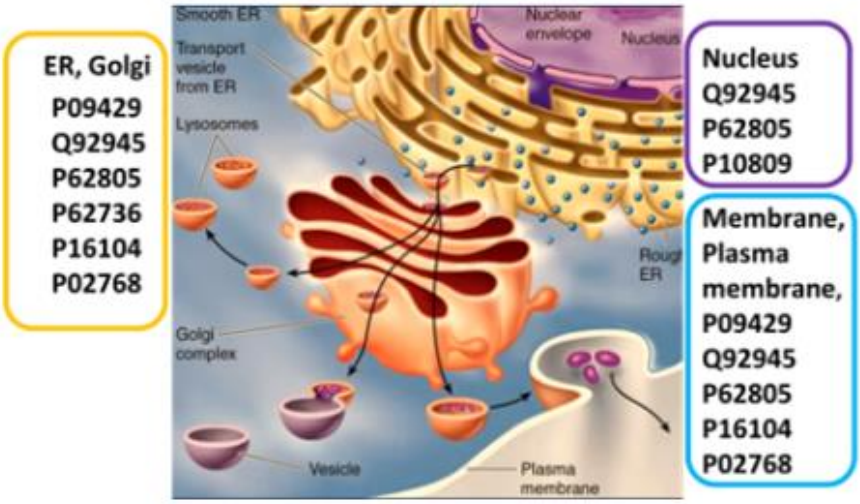


Figure 4-16 Proteomics analysis of microsomal sample after incubation in the absence or presence of reduced glutathione (GSH). Cartoon illustrating the final cellular location of proteins whose redox status changed significantly upon incubation of microsomes with the reduced Glutathione. Proteins that enter the secretory pathway are in blue and orange boxes that are distinguished by Accession number, whereas those that do not enter the ER are in purple ones.

Table 4-4 List of proteins demonstrating a significant change to their oxidized thiols along with information on their subcellular location, position, and identity of the modified cysteine(s).

Location of cysteine modification	Associate no	Protein location	Type	Cysteine modified	Red/Ox
ER	P08670	Association response	Soluble	Iodo-TMT	red
ER	P62437		soluble	Iodo-TMT	red
Cytosol	P08708		Soluble	Iodo-TMT	red
cytosol	P60174		Soluble	Iodo-TMT	red
ER	P35613	Membrane transport	integrate	Iodo-TMT	ox
Cytosol	P6017		Soluble	Iodo-TMT	-GSH
Nucleus	Q92945		Soluble	Iodo-TMT	-GSH
cytosol	Q92945		Soluble	Iodo-TMT	-GSH
cytosol	Q92945		Soluble	Iodo-TMT	-GSH
ER	Q92945		Soluble	Iodo-TMT	-GSH
Nucleus	Q92945		Soluble	Iodo-TMT	-GSH
cytosol	Q92945		Soluble	Iodo-TMT	-GSH
cytosol	Q92945		Soluble	Iodo-TMT	-GSH
mitochondria	Q92945		Soluble	Iodo-TMT	-GSH
ER	P35613	Membrane transport	integrate	Iodo-TMT	+GSH
Nucleus	P60174		Soluble	Iodo-TMT	+GSH
Cytosol	P08708		Soluble	Iodo-TMT	+GSH
Cytosol	P40926		Soluble	Iodo-TMT	+GSH
Nucleus	P00338		Soluble	Iodo-TMT	+GSH
ER	P04080		Soluble	Iodo-TMT	+GSH
Cytosol	P356234		Soluble	Iodo-TMT	+GSH
Cytosol	P631041		Soluble	Iodo-TMT	+GSH
Cytosol	P178527		Soluble	Iodo-TMT	+GSH
Nucleus	P31930		Soluble	Iodo-TMT	+GSH
ER	P62937		Soluble	Iodo-TMT	+GSH
Cytosol	Q96AE4		Soluble	Iodo-TMT	+GSH
ER	P17987		Soluble	Iodo-TMT	+GSH
cytosol	QP0839		Soluble	Iodo-TMT	+GSH
Nucleus	P33240		Soluble	Iodo-TMT	+GSH
Cytosol	P23369		Soluble	Iodo-TMT	+GSH
Cytosol	P54577		Soluble	Iodo-TMT	+GSH
Cytosol	P54577		Soluble	Iodo-TMT	+GSH

ER	P10768		Soluble	Iodo-TMT	+GSH
Cytosol	Q13151		Soluble	Iodo-TMT	+GSH
ER	Q02543		Soluble	Iodo-TMT	+GSH

Ox; oxidised, Red; reduced.

4.3 Discussion and Conclusion

Members of the CLIC family are soluble proteins capable of spontaneously inserting into lipid membranes and intracellular membranes - to form chloride selective ion channels (Shanks RA, et al., 2002). To date, characterization of these proteins has focused on their membrane insertion and their ion channel activity, with no distinct function assigned to their soluble form. The glutaredoxin-like activity of CLIC4 was further supported by its lack of activity in the common Trx disulfide reductase (Ferofontov A, et al., 2018). Grx2 and Grx3 dithiol glutaredoxin have been found to being reduced by thioredoxin reductase (TrxR) as well as GSH and glutathione reductase (GrxR). However, CLIC4, although belonging to Glutathione reductase family, cannot reduce by the selenonzyme, thioredoxin reductase (TrxR) (Alkameci H, et al., 2015). Given the lack of activity by CLIC4 protein, it could be speculated that CLIC4 has GSH-dependent enzymatic activity. The functional role of CLIC4 as a glutaredoxin protein which acts as enzymes by deglutathionylating the mixed disulphide between glutathione (GSH) and HEDS reagent suggesting soluble form of CLIC4 with Grx-like activity may act to catalyse the reduction of disulfide and thus function as oxidoreductases in mammalian cells (Alkhameci H, et al 2015).

We demonstrated that GSH is sufficient for reduction of CLIC4. Hence, CLIC4 has a potential ability in participating in reductive pathway due to its oxidoreductase. Moreover, we showed that CLIC4 exists in a predominantly reduced form in vivo, suggesting that it might be mostly present in soluble form and has enzyme catalytic activity. The reduction of CLIC4 in oxidized microsomes only occurs in the presence of cytosol or, more specifically, GSH. We demonstrated that when glutathione reductase from cytosol depleted of small molecules is supplemented with NADPH is not able to produce sufficient GSH to bring about the reduction of CLIC4 (Van Lit M, et al., 2011). In addition, the inhibition of glutathione reductase with Carmustin prevents the reduction of CLIC4 in cells, suggesting that cytosolic glutathione reductase is the main provider of reduced glutathione for reduction of CLIC4

and another ER oxidoreductase, hence, CLIC4 might be play an important role in the reductive pathway. However, this provides us to raise two main questions. First, how can CLIC4 eliminate the effect of oxidative stress in the ER? we have shown that GSH are able to cross the ER membrane to reduce CLIC4; thus, second how GSH crosses the ER membrane?

