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Regulation of protein cysteinylation and the inflammatory cascade by thioredoxin-related protein of 14 kDa in pancreas

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CERTIFICAN:

Que la presente memoria, titulada "**Regulation of protein cysteinylation and the inflammatory cascade by thioredoxin-related protein of 14 kDa in pancreas**", corresponde al trabajo realizado bajo su dirección por D. **PABLO MARTÍ ANDRÉS**, para su presentación como Tesis Doctoral en el Programa de Doctorado en Fisiología de la Universitat de València.

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INDEX

ABBREVIATIONS

–SH	Thiol group
–SO_2H	Sulfinic acid
–SO_3H	Sulfonic acid
–SOH	Sulfenic acid
γ-GT	γ-glutamyltranspeptidase
ADA	Adenosine deaminase
AK	Adenosine kinase
AMP	Adenosine monophosphate
AP	Acute pancreatitis
Bcl-2	B-cell lymphoma 2
BECN1	Beclin-1
BSO	Buthionine sulfoximine
CBS	Cystathionine beta-synthase
CDK5	Cyclin-dependent kinase 5
CKK	Cholecystokinin
CXCL1	C-X-C motif chemokine ligand 1
Cys	Cysteine
CysBIO	Biotinylated cysteine
Cys_P	Peroxidatic cysteine
Cys_R	Resolving cysteine
DAMPs	Damage associated molecular patterns
DSP	Dual specificity phosphatases
GCL	Glutamate-cysteine ligase
GCL	Cystathionine gamma-lyase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GR	Glutathione reductase
Grx	Glutaredoxin
Grx2	Glutaredoxin 2
GS(O)SG	Thiosulfinate
GSH	Reduced glutathione
GSK-3β	Glycogen synthase kinase 3 beta

GSNO	*S*-nitrosoglutathione
GSS	Glutathione synthetase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HEAT	Huntington elongation-A-subunit TOR
HMBG1	High mobility group box 1
ICAM1	Intercellular adhesion molecule 1
IL-1	Interleukin 1
IL-1β	Interleukin 1β
IL-1α	Interleukin 1α
IL-10	Interleukin 10
IL-18	Interleukin 18
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
k_{cat}	Catalytic constant
kDa	Kilodaltons
K_m	Michaelis constant
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAT	Methionine adenosyltransferase
MCP1	Monocyte chemoattractant protein 1
MLKL	Mixed lineage kinase domain-like
MPO	Myeloperoxidase
MS	Methionine synthase
Msr	Methionine sulfoxide reductase
MTs	Methyltransferases
NAC	N-acetylcysteine
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NO	Nitric oxide

$O_2^{\bullet -}$	Superoxide anion
OH•	Hydroxyl radical
PAMPs	Pathogen associated molecular patterns
PDI	Protein disulfide isomerase
PIP	PP1-interacting proteins
PP1	Protein phosphatase 1
PP2	Type 2 protein phosphatase
PP2A	Protein phosphatase 2A
PPP	Protein serine/threonine phosphatases
Prx	Peroxiredoxin
Prx1	Peroxiredoxin 1
Prx2	Peroxiredoxin 2
Prx3	Peroxiredoxin 3
Prx4	Peroxiredoxin 4
Prx5	Peroxiredoxin 5
Prx6	Peroxiredoxin 6
PTK	Protein tyrosine kinases
PTP	Protein tyrosine phosphatases
PTP1B	Protein tyrosine phosphatase 1B
RIP	Receptor-interacting protein kinase
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$RS^{\bullet}$	Thiyl radical
SAH	*S*-adenosylhomocysteine
SAHH	SAH hydrolase
SAM	*S*-adenosylmethionine
Sec	Selenocysteine
SOD	Superoxide dismutase
SOFA	Sepsis-related organ failure assessment
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
TGR	Thioredoxin glutathione reductase
THF	Tetrahydrofolate
TNF-α	Tumor necrosis factor
TRP14	Thioredoxin-related protein of 14 kDa

Trx	Thioredoxin
Trx1	Thioredoxin 1
Trx2	Thioredoxin 2
TrxR	Thioredoxin reductase
TrxR1	Thioredoxin reductase 1
TyrR	Trypanothione reductase
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

TABLE INDEX

FIGURE INDEX

ABSTRACT

Although under physiological conditions ROS are essential for normal cell signaling, ROS overproduction under oxidative stress conditions may cause damage to biomolecules. Cysteine residues within proteins are often easily oxidized. Reversible cysteine oxidation is used as a mechanism of redox signaling and control of protein function. However, oxidative stress promotes oxidation of protein thiols, leading to the formation of mixed disulfides between proteins and low-molecular-weight thiols. Disulfide reductases such as thioredoxins can reduce disulfide bonds back to free thiols.