In addition, we showed that following oxidative stress, CLIC4 is immediately reduced and recovered, demonstrating that a pathway is in place to maintain CLIC4 in a reduced state. However, following oxidation, CLIC4 is not reduced in microsomes unless GSH is present. We showed that the cytosolic component capable of reducing CLIC4 could be GSH, which in turn is reduced by cytosolic glutathione reductase. We have shown that GSH is essential in the formation of native disulfide bonds by maintaining the oxidoreductases in a reduced state. The reduction of CLIC4 in oxidized microsomes only occurs in the presence of GSH. We demonstrated that when glutathione reductase from cytosol depleted of small molecules including NADPH it is not able to produce sufficient GSH for CLIC4 reduction. In addition, the inhibition of glutathione reductase with Carmustin prevents the reduction of CLIC4 in cells, suggesting that cytosolic glutathione reductase is the main provider of reduced glutathione for the ER and hence plays an important role in the reductive pathway. However, We show that biotinylated GSH and GSH able to reduce CLIC4 by crossing to the ER membrane; however, the question of how GSH crosses the ER membrane has been an area of much speculation. Several possibilities can be occurred, 1) through a membrane binding protein with oxidoreductase activity through a catalytic cysteine in its active site (Cao X, et al., 2020), 2). The import of GSH could occur by either a specific transporter by inserting to the ER membrane or through pores in the membrane (Duncan R.R, et al., 1997). 3) or through interaction with sec61 probably “Beta subunit” for its anti-toxic function (Abada et al 2012) and maybe involved with GSH conduction and might have an interaction of CLIC4 with Sec61 subunits to find a clear answer for transporting of GSH from the cytosol into the ER by CLIC4, we estimated CLIC4 due to ion channel activity may be involved with GSH transport.

The second question is what is the fate of GSH in the lumen once it has been oxidized? Because we have shown that the source of GSH for reduction of the ER oxidoreductases or ER placed CLIC4 is in cytosol, there are many options, which would be suggested. First,

GSSG may return to the cytosol via a specific unknown factor or transporter where it would be quickly reduced by glutathione reductase. However, this amount may not be significant because CLIC4 is not reduced in cells following oxidative stress when glutathione reductase is inhibited. Because by depletion of (GR) via Carmustin as a GR inhibitor, we showed deplation of CLIC4 be stopped and conversely might be the transport of GSSG be abolished, suggesting CLIC4 can be involved with this transport. Moreover, I show in 60 min after removal of TNF-α CLIC4 not able to be recovered, suggesting after consumption of GSH to recover of CLIC4 from extra oxidizing agent in 30 min the amount of GSSG increased and through CLIC4 might be transported to cytosol. In summary, I have shown that GSH makes a reduction for CLIC4 and thus GSH is reduced in the cytosol and transported into the ER; however, much is still unknown about the details of the trafficking of glutathione in eukaryotic cells. We estimated that CLIC4 can play a significant role in the trafficking of glutathione due to ion channel activity and reduction of disulfide bonds due to its oxidoreductase activity. We designed a mutant (cysteine 35 to Ala) to investigate the interaction of CLIC4 to GSH as a mutant trap approach; the result indicated that although the amount of GSH was decreased after 5 min of removal of TNF-α in order to recovery of oxidoreductase protein CLIC4 in the intact cells, the decrease of the amount of GSH with compared to wild type was less, suggesting cysteine 35 plays significant role in reduction of CLIC4 through GSH. As by replacing it to Alanine, GSH not able to make a transient structure with CLIC4 for reduction. Hence, in the next experiment to make sure GSH has a direct effect on CLIC4 or reduction of overall membrane protein, I designed a workflow with TMT-labelling.

Upon previous study (Prakash A.S, et al., 2018) Iodo-TMT has been designed to monitor cysteine redox status in biological samples (Giese J et al., 2022). This study demonstrates the advantage of utilizing iodo-TMT multiplexing to directly relate the redox state to changes in protein expression for complex proteomic samples providing more depth and information from a single set of samples. Applying this multi-quantitation method to samples originating from a single source (Human cell line) allows inconsistencies in redox conditions and sample variability to be minimized. In this project, the microsomal fractions were prepared from the HEK293T cell line, which stably overexpressed a CLIC4-V5. The results acquired have not only demonstrated the practicality of this method but also provided a list of proteins that have shown significant oxidation changes between minus GSH and GSH. Firstly, reduced glutathione (GSH) was used to incubate the microsomal

protein to investigate whether GSH would be able to reduce microsomal membrane proteins. Hence, by preparing the redox microsomal membrane in the presence or the absence of GSH, immunoprecipitation of CLIC4-V5, enzymatic digestion and LC/MS/MS (liquid chromatography and mass spectrometry), it was demonstrated that GSH can reduce microsomal CLIC4 in response to redox exchange reaction.

It has been illustrated that cytosolic GSH was required for the reduction of the disulfide bond of oxidoreductases (ERp57) in the ER (Bulleid N, et al., 2005); furthermore, the cytosolic NADPH was shown as a good candidate for the ultimate electron donor for the reduction in the ER (Robinson Ph. et al., 2020). The role of other components needed to reduce non-native disulfide in the ER and how to be generated in the cytosol and transferred into the ER remain to be addressed. Here, we used LC/MS/MS to indicate the potential reductive effect of GSH on CLIC4 cysteine residues to determine the ability of CLIC4 in participating in the reductive pathway either as a membrane-binding protein for transferring the reduced equivalents from the cytosol into the ER or as an oxidoreductase enzyme to reduce or isomerase of non-native disulfide bond within folding proteins entering the mammalian secretory pathway. Hence, indicating the redox status of CLIC4 with GSH and without GSH could be a requirement, and Iodo-TMT labelling following LC/MS/MS can provide us with essential information on the functional role of CLIC4 or other microsomal membrane proteins.

I have applied this workflow upon previous study by to compare the redox state of CLIC4 in response to +/-GSH treatment in microsomal membrane with respect to protein expression. In chapter 3, we demonstrated that the redox state of CLIC4 in vivo by modification of free cysteines with the alkylating agent AMS, demonstrating in response to oxidative stress GSH is capable to reduce CLIC4 in intact cells. In chapter 4, also, we show that following oxidative stress, CLIC4 is quickly reduced, demonstrating that a pathway is in place to maintain the oxidoreductase activity of CLIC4 in a reduced state. However, following oxidation, CLIC4 is not reduced in microsomes unless cytosol is present. We show that the only cytosolic component capable of reducing CLIC4 is GSH, in turn, reduced with glutathione reductase. In the end of this chapter, to uncover the important role of GSH in the reduction of CLIC4, we first examined the abundance of CLIC4 cysteine residues in the microsomal membrane in the absence or in the presence of GSH by subjecting them to LC/MS/MS at different ion reporters. We show that in the presence of GSH, most of the

CLIC4 cysteine residues are partially reduced, and CLIC4 exists in a predominantly reduced form in human cell line, suggesting that CLIC4 might act as isomerases or reductase. To investigate whether CLIC4, due to its ion channel activity, could be involved in the electron-shuttling or GSH transporting in the ER, we have developed a proteomic workflow with IodTMT-labeling to analyze the redox status of proteins in the microsomes before and after GSH treatment. Due to lack of coverage of CLIC4, Wecannot detect CLIC4 on TMT-labelling but we considered the effect of GSH on the overall redox state of microsomal membrane protein.

To identify overall protein oxidative changes between two (+GSH) and (-GSH) and normalize these changes concerning changes in protein expression, three redox microsomal replicates of each group, plus GSH and minus GSH, were isolated, and each sample I divided and treated separately to create two different sample sets. Half of each sample was incubated with N-ethylmaleimide during cell lysis to irreversibly block any free cysteines (SH) and prevent any further redox reactions. Any reversibly modified cysteines were then reduced and labeled with iodoTMT reagents. To quantify protein expression levels for cysteine-containing peptides of detected proteins, the other half of each sample was fully reduced, and all cysteine residues were labeled with iodoTMT. Affinity purification was carried out after trypsin digestion, allowing unlabelled peptides to be depleted while iodoTM-labeled peptides were enriched before MS analysis. Relative quantitation for each sample has been calculated from the average reporter ion abundances from each group of three replicates, investigating the abundance and reduction state of cysteine residues in the +GSH sample about -GSH sample. In this data set, an additional 326 proteins can be identified and quantified from peptides observed in any three out of the four samples and the lack of coverage of CLIC4 cysteine residues in the four samples could be a result of either instability of CLIC4 during sample preparation for isobaric labeling with several overnight incubations or lack of multidomain signal chromatography. The result demonstrated the reduction of cysteine residues in +GSH samples and provided us with a list of proteins that have shown significant oxidation changes under glutathione incubation, suggesting that GSH is able to reduce microsomal protein.

In addition to overcome to lack of coverage of CLIC4 in the TMT-labelling, we applied the MS/MS analysis of microsomal fractionation in three replicates of redox result of reduction

of CLIC4 show that GSH is responsible for reducing CLIC4 during oxidative stress induced by TNF-alpha and the partial reduction of microsomal CLIC4 was dependent on the catalytic cysteine (cysteine 35) in the CLIC4 motif (CXXS). Furthermore, the previous study by van Haaften indicated that reduced Trx could reduce disulfide bonds within the membrane protein that contains redox-active cysteine residues as part of their active sites (Cao X. et al 20 20). Reduced CLIC4 as a membrane-binding protein with this (CXXS) motif in its active site, can be considered as a candidate for a missing membrane protein in the ER to transfer reduction equivalent from the cytosol into the ER.

We assumed that the GSH as one of the essential reductive cytosolic powers which were generated in the cytosol was finally transported into the ER by disulfide exchange in a membrane protein that contains redox-active cysteine residues as part of their active sites. Based on this assumption, a membrane protein could be reduced to be capable to transfer the reduced equivalents into the ER (Holmgren, 1989). Hence, a proteomic experiment was carried out to identify the membrane proteins whose redox status was changed after incubation with a microsomal buffer with or without GSH. The redox status of several peptides changed during the incubation with the reduced Glutathione. The analysis of protein location showed that many of the corresponding proteins were placed in the ER. The results demonstrated that several thiols within endogenous proteins that were synthesized in the ER can be reduced by the reduced glutathione that could be investigated in our future work.

Chapter 5

5.1 General discussion

5.1.1 CLIC4 in response to TNF-α translocate from the cytosol into the ER and maintains in a reduced state.

To form chloride selective ion channels, embers of the CLIC family are soluble proteins capable of spontaneously inserting into lipid membranes and intracellular membranes - To date, despite the growing evidence supporting the role of cellular ion channels for the CLIC4 with the ability to auto-insert into several intracellular organelles upon the percentage of membrane cholesterol (Littler RD, et al., 2010) and its contribution to main signaling pathways (Valenzuela SM, et al., 2013), its enzyme catalytic activity and ion channel regulatory under redox stimuli in response to oxidative stress still need to be documented. Furthermore, the characterization of CLIC4 has focused on insoluble form and its ability for membrane insertion with ion channel activity, and no distinct function is assigned to a soluble form (Singh H, et al., 2007). The soluble form upon the glutaredoxin-like activity of CLIC4 was further supported by its lack of activity in the common Trx disulfide reductase. While Grx2 and Grx3 dithiol glutaredoxin can be reduced by (TrxR), (GrxR), and (GSH), and no evidence observed for reducing CLIC4 by (TrxR) in the absence of GSH (Littler RD, et al., 2010), because CLIC4 has identified for GSH-dependent enzymatic activity (Zanivan S, et al., 2017). The functional role of CLIC4 as a glutaredoxin protein which acts as an enzyme by deglutathionylating the mixed disulfide between glutathione (GSH) and HEDS reagent suggested the soluble form of CLIC4 with Grx-like activity might act to catalyze the reduction of disulfide bonds with potential as an oxidoreductase enzyme in mammalian cells (Alkhameci H, et al., 2015, and 2016).

In this project, we showed that CLIC4 is expressed and localized to several intracellular organelles and confocal microscopy confirmed the co-localization of CLIC4 with the specific organellular markers in each compartment. In addition, we showed that CLIC4 localized into the ER membrane and ER lumen due to colocalization with Calnexin (CNX) and Protein Disulfide Isomerase (PDI) as both protein resistance in the ER and lumen, respectively, and in response to TNF-α, not H_2O_2, CLIC4 can translocate into the ER from the cytosol. Further support for the localization of CLIC4 as a membrane-binding protein with an ion channel

function can be provided by translocating CLIC4 from the cytosol to the ER by inserting its putative binding site into the ER membrane as the cytoplasmic domain (*22.7 kDa*) remain in the cytosol, and the rest of CLIC4 stayed at the ER membrane as the ER luminal and membrane domains (*6.2 kDa*) (Duncan RR, et al., 1997). The soluble form of CLIC4 is indicated with enzyme catalytic activity (Venezuela S, et al., 2016), and is localized into the ER lumen (Hsu K, et al., 2019).

ER is the host of disulfide formation, and the formation of disulfide bonds is a rate-limiting step in protein folding and does not always result in the correct pairing of cysteine residues and might result in the accumulation of non-native disulfide or misfolded proteins in the ER (Chakravarthi S, et al., 2006). In addition, under oxidative stress induced by TNF-α, the rate of disulfide formation can be increased, which in turn can result in the accumulation of ROS and consequently accumulation of unfolded and misfolded protein in the ER (Xue X et al., 2005). Accumulation of unfolded protein consisting of newly synthesized secretory and membrane-associated proteins in the ER under oxidative stress induced by TNF-α is resulting in ER stress, and mammalian cells trigger a specific response termed the unfolded protein response (UPR) (Read A, et al., 2021). The UPR is characterized by three distinct signaling pathways that are triggered in response to ER stress, mediated by PERK, ATF6, and IRE1 to inhibit and remove unfolded protein from the ER. But in the case of misfolded proteins that result from non-native disulfide bond formation, ROS accumulation, and consequently ER oxidative stress, mammalian cells characterized by reductive pathways to form native and remove/edit non-native disulfide bonds with reduction and isomerization of disulfide bonds. Therefore, the ER is characterized by an entire family of oxidoreductases, which is dedicated to forming native disulfide bonds. The ER oxidoreductase family is essential for the formation of native disulfide bonds, and these enzymes must be either oxidized to be capable of forming disulfide bonds or reduced to reduce or isomerize non-native disulfide (Chakravarthi S, et al., 2003 and Board, P, et al., 2021).

We demonstrated that in the SP cells as a source of the ER (Wilson et al., 1995), CLIC4 is expressed in a reduced state in the normal physiological condition when no reducing or oxidizing agent was added to emphasize that although the ER lumen is optimized for the formation of disulfide bonds in secretory proteins, the active site of CLIC4 is either partially or completely reduced. Such a reduced state would be required if these proteins are

involved in disulfide bond reduction of isomerization. The presence of a reductive pathway should enable CLIC4 to recover from the addition of oxidizing agent to re-establish free thiols. In addition, we indicated that CLIC4 was partially reduced after 5 min and then completely reduced by 20 min following the removal of oxidative stress induced by TNF-α. Similar results were obtained for the other ER oxidoreductases (Jossep C, et al., 2005), thus establishing that in intact cells a robust reductive pathway exists to maintain this CLIC4 in a reduced state. Hence, CLIC4 as an oxidoreductase must be reduced to reduce or isomerize non-native disulfide. In addition, we examined the oxidoreductase activity of CLIC4 when enhancing the time point of removal of TNF-α, we indicated that CLIC4 was partially reduced after 10 min and was completely oxidized by 60 min following the removal of the oxidizing agent, suggesting that the reduction equivalent was consumed and CLIC4 be reduced and activated owning to recover from extra oxidized reagent. However, by consumption of GSH, the amount of GSSG to be increased suggesting CLIC4 might be responsible for removing GSSG from ER to cytosol where immediately be reduced by GR and returned to the reduced state to form disulfide bonds with another protein or peptides, notably GSH or even ER oxidoreductase, and after oxidizing, it needs to return back to the original state or reduced form; this recycling required the existence of an oxidative and reductive pathway in the mammalian cells (Chakavarthi s. et al.,2005).