Acute pancreatitis is currently one of the leading causes of hospital admission for gastrointestinal disorders, and its incidence has increased considerably over the last decade. Acute pancreatitis is an acute inflammatory process of the pancreas, which can affect other organs and systems. Our laboratory described disulfide stress associated with acute inflammatory processes, which is characterized by a decrease in GSH levels as well as protein cysteinylation and γ-glutamylcysteinylation, without changes in protein glutathionylation.

We found an increase in protein cysteinylation levels in TRP14 knockdown cells, and identified 42 proteins whose cysteinylation levels increased in TRP14 deficient cells, such as elongation factor 2 and peroxiredoxin 2. Besides, we demonstrated that cysteinylated Prx2 is inactive, and both TRP14 and Trx1 systems can reduce it and restore its activity.

Unexpectedly TRP14 knockout mice showed lower levels of cysteinylated proteins than wild type sham mice in pancreas under basal conditions. However, upon acute pancreatitis a marked increase in protein cysteinylation

levels together with cystine levels was observed in TRP14 knockout mice, while there was a decrease in protein cysteinylation levels in wild type mice. Protein γ-glutamylcysteinylation also increased in TRP14 knockout mice with pancreatitis as well, but without changes in protein glutathionylation. Furthermore, the transsulfuration pathway and GSH synthesis were both induced in TRP14 knockout mice with pancreatitis avoiding GSH depletion.

The mRNA expression of *Trx1*, *TrxR1*, *Gr*, as well as other NRF2 targets such as *Nqo-1*, *Gclc*, and *Ho1* increased in pancreas from wild type mice upon acute pancreatitis, with further increases in pancreas from TRP14 knockout mice with pancreatitis correlated with nuclear translocation of NRF2.

At the histological level, TRP14-deficient mice showed less tissue edema and inflammatory infiltrate in pancreas upon pancreatitis induction. Decreased pancreatic MPO activity was also observed in this group.

Therefore, TRP14 and thioredoxin 1 exhibit de-cysteinylation activity, and TRP14 deficiency induces protein cysteinylation and γ-glutamylcysteinylation, NRF2 activation as well as up-regulation of the transsulfuration pathway and GSH synthesis upon acute pancreatitis.

I. INTRODUCTION

1.1. OXYGEN AND LIFE

Oxygen is one of the critical elements which define the existence of eukaryotic life on the Earth. Its fluctuations in concentration along the different Earth periods have shaped the development of the different life forms we know nowadays, specially thanks to the development of the ability of the organisms to efficiently produce their own energy through oxidative phosphorylation, allowing unicellular organisms to evolve into more complex multicellular organisms (Stamati, Mudera and Cheema, 2011). The change from unicellular to multicellular life implied a dramatic increase in the energy needs of multicellular organisms just to maintain their cell function. Using oxygen as the final electron acceptor in oxidative phosphorylation as well as the development of appropriated antioxidant defense systems allowed multicellular organisms to obtain as much energy as they required for maintaining life from the available nutrients (Semenza, 2009).

However, as essential as oxygen is for life, it can be as toxic. As a consequence of aerobic life, reactive oxygen species (ROS) are formed. Although these molecules play key roles in normal cell signaling and physiological processes such as the activation of certain transcription factors or the dynamics of the redox status of the cell, they have been related to abnormal processes like impaired mitochondrial function, damage to cell membranes and structures, or enzyme malfunction (Halliwell, 1996). As stated before, evolution lead to the development of mechanisms and strategies to cope with ROS production and to avoid extended damage under oxidative stress conditions (Semenza, 2009).

1.1.1. Free radicals and reactive oxygen species

A free radical is any chemical species containing one or more unpaired electrons in their outer orbital, making it highly reactive towards a wide range of compounds (Halliwell, 2011). Reactive oxygen species (ROS) are pro-oxidant agents derived from oxygen found in a biological setting, which include among them oxygen-derived free radicals. They play a role in numerous physiological processes, from controlling cell function and signaling (Dröge, 2002) to participating in physical exercise (Sastre *et al.,* 1992; Magherini *et al.,* 2019) and aging (Liochev, 2013; Santos, Sinha and Lindner, 2018). However, under oxidative stress conditions, when the levels of ROS rise considerably, they are responsible for oxidative damage to macromolecules such as nucleic acids, lipids, carbohydrates, and proteins (Lambeth, 2007). In this regard, a wide range of diverse diseases have been related to increased levels of ROS, although it is still not clear whether ROS are cause or consequence of the pathological process (Lugrin *et al.,* 2014; Kudryavtseva *et al.,* 2016; Krata *et al.,* 2018). Anyway, upon tissue damage, cell rupture may take place leading to the release of intracellular content within the affected area. This includes transition metals, which catalyze the generation of small amounts of free radicals that can saturate the physiological antioxidant capacity of the organism, aggravating in this way the pathological process that caused the damage at the beginning (Del Maestro *et al.,* 1980; Bencini *et al.,* 2010).

1.1.2. Types of reactive oxygen species

The most biologically relevant reactive oxygen species in the context of this Thesis are hydroxyl radical, superoxide anion, and hydrogen peroxide.

Each of them shows different properties which will be briefly discussed below.

1.1.2.1. Hydroxyl radical

Hydroxyl radical (OH•) is the highest-reacting species that can be found in biological systems, and it reacts with virtually any cell component, giving rise to other species such as superoxide anion and hydrogen peroxide (Aruoma *et al.,* 1989; Sies, Berndt and Jones, 2017). Hydroxyl radicals are mainly formed from hydroperoxides in the presence of transition metals that act as catalysts as a result of Fenton's reaction (Goldstein, Meyerstein and Czapski, 1993; Sies, Berndt and Jones, 2017). Iron is the main metal involved in this reaction, although copper, chromium, vanadium, and cobalt may as well play a role in OH• production (Valko, Morris and Cronin, 2005).

1.1.2.2. Superoxide anion

Superoxide anion ($O_2^{\bullet -}$) is formed when an oxygen molecule acquires an extra electron in its outer orbital as a result of the activity of a wide range of enzymes or due to autoxidation processes and non-enzymatic electron transfer. Superoxide anion shows a very short half-life –in the range of milliseconds– since it is rapidly cleared by superoxide dismutases (SODs), rendering oxygen and hydrogen peroxide as products of the reaction (Bresciani, da Cruz, I. B. M. and González-Gallego, 2015). Furthermore, superoxide anion may be converted to hydrogen peroxide through other non-enzymatic mechanisms.

The role of superoxide anion is double: not only it is an oxidant species itself –although not very strong–, but also it is the precursor of numerous reactive oxygen species, being involved in the establishment and maintenance of oxidative cascade reactions (Turrens, 2003). Furthermore, it has been reported that $O_2^{\bullet -}$ can directly react with thiols, although the reaction rate of this process is generally low (Aruoma *et al.,* 1989). Interestingly, when $O_2^{\bullet -}$ specifically reacts with GSH, sulfur radicals such as thiyl radicals are produced. These radicals are much more hazardous for the cell than $O_2^{\bullet -}$ itself (Schöneich *et al.,* 1989).

1.1.2.3. Hydrogen peroxide

Although it is not a free radical, hydrogen peroxide (H_2O_2) is a major pro-oxidant metabolite formed upon $O_2^{\bullet -}$ dismutation –either by spontaneous processes or as a result of the catalytic activity of SODs or other oxidases that take part in redox reactions– as a consequence of normal cell metabolism (Winterbourn, 2018).

Hydrogen peroxide is considered a strong oxidizing agent; however, in practice it is unreactive towards many biomolecules due to its high activation energy barrier that must be overcome in order to release its oxidizing power (Winterbourn, 2018). Nevertheless, it shows strong reactivity towards transition metal centers. Thus, iron (II) and copper (I) are able to cleave the oxygen-oxygen bond in hydrogen peroxide leading to the production of hydroxyl radicals or activated metal complexes (Imlay, 2008). Besides its affinity for transition metals, H_2O_2 functions in signaling by controlling protein function through the targeted oxidation of protein thiol groups into sulfenic acids (Winterbourn, 2018), which can be further oxidized or react with low-

molecular-weight thiols or neighboring groups in order to avoid further oxidation.