However, although the oxidative pathway for the formation of disulfide bonds with two main role players such as Ero1 and PDI has been well characterized, a reductive pathway had not been clearly defined until now although in 2004 Jessop showed that the ER oxidoreductase enzyme is essential for the reduction of non-native disulfide bonds and maintained in a reduced state, and this reduction can occur by the reductive cytosolic components mainly reduced glutathione. Furthermore, in 2017 Poet demonstrated that the reduction in non-native disulfide requires NADPH as the ultimate electron donor, a robust cytosolic thioredoxin system, driven by thioredoxin reductase 1 (TrxR1 or TXNRD1), and inhibition of this reductive pathway prevents the correct folding and secretion of proteins that are known to form non-native disulfide during their folding and suggested the mammalian cells must contain a pathway for transferring reducing equivalents from the cytosol to the ER, which is required to ensure correct disulfide formation in proteins entering the secretory pathway. In addition, in 2019, Cao indicated that the thioredoxin reductase system provides the minimal cytosolic components required for reducing proteins within the ER lumen and required a membrane protein to shuttle electrons from

the cytosol to the ER. Hence, we assume that since CLIC4 structurally belongs to the glutaredoxin family with a binding site for GSH and a diverse function with oxidoreductase activity and ion channel responsibility, it can be considered as a good candidate to participate in the reductive pathway either directly with reduction and isomerization of non-native disulfide bonds or indirectly with transferring electron or other reductive cytosolic equivalent components from the cytosol into the ER. If this assumption is correct, then we can consider CLIC4 as a mis-component in this pathway either as a membrane protein for transferring cytosolic reductive equivalent or a reductase/isomerase for reduction of non-native disulfide bond and even for reduction and isomerization of ER oxidoreductase.

In this way, we first show the site and abundance of cysteine residues with mass spect analysis. The mass spectrum will usually be presented as a vertical bar graph and each bar represents an ion having a specific mass-to-charge ratio (m/z); the length of each bar determines the relative abundance of the ion, and the base peak is the most intense ion and is assigned an abundance of 100. The ion abundances and ion versus (m/z) of oxidized and reduced cysteine containing-peptide of CLIC4 in the two-dimensional representation are reflected by the signal area, and more simply by signal height. The height signal for cysteine residues can indicate the reduced form of cysteine-containing peptide and the intensive peak of a mass spectrum indicated the oxidized state of cysteine-containing-peptide and the intensity of the others is represented by relative to the base peak (Dourado A, et al., 2019). Since as much as CLIC4 was identified for 4 cysteine residues including cysteine 35, cysteine 100, cysteine 189, and cysteine 236 in 4 separate peptide fragmentation with no interaction or disulfide bridge formation between cysteine residues, this property could be considered as a potential for CLIC4 to identify cysteine site abundance via ion chromatograms without any overlapping of cysteine ion chromatogram signals (Yin Luo, et al., 2011). We showed that the reduction and alkylation of cysteine residues can improve the efficiency and robustness of proteolytic digestion of cysteine residues, which in turn, can be complicated for either disulfide scrambling or for structural hindrance to proteolytic sites. We showed that improving the optimal conditions for reduction and alkylation (achieving complete reduction and alkylation, without over-alkylating) can be evaluated by LC/MS with optimal coverage for cysteine residues, and after reduction and alkylation, excess alkylate was neutralized by another addition of a reducing agent to quench the alkylation reaction and avoid overalkylation (Joedicke L, et

al., 2016). The reduced state of CLIC4 with modification of free thiol of cysteine residues with N-ethylmaleimide (NEM) to trap free (-SH) to keep any further oxidation and be a specific identification for reduced cysteine residues. To identify the oxidized state of CLIC4, after reducing with a common reductant dithiothreitol (DTT), we slightly alkylated it with (IAA) iodoacetamide to characterize oxidized cysteine. Our result clearly indicated that CLIC4 is in place in a reduced position in the ER, possibly in a soluble form, and mostly in the ER for isomerization and reduction of non-native disulfide bonds.

The formation of disulfide bonds is an indispensable step in the folding of many glycoproteins and secretory proteins. Non-native disulfide bonds are often formed between incorrect cysteine residues, and thus the cell has dedicated a family of oxidoreductases that are thought to isomerize non-native bonds. For CLIC4 to be capable of performing isomerization or reduction reactions, it must be maintained in a reduced state. To further support oxidoreductase activity of the CLICs family, we examined the redox status of two family members of the CLICs family (CLIC1 and CLIC4), which are predominantly reduced in vivo and are quickly reduced following oxidative stress induced by TNF-α, confirming CLICs family have oxidoreductase activity and a robust reductive pathway is in place in mammalian cells. Using CLIC4 as a model of the CLICs family to investigate the oxidoreductase activity of CLIC4 and the possibility to participate in this pathway. But the next question will be raised; how does the reductive pathway make the reduction of CLIC4? And which components can be involved with this pathway? And how can this component be delivered into the ER? The previous studies indicated that the reductive pathway is cytosol-dependent (Chakravarthi S, et al., 2006) and the component responsible for the reduction of the oxidoreductases is the low molecular mass thiol glutathione. For isomerization and reduction of disulfide bonds, an oxidoreductase must be in a reduced state to be active for donating the two-electron and reduction of disulfide bonds. Hence, the existence of GSH can play a significant role in this process.

In addition, in chapter 3, our results demonstrated that the main reason for the translocation of CLIC4 from the cytosol into the ER is related to the catalytic cysteine of the redox motif (CXXS) within the thioredoxin-like motif in the N-terminal (Littler RD, et al., 2010). The oxidized cytosolic wild-type CLIC4 was reduced after the removal of TNF-α and with the enhancement of the time point, CLIC4 was totally depleted from the cytosol towards ER; by contrast, the oxidized cytosolic mutant CLIC4 (AXXS) did not recover from

the additional oxidized agent and no deplation was observed. Our result clearly demonstrated that catalytic cysteine residue can be involved in the reduction and translocation of CLIC4 from the cytosol into the ER and can confirm glutathione-dependent oxidoreductase activity and glutathione transferase function of CLIC4.

5.1.2 GSH can directly reduce CLIC4 in the ER

In chapter 3, we demonstrated that CLIC4 can be involved in the reductive pathway with oxidoreductase activity by maintaining a reduced position in the ER. In chapter 4, we examined several cytosolic components and we demonstrated that GSH is the only cytosolic component with potential for reduction and recovery of CLIC4 from oxidative stress induced by TNF-α in mammalian cells. This stage raises the question of how GSH has the ability for reduction. Further supporting evidence for the ability of GSH for reduction can refer to many years while it was assumed that oxidized glutathione (GSSG) can able to provide an oxidizing equivalent for disulfide bond formation in newly synthesized proteins in the ER (Chcavarthi S. et al., 2006). However, it has now been shown that the inhibition or depletion of GSH from the yeast cells could be resulted in hyper-oxidation of proteins entering the secretory pathway in mammalian cells, suggesting that glutathione can be the main provider of the reducing equivalents for reduction of non-native disulfide in the ER compartments (Cuozzo J, et al., 1999). In fact, that the efficient amount of GSH level not only plays an important role in disulfide bond f formation, (Molteni S, et al., 2004) but also can be involved in a correct disulfide formation, suggesting GSH could be required for isomerization (Chakravarthi S, et al., 2004).