Under physiological conditions, peroxiredoxins and glutathione peroxidases, in combination with the thioredoxin system and reduced glutathione as well as catalase, are in charge of removing the hydrogen peroxide produced in cells in order to avoid any oxidative damage to biomolecules or cell structures (Marinho *et al.,* 2014; Winterbourn, 2018).

1.1.3. Generation of ROS

Reactive oxygen species production can be either endogenous or exogenous (Freeman and Crapo, 1982). The ROS described above are endogenously formed species. Among endogenous ROS sources we can highlight the mitochondrial electron transport chain (Srinivasan and Avadhani, 2012), NADPH oxidases (Kawahara, Quinn and Lambeth, 2007), xanthine oxidase (Vergeade *et al.,* 2012), peroxisomes (Del Río and López-Huertas, 2016), and extracellular hemoglobin released as a consequence of hemolysis which may take place during inflammatory processes (Pérez *et al.,* 2015a). In addition, there are reactive oxygen species that are generated *in vivo* in order to fulfil a specific function, as it is the case of $O_2^{-\bullet}$ and H_2O_2 produced by active phagocytes (Curi *et al.,* 2017).

On the other hand, the organism is exposed to ROS that are exogenously produced. For instance, we ingest daily numerous pro-oxidant compounds that can lead to the production of free radicals. Environmental factors such as air pollutants, hyperoxia, pesticides, tobacco smoke, organic solvents, anesthetics and aromatic hydrocarbons are among other exogenous ROS sources (Banerjee, Seth and Ahmed, 2001; Strzelak *et al.,* 2018; Rao *et al.,*

2018; Vogel *et al.,* 2020). Furthermore, exogenous ROS production can be result of electromagnetic radiation –mainly X and γ rays– or particle radiation –electrons, protons, neutrons, deuterons, as well as α and β particles (von Sonntag, 1994; Kammeyer and Luiten, 2015; Alwood *et al.,* 2017).

1.1.4. Antioxidants

As a result of cell metabolism, reactive oxygen species are constantly produced inside the cell. Hence, cells developed defense systems against these prooxidant species: antioxidants. In 1995, Halliwell and Gutteridge defined the term antioxidant as 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate' (Halliwell and Gutteridge, 1995). According to their chemical properties, antioxidants can be classified as enzymatic antioxidants or non-enzymatic antioxidants.

1.1.4.1. Enzymatic antioxidants

Examples of enzymatic antioxidants are catalase –which takes part in hydrogen peroxide clearance, leading to water and molecular oxygen– (Chance, Sies and Boveris, 1979), glutathione peroxidases –which convert hydrogen peroxide and lipid peroxides in non-reactive molecules– (Chance, Sies and Boveris, 1979), and superoxide dismutases –which transform superoxide radical into hydrogen peroxide– (McCord, Keele and Fridovich, 1971; Fridovich, 1978). For the purpose of this Thesis, the thioredoxin/peroxiredoxin system is described later in this chapter.

1.1.4.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants are molecules that do not require enzymatic support in order to react directly with oxidant species, although they may sometimes participate in enzymatic antioxidant processes. Examples of non-enzymatic antioxidants are reduced glutathione, vitamin C, vitamin E, β-carotenes, and uric acid.

Due to its cellular abundance and its importance in maintaining redox homeostasis in cell, we will focus on glutathione.

1.1.4.2.1. Reduced glutathione

Reduced glutathione (GSH) is the most abundant non-protein thiol in mammalian cells (Meister, A. and Anderson, 1983a). Its intracellular concentration is as high as glucose's (Viña, Hems and Krebs, 1978).

The multiple and important functions of GSH derive from its peculiar structure (**Figure 1**). It contains a free thiol group (–SH) corresponding to the cysteine amino acid, which is the one through which glutathione redox reactions take place (Lu, S. C., 2013). Furthermore, its γ-glutamyl bond renders it resistant to degradation by common peptidases present in the cell (Lu, 2013). In fact, it is only known one enzyme able to hydrolyze GSH: γ-glutamyltranspeptidase, which is expressed on cell membranes (Viña *et al.,* 1992).

According to its redox status, glutathione can be present as reduced glutathione (GSH) or as oxidized glutathione (GSSG) (Lu, 2013). GSSG is

formed by two GSH molecules bound together through a disulfide bond between the two cysteines (**Figure 1**).

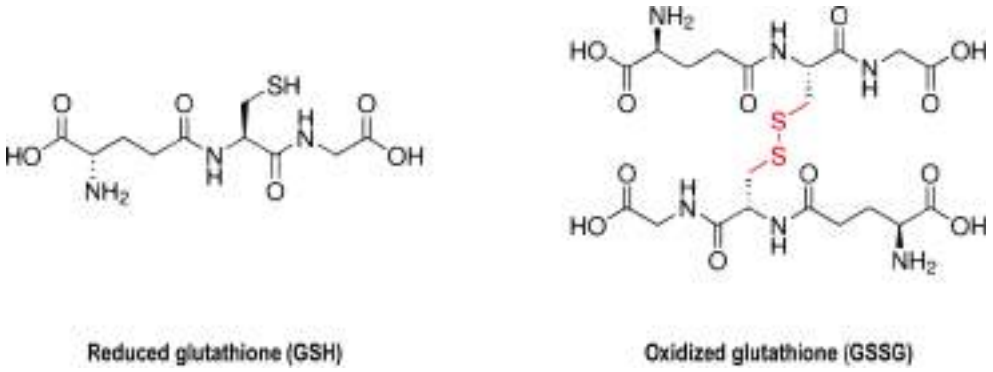

Figure 1. Chemical structure of reduced and oxidized glutathione.

a) Glutathione synthesis

GSH is synthesized in the cytosol of every mammalian cell from its precursor amino acids (Meister, A., 1988). Glutathione synthesis is catalyzed by two enzymes. The first one is glutamate-cysteine ligase (GCL), which catalyzes the synthesis of γ-glutamylcysteine from L-glutamic acid and L-cysteine (Lu, 2013). Under physiological conditions, the rate limiting step in glutathione synthesis is cysteine availability (Tateishi *et al.,* 1974; Tateishi *et al.,* 1977). The second step in GSH synthesis is catalyzed by glutathione synthetase (GSS), which links glycine to γ-glutamylcysteine to form the tripeptide (Lu, 2013). Glutathione synthesis is controlled by negative feedback as GSH inhibits GCL, and thus, glutathione synthesis (Richman and Meister, 1975).

b) Glutathione degradation: the Meister cycle

Newly synthetized GSH can be either consumed exerting its function or degraded. For this latter purpose, GSH is exported outside the cell. Once outside, γ-glutamyltranspeptidase (γ-GT) present on the cell membrane hydrolyzes the γ-glutamyl bond of GSH, rendering glutamic acid –which may enter into the cell– and cysteinylglycine –which is then hydrolyzed by a dipeptidase (Meister, A. and Anderson, 1983b). This set of reactions involved in GSH degradation is known as the Meister cycle.

c) The transsulfuration pathway

As it has been previously mentioned, L-cysteine availability is the limiting step in glutathione synthesis under physiological conditions (Lu, 2013). Cysteine is an amino acid that may be obtained from different sources such as diet, proteolysis, or by synthesis from methionine through the so-called transsulfuration pathway (Sbodio, Snyder and Paul, 2019).

The transsulfuration pathways occurs in many tissues, but mainly in the liver (Sbodio, Snyder and Paul, 2019). It comprises five steps (**Figure 2**) (Sbodio, Snyder and Paul, 2019):

1. Activation of methionine to *S*-adenosylmethionine
2. Demethylation of *S*-adenosylmethionine
3. Elimination of the *S*-adenosyl fraction to form homocysteine
4. Cystathionine formation from homocysteine and serine