We demonstrated that GSH is sufficient for the reduction of CLIC4. Hence, CLIC4 has a potential ability in participating in the reductive pathway due to its oxidoreductase. Moreover, we showed that CLIC4 exists in a predominantly reduced form in vivo, suggesting that CLIC4 might be mostly present in soluble form and showed the enzyme catalytic activity. The reduction of CLIC4 in oxidized microsomes only occurs in the presence of cytosol or, more specifically, GSH. We demonstrated that when glutathione reductase from cytosol depleted of small molecules is supplemented with NADPH is not able to produce sufficient GSH to bring about the reduction of CLIC4. In addition, the inhibition of glutathione reductase with Carmustin prevents the reduction of CLIC4 in cells, suggesting

that cytosolic glutathione reductase is the main provider of reduced glutathione for the reduction of CLIC4; hence, GSH plays an important role in the reductive pathway. However, still we need to know how GSH can pass through the ER membrane? and how oxidized GSSG conversely can pass from the ER to the cytosol? we assumes that the answer for both of questions could be CLIC4 because we show the mutant of CLIC4 not be reduced and not able to re-localized from the ER to the cytosol, hence possibility of interaction of GSH and CLIC4 for transferring GSH cross the ER might be suggested.

In addition, we showed that following oxidative stress, CLIC4 is immediately reduced and recovered, demonstrating that a pathway is in place to maintain CLIC4 in a reduced state. However, following oxidation, CLIC4 is reduced in microsomes unless GSH is present. We showed that the cytosolic component capable of reducing CLIC4 could be GSH, which in turn is reduced by cytosolic glutathione reductase. Also, GSH is essential in the formation of native disulfide bonds by maintaining the oxidoreductases in a reduced state. The reduction of CLIC4 in oxidized microsomes only occurs in the presence of GSH. We demonstrated that when glutathione reductase from cytosol depleted of small molecules, including NADPH, it is not able to produce sufficient GSH for CLIC4 reduction. However, the question of how GSH crosses the ER membrane has been an area of much speculation. The import of GSH could occur by either a specific transporter or through pores in the membrane. We estimated CLIC4 due to ion channel activity may be involved with GSH transport. The second question is what is the fate of GSH in the lumen once it has been oxidized? Because we have shown that the source of GSH for reduction of the ER oxidoreductases is in the cytosol, thus, GSSG may be transport to the cytosol via a specific transporter where it would be quickly reduced by glutathione reductase. But this amount is not enough for reduction of CLIC4 or even other oxidoreductase following oxidative stress if glutathione reductase be inhibited with BCUN (Joosep C, et al 2005).

However, whether the role of GSH as a redox buffer (Chakravarti S, et al., 2004) against oxidative stress or as a reductant for the reduction of CLIC4 is described, the existence of a membrane-binding protein with oxidoreductase activity with catalytic cysteine in its active site would be necessary. In chapter 4, we showed that CLIC4 as a membrane-binding protein with ion channel activity and as an oxidoreductase with (CXXS) motif is totally involved with GSH function because in the mutant form of CLIC4 no observation of reduction and translocation to the ER was apparent, suggesting CLIC4 as a membrane-

binding with oxidoreductase activity, and ion channel activity might be involved with GSH translocation and reduction. Moreover, we showed in both functional forms of CLIC4 in the ER, CLIC4 must be located in a reduced form to be activated for reduction or isomerization. In addition, we demonstrated that the cytosol is the source of reduced glutathione, which is imported into the ER and directly reduces the CLIC4. Such a direct role for GSH in the reduction of disulfide within the ER via reductive pathway would be considered for DsbD/DsbC pathway present in the prokaryotic cells (periplasm of E. coli) (Molteni S, et al., 2004), which is involved to provide electrons for the bacterial reductive pathway with the aid of Trx (Thorpe C, et al., 2002).

5.1.3 CLIC4 as a cross-link between oxidative and reductive pathways in the ER

The separation of the oxidative and reductive pathways is achieved with consideration of GSH as a not suitable or a poor substrate for Ero1 (Tu B, et al., 2002), while Ero1 can oxidize proteins at a high level of GSH concentration (even at 2.5 mM GSH) (Tu B, et al., 2000). Thus, whereas Ero1 is essential for providing oxidizing equivalents, GSH is essential for providing reducing equivalents in mammalian cells (Shi Z, et al., 2000), suggesting GSH is essential for making a balance between oxidative and reductive pathways.

We showed in addition to glutathione reductase which is essential for the reduction of glutathione to provide enough reduced glutathione (GSH) for the reduction of CLIC4 via the reductive pathway, glutathione synthetase also plays a significant role in providing sufficient glutathione from the synthetase process of three amino acids. We showed by depleting the cytosolic and membrane intracellular pool of GSH by inhibiting the initial enzyme (γ-glutamyl cysteine synthetase) for synthesizing GSH through BSO (Griffith O.W, et al., 1982), the reduction of CLIC4 was abolished, although BSO makes a reduction of cellular GSH from the 10 mM to 2 mM concentration and it would be sufficient for reduction of ER oxidoreductase at a steady state when incubated with BSO. We showed the effect of this decreased pool of GSH on the recovery of CLIC4 from oxidation was totally different. AS no recovery was absorbed of oxidized CLIC4 even after 60 min of incubation following the removal of the oxidizing agent, and also, we showed that the inhibition of GSH recovery also prevented any reduction of CLIC4 following the removal of an oxidizing agent, clearly demonstrating that for CLIC4 to remain in a reduced state, it requires the

presence of intracellular GSH. In addition, the time course of recovery of the intracellular pool of GSH following treatment with TNF-α correlates with the reduction of CLIC4. Moreover, we indicated that the reduction of CLIC4 in the cytosolic fraction in the absence of a glutathione inhibitor is faster than the membrane fraction, indicating glutathione has a cytosolic source for synthesis and reduction. In addition, we demonstrated that the reduction and recovery of CLIC4 from the oxidizing agent (TNF-α) in the presence of glutathione synthetase and reductase inhibitors was abolished and doesn't matter in the ER membrane or even in the cytosolic fraction. It is clear that glutathione plays a central role in donating electrons to maintain the CLIC4 in a reduced form, active state, to reduce non-native disulfide (Hansen H.G, et al., 2012). Also, we showed in the knockout CLIC4, that PDI is located in an oxidized state, suggesting CLIC4 might be involved with the reduction of ER oxidoreductase and could be considered as a cross-link between oxidative and reductive pathways.

5.1.4 CLIC4 is a membrane-binding protein with oxidoreductase activity that might be involved in GSH transport

In chapter 4 we have shown that the source of GSH for the reduction of the CLIC4 in the ER is in the cytosol. GSH cross the ER membrane either through a specific transporter (Banhegyi G, et al., 1999) or through pores in the membrane (Le Gall S, et al., 1999). In the cytosol we assume that GSSG would be quickly reduced by glutathione reductase. Also, a little amount of GSSG may be reduced by free thiols in nascent polypeptides because the nascent are translocated from the ribosome into the ER lumen. However, this proportion appears not to be significant because CLIC4 is not reduced in cells following oxidative stress when glutathione reductase is inhibited hence, the possibility of CLIC4 in transferring GSH due to ion channel activity and inserting to the ER lumen (Duncan RR, et al., 1997), and glutathione binding site in the G-site within redox motif. In chapter 4, we have demonstrated that GSH directly reduces CLIC4 at physiological concentrations in vitro, and biotinylated glutathione forms a mixed disulfide with CLIC4 in the microsomes. Our results demonstrate that glutathione plays a direct role in the isomerization of disulfide bonds by forming a transient intermediate structure and maintaining CLIC4 in a reduced state and a large proportion of ER luminal glutathione is found as mixed disulfide with proteins (Bulleid N, et al., 2006); hence, GSH could reduce substrate proteins directly. CLIC4 needs to form

a transient intermediate with GSH to transient from the oxidized state to the reduced state. Our mass spec analysis supported this transient structure, which could have belonged to catalytic cysteine within the CXXS motif, under TNF-α induced oxidative stress.