5. Excision of cystathionine to render cysteine and α-ketobutyrate

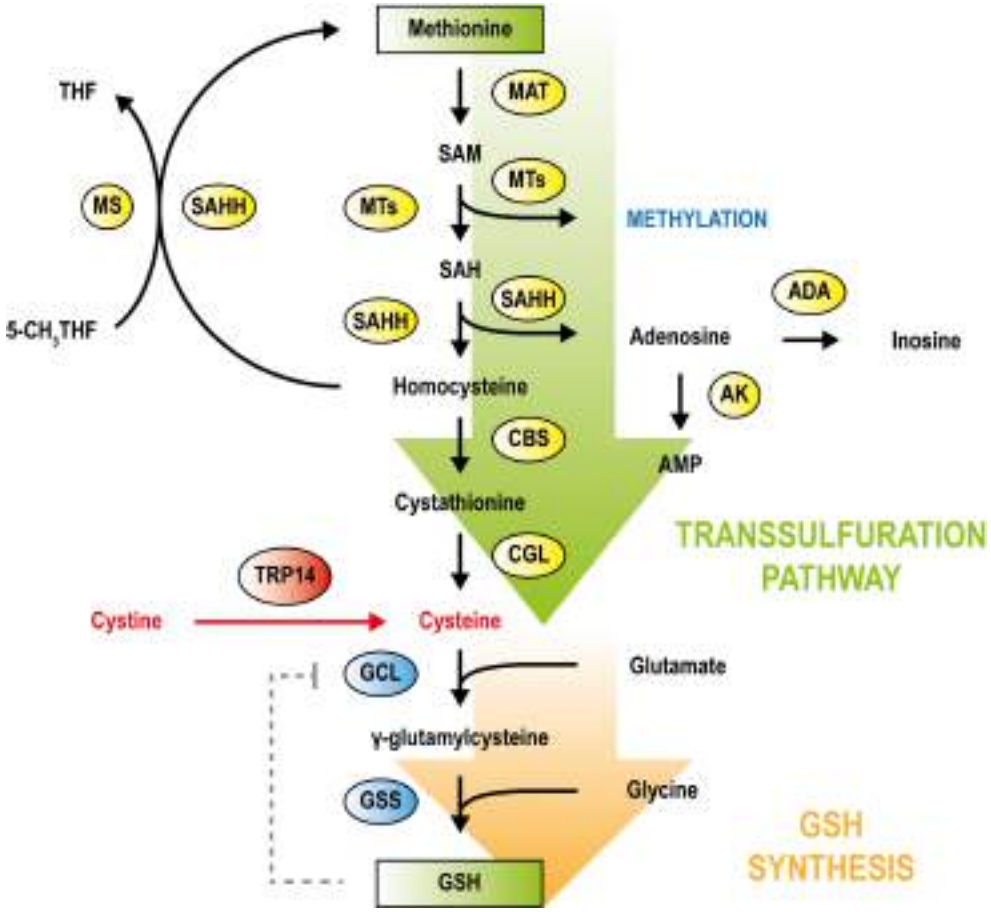

Figure 2. Transsulfuration pathway and GSH synthesis. For the purpose of this Thesis, cystine reduction by TRP14 has been included as a source of cysteine, as it will be discussed later. *ADA: adenosine deaminase; AK: adenosine kinase; AMP: adenosine monophosphate; CBS: cystathionine beta-synthase ; CGL: cystathionine gamma-lyase; GCL: glutamate-cysteine ligase; GSH: reduced glutathione; GSS: glutathione syntethase; MAT: methionine adenosyltransferase; MS: methionine synthase; MTs: methyltransferases; SAH: S-adenosylhomocysteine; SAHH: SAH hydrolase; SAM: S-adenosylmethionine; THF: tetrahydrofolate; TRP14: thioredoxin-related protein of 14 kDa.*

The most important steps in the transsulfuration pathway are the first one, which is catalyzed by methionine *S*-adenosyltransferase, and the

last one, which is irreversible and is catalyzed by γ-cystathionase (Sbodio, Snyder and Paul, 2019).

1.1.5. Oxidative stress and redox signaling

Under physiological conditions, the production of small amounts of free radicals as a result of cell metabolism is considered normal (Slater, 1984). Under these conditions when antioxidant mechanisms can cope with the amount of ROS produced within the cell, ROS act as redox signaling agents promoting physiological processes such as cell proliferation and survival (Sies, 2015). However, under certain conditions antioxidant systems cannot face the amount of ROS generated within the cell or exogenously, which can lead to oxidative damage. The term oxidative stress was coined by Sies in 1986, and it can be described as the disturbance in the balance between oxidant species and antioxidants in favor of the formers (Sies, 1986). It is important to note that oxidative stress can be originated by an increase in oxidant species, a decrease in antioxidant agents, or both at the same time (Sies, 2015). The two faces of oxidative stress and redox signaling are depicted in **Figure 3**.

Oxidative stress has been related to cell death and development of a great variety of pathologies such as cancer, type 2 diabetes mellitus, atherosclerosis, myocardial infarction, and acute pancreatitis among others (Ghezzi *et al.,* 2017). However, whether oxidative stress is cause or consequence of these diseases is still a matter of debate.