We showed the biotinylated GSH is able to cross the ER membrane to reduce CLIC4, suggesting this intermediate non-covalent structure provided the isomerase potential activity for CLIC4 for thiol exchange reaction across the ER. Moreover, we showed that GSH directly makes a reduction of CLIC4 in vitro conditions. To support data, we demonstrated that GSH directly reduced CLIC1 in vitro condition as well, suggesting GSH has significant potential in the reduction of CLICs protein in vivo, and CLICs family owing to membrane binding ability, and G-site for GSH binding could be involved with GSH transport from the cytosol into the ER. In addition, we demonstrated with block of ion channel activity with IAA-94, the reduction of CLIC4 is inhibited, suggesting ion channel activity can regulate oxidoreductase activity of CLIC4, the catalytic activity of CLIC4 involved with GSH and we can conclude that IAA-94 can inhibit GSH transferring via inhibition ion channel activity, hence, CLIC4 can be involved with GSH transporting.

To further support using fractionation study we showed that CLIC4 in response to TNF-α with respect to concentration (10 uM) can translocate from the cytosol to the ER and a mis-localization in the cytosol and an increase of CLIC4 expression in the ER had been absorbed. In addition, we showed that this mis-localization can be related to the ion channel activity of CLIC4 because by using IAA-94 inhibitor, no depletion of CLIC4 was observed in the cytosol, suggesting CLIC4 in response to oxidative stress is not only maintained in a reduced state or active form as an oxidoreductase but also can be translocated to the cytosol via its ion channel activity property. This translocation activity can be absorbed with depletion from the cytosol towards ER or possibly the existence of a membrane protein with an isomerization activity and containing a redox motif with two or even one catalytic cysteine is a good candidate for its active site for GSH translocation, hence, CLIC4 could be a good candidate for GSH translocation or transferring of cytosolic reductive equivalents.

5.1.5 CLIC4 as a protein membrane has the potential to participate in the reductive pathway

To reduce the non-native disulfide in the ER, CLIC4 needs to stay in a reduced form to donate electrons to the substrate proteins. In chapter 4, we noticed that incubation of CLIC4 with GSH, which is required for redox homeostasis in the ER (Appenzeller-Herzog et al., 2010), can fully reduce oxidized CLIC4 in the SP cells. This result is consistent with a previous study by Jessop (Jessop C, et al., 2004), who showed that cytosolic GSH is required for the ER reductive pathway by maintaining the CLIC4 in a reduced state. We showed further support for GSH for its potential ability for reducing CLIC4 by using TMT labelling and MS/MS analysis (Prakash S.A, et al., 2018). The proteomic experiment confirmed the reduction of CLIC4 by GSH. In addition, we indicated the redox state of the overall protein in the absence and presence of GSH with respect to protein expression.

5.2 Future experiments:

5 .2.1 Investigate the ability of CLIC4 for transporting GSH from the Cytosol to the ER via sec61

In chapter 3 we showed CLIC4 is involved in the interaction with GSH and using IAA-94 and mutant trap (CXXS-AXXS) can inhibit the reduction of CLIC4, suggesting CLIC4 as a membrane-binding protein can be involved with GSH transporting. GSH is originally produced in the cytosol and can be transferred into mitochondria (Meredith and Reed, 1982) and, the ER (Hwang et al., 1992). GSH can be transported across the ER membranes (Molteni et al., 2004, Chakravarthi et al., 2006), and might be involved in several ER pathways, notably the reductive pathway. In chapter 4 also our result supports that GSH can cross the ER membrane and lead to the reduction of CLIC4 and PDI as an ER oxidoreductase. However, still, there are many points not understood about transporting GSH across the ER membrane. Recently, it was indicated that the main functional role of the Sec61 channel is facilitating GSH circulation to the ER and the study also showed that in S. cerevisiae the transportation of GSH crossing the ER membrane is regulated by the BiP and ERO1 (Ponsero et al., 2017). Thus, investigating Sec61 for transporting GSH-dependent

ER in the reductive pathway could be valuable and pave the way for understanding many aspects of the reductive pathway.

To understand the functional role of Sec-61 in GSH transportation, we will apply some sec61 inhibitors, such as mycolactone, to block sec-61 channel function (Baron et al., 2016, McKenna et al., 2017). Then we can check the amount of GSH in the two types of stable cell lines Sec-61 (+/-mycolactone) and CLIC4-V5(+/-IAA-94), to determine the functional role of Sec-61 and CLIC4-V5 on GSH transport. The result will suggest that reduction is observed in the SP cells untreated with mycolactone/ IAA-94 but no reduction is observed in the sample treated with mycolactone. In addition, to investigate whether Sec-61-His has an interaction with CLIC4-His, we need to purify CLIC4-His and Sec-61-His in vitro conditions using HPLC, in order to investigate the interaction of Sec-61-His and CLIC4-His in the presence and in the absence of GSH. We think our result indicated that CLIC4 and Sec-61 have interaction and both of them are involved with GSH transport.

In addition, the determination of the redox state of CLIC4-V5 can be used in the evaluation of the role of Sec61. The SP cells are generated from the HEK293Tstable cell line, after full oxidation, the SP cells are treated with or without mycolactone. Then the SP cells will be incubated with GSH. The redox state of CLIC4 will be visualized by differential alkylation. If no reduction occurs in the SP cells treated with mycolactone, it will demonstrate that the GSH is transported across the ER membrane by the Sec61 channel.

5.2.2 Evaluation the role of CLIC4 as a membrane protein in the ER reductive pathway

In chapter 5 we already screened some ER membrane proteins, which might be their redox state can be influenced by GSH. In addition, we succeed to identify more membrane protein with utilizing GSH trapping and proteomic analyse, it is necessary to have an assay to evaluate if the proteins are involved in the ER reductive pathway. We need to characterise is CLIC4 as a membrane protein required for the ER reductive pathway? our future work mostly would be focused on identifying binding affinity CLIC4 to GSH involved in GSH trafficking from the cytosol to the ER (structural study) through calorimetry methods and investigating the effect of CLIC4 oxidoreductase activity on disulfide formation under oxidative stress induced by TNF-α in KO and KW stable cell line.

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Appendix 1 Primers

CLIC4-wild type primer Forward 5’-AAAAAATCTAGAACCATGGCGTTGTCGATGCCG-3’ Revers 3’AAAAAAGAATTCTTAAGTAGAATCAAGACCAAGAAGAGGATTAGGAATAGGTTTACCCTTGGTGA GTCTTTTGGCTACATCA-5’

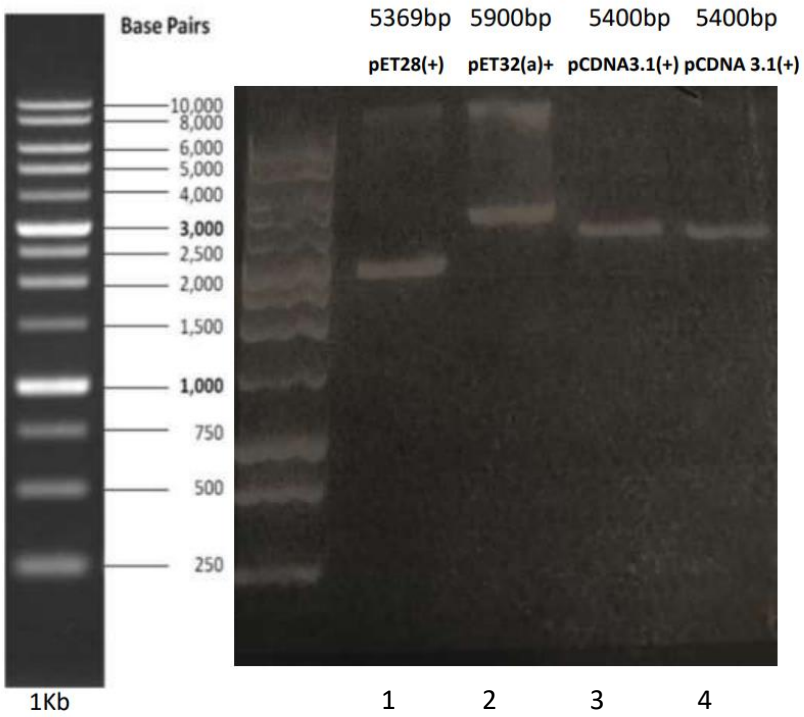
CLIC1 wild type primer Forwards 5’-AAAAAACTTAAGACCATGGCGTTGTCGATGCCG-3’ Revers 3’AAAAAATCTAGATTAAGTAGAATCAAGACCAAGAAGAGGATTAGGAATAGGTTTACCCTTGGTGA GTCTTTTGGCTACATC-5’

Mutant CLIC4 Primer sequences

(5’to 3’) 5’-CTCTGGGAAAAGGGGGCGTTTCCTATGCTTTCACCATCACT-3’

5’-AGTGATGGTGAAAGCATAGGAAACGCCCCCTTTTCCCAGAG-3

Appendix 2: Vector was applied in this project



Appendix 3 DNA sequencing for this project

Chloride intracellular ion channel 4 (CLIC4) – 256 aa (+TEV +6xHis)

MALSMPLNGLKEEDKEPLIELFVKAGSDGESIGNCPFSQRLFMILWLKGVVFSVTTVDLK

RKPADLQNLAPGTHPPFITFNSEVKTDVNKIEEFLEEVLCPPKYLKLSPKHPESNTAGMD

IFAKFSAYIKNSRPEANEALERGLLKTLQKLDEYLNSPLPDEIDENSMEDIKFSTRKFLD

GNEMTLADCNLLPKLHIVKVVAKKYRNFDIPKEMTGIWRYLTNAYSRDEFTNTCPSDKEV

EIAYSDVAKRLTKENLYFQSAHHHHHHH

DNA sequence

GGATCCatggcgttgtcgatgccgctgaatgggctgaaggaggaggacaaag
agcccctcatcgagctcttcgtcaaggctggcagtgatggtgaaagcatagg
aaactgccccttttcccagaggctcttcatgattctttggctcaaaggagtt
gtatttagtgtgacgactgttgacctgaaaaggaagccagcagacctgcaga
acttggctcccgggacccacccaccatttataactttcaacagtgaagtcaa
aacggatgtaaataagattgaggaatttcttgaagaggtcttatgccctccc
aagtacttaaagctttcaccaaaacacccagaatcaaatactgctggaatgg
acatctttgccaaattctctgcatatatcaagaattcaaggccagaggctaa
tgaagcactggagaggggtctcctgaaaaccctgcagaaactggatgaatat
ctgaattctcctctccctgatgaaattgatgaaaatagtatggaggacataa
agttttctacacgtaaatttctggatggcaatgaaatgacattagctgattg
caacctgctgcccaaactgcatattgtcaaggtggtggccaaaaaatatcgc
aactttgatattccaaaagaaatgactggcatctggagatacctaactaatg
catacagtagggacgagttcaccaatacctgtcccagtgataaggaggttga
aatagcatatagtgatgtagccaaaagactcaccaagGAAAACCTGTACTTC
CAAAGCGCGCACCATCACCATCACCATTAACTCGAG

Chloride intracellular ion channel 1 (CLIC1) – 241 aa

MAEEQPQVELFVKAGSDGAKIGNCPFSQRLFMVLWLKGVTFNVTTVDTKRRTETVQKLCPGGQLPFLLYGTEV
HTDTNKIEEFLEAVLCPPRYPKLAALNPESNTAGLDIFAKFSAYIKNSNPALNDNLEKGLLKALKVLDNYLTSPLPEE
VDETSAEDEGVSQRKFLDGNELTLADCNLLPKLHIVQVVCKKYRGFTIPEAFRGVHRYLSNAYAREEFASTCPDD
EEIELAYEQVAKALK

XX – Required for membrane protein integration

XX – Potential Helical transmembrane domain

XX – Disulfide bond formation

XX – GST C-terminal domain

XX – Glutathione binding sites

```
atg gct gaa gaa caa ccg cag gtc gaa ttg ttc gtg aag gct ggc agt gat ggg gcc aag
att ggg aac tgc cca ttc tcc cag aga ctg ttc atg gta ctg tgg ctc aag gga gtc acc
ttc aat gtt acc acc gtt gac acc aaa agg cgg acc gag aca gtg cag aag ctg tgc cca
ggg ggg cag ctc cca ttc ctg ctg tat ggc act gaa gtg cac aca gac acc aac aag att
gag gaa ttt ctg gag gca gtg ctg tgc cct ccc agg tac ccc aag ctg gca gct ctg aac
cct gag tcc aac aca gct ggg ctg gac ata ttt gcc aaa ttt tct gcc tac atc aag aat
tca aac cca gca ctc aat gac aat ctg gag aag gga ctc ctg aaa gcc ctg aag gtt tta
gac aat tac tta aca tcc ccc ctc cca gaa gaa gtg gat gaa acc agt gct gaa gat gaa
ggt gtc tct cag agg aag ttt ttg gat ggc aac gag ctc acc ctg gct gac tgc aac ctg
ttg cca aag tta cac ata gta cag gtg gtg tgt aag aag tac cgg gga ttc acc atc ccc
gag gcc ttc cgg gga gtg cat cgg tac ttg agc aat gcc tac gcc cgg gaa gaa ttc gct
tcc acc tgt cca gat gat gag gag atc gag ctc gcc tat gag caa gtg gca aag gcc ctc
aaa
```

CLIC4 mutant (C35>A)