What it is clear is that oxidative stress may cause oxidative damage to biomolecules which, in turn, leads to tissue damage (**Figure 3**). Oxidation takes place targeting all four major biomolecules: lipids, carbohydrates,

nucleic acids, and proteins. Free radicals attack these biomolecules oxidizing them, leading to loss of their functions, accumulation of oxidized molecules, and eventually development of mutations (Gebicki, 2016).

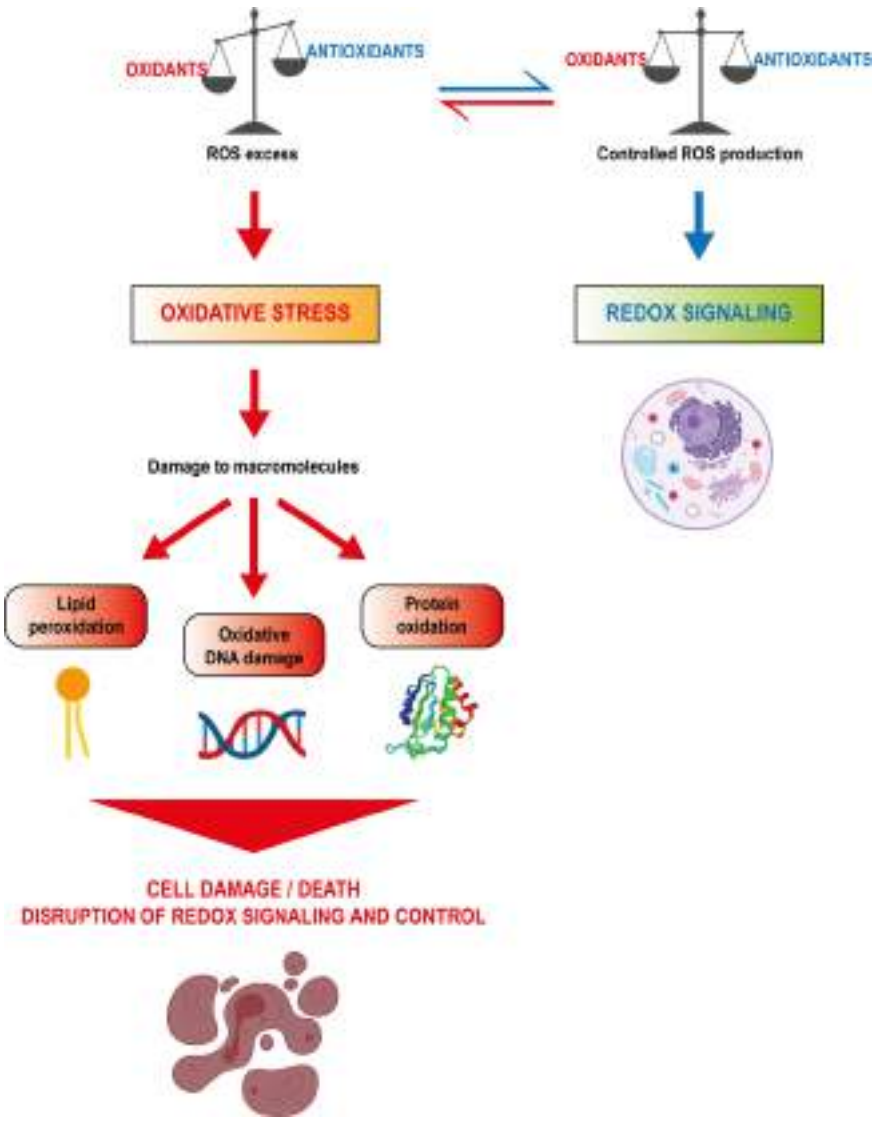

Figure 3. The two faces of reactive oxygen species. When there is a physiological balance between oxidants and antioxidants, ROS production contributes to normal cell signaling and functioning (right). However, when oxidants overcome the antioxidant defense, oxidative stress arises leading to oxidative damage to macromolecules, eventually causing cell damage and death.

Taking into account the aim of this Thesis, we will focus on oxidative protein modifications.

1.1.6. Oxidative damage to proteins

Proteins are one of the main cellular targets of reactive oxygen species, (Winterbourn and Kettle, 2013). The reason why proteins are such good targets of oxidative stress is due to the numerous oxidation