Chloride intracellular ion channel 4 (CLIC4) +V5 tag

MALSMPLNGLKEEDKEPLIELFVKAGSDGESIGAPFSQRLFMILWLKGVVFSVTTVDLK

RKPADLQNLAPGTHPPFITFNSEVKTDVNKIEEFLEEVLCPPKYLKLSPKHPESNTAGMD

IFAKFSAYIKNSRPEANEALERGLLKTLQKLDEYLNSPLPDEIDENSMEDIKFSTRKFLD

GNEMTLADCNLLPKLHIVKVVAKKYRNFDIPKEMTGIWRYLTNAYSRDEFTNTCPSDKEV

EIAYSDVAKRLTKGKPIPNPLLGLDST

DNA sequence

aaaaaatctagaaccatggcgttgtcgatgccgatggcgttgtcgatgccgctgaatgggctgaag
gaggaggacaaagagcccctcatcgagctcttcgtcaaggctggcagtgatggtgaaagcatagga
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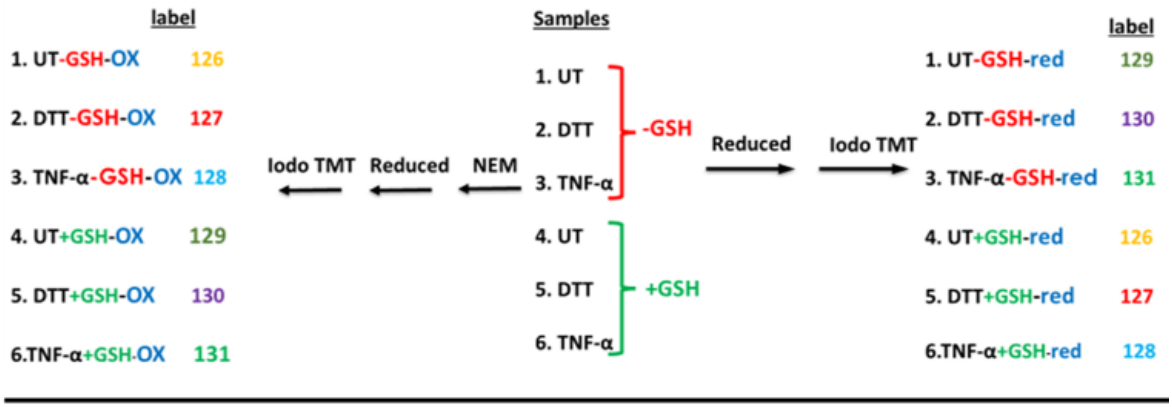
Appendix 4 TMT-workflow

(A)



(B)

Red		
	UT+GSH-*red*	126
	DTT+GSH-*red*	127
	TNF-α+GSH-red	128
	UT-GSH-*red*	129
	DTT-GSH-*red*	130
	TNF-α-GSH-*red*	131

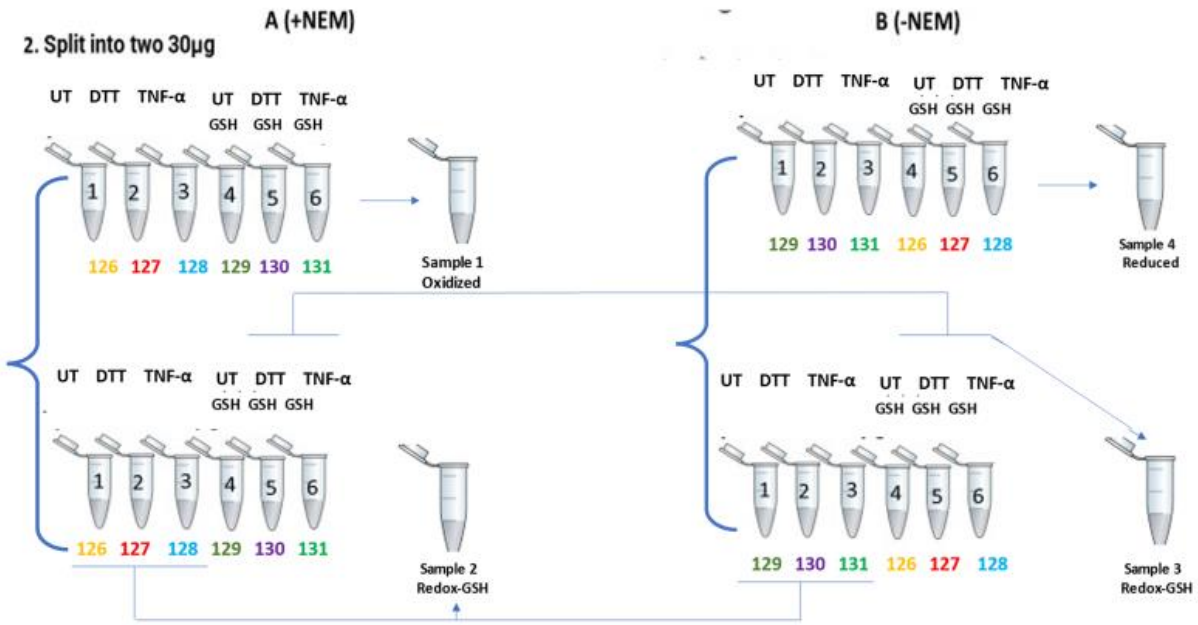
Ox		
	UT-GSH-*Ox*	126
	DTT-GSH-*Ox*	127
	TNF-α-GSH-*Ox*	128
	UT+GSH-*Ox*	129
	DTT+GSH-*Ox*	130
	TNF-α*+GSH-Ox*	131

+GSH		
	UT+GSH-*red*	126
	DTT+GSH-*red*	127
	TNF-α+GSH-*red*	128
	UT+GSH-*Ox*	129
	DTT+GSH-*Ox*	130
	TNF-α*+GSH-Ox*	131

-GSH		
	UT-GSH-*Ox*	126
	DTT-GSH-*Ox*	127
	TNF-α-GSH-*Ox*	128
	UT-GSH-*red*	129
	DTT-GSH-*red*	130
	TNF-α-*GSH-red*	131

Labelling workflow for redox state without GSH (-GSH) and with GSH (+GSH) of human cell line (A). Each sample was split into two equal parts and either fully reduced (red subscript) or blocked with NEM (ox subscript) before iodoTMT labeling. (b) By using strategic iodoTMT labeling, the individually labeled samples could then be combined in different groups to create four individual simple samples, providing relative quantitation for protein expression (Red), quantitation of cysteine oxidation (Ox), percentage of cysteine oxidation in group 1 (redox state-GSH), and percentage of cysteine oxidation in (redox +GSH). Thus, this study shows that the comparative proteomic analyses can indicate greater response to oxidative stress for +GSH compared with the -GSH condition. Therefore, this potential leads us to investigate the ability of membrane proteins to participate in reductive pathways as a mis essential component, this workflow and labelling is deigned upon previous work (Prakash A.S, et al., 2018).

Appendix 5 TMT-workflow and samples divisions



Workflow of Mix-and-Match Proteomics Each sample was split into two 30 µg aliquots (24 samples in total) and made up of equal volumes with HEPES buffer. Reduction of reversibly modified cysteine residues was performed with TCEP-HCl (final concentration ~5 mM) and incubated at 37 °C for 1 h. IodTMT reagents were resuspended in MeOH to a concentration of 10 mg mL−1 and samples were labeled with 10µL of the appropriate iodoTMT reagent, as described in Figure 5-3. Individual samples were combined in equal protein concentrations into four simple samples; a fully reduced (Red) sample with all cysteines labeled with iodoTMT, a NEM-blocked (Ox) sample with only modified cysteines labeled with iodoTMT, and (-GSH) and (+GSH) samples comparing modified cysteines with total cysteine content for each group.

Appendix 6 Furmola for Calculation OX/red, +GSH/-GSH(the furmula just come from previous work by (Prakash A.S, et al., 2018)).

$$\frac{\mathbf{Ox}}{\mathbf{Red}} = \frac{\frac{\text{+GSH(average)-ox}}{\text{-GSH(average)-ox}}}{\frac{\text{+GSH(average)-red}}{\text{-GSH(average)-red}}} = \frac{\text{(+GSH(average)-ox) X (-GSH(average)-red)}}{\text{(-GSH(average)-ox) X (+GSH(average)-red)}} = \frac{\frac{\text{+GSH(average) -ox}}{\text{+GSH(average)-red}}}{\frac{\text{-GSH (average)-ox}}{\text{-GSH (average)-red}}} = \frac{\mathbf{+GSH}}{\mathbf{-GSH}}$$

$$\frac{\mathbf{Ox}}{\mathbf{Red}} = \frac{\mathbf{+GSH}}{\mathbf{-GSH}}$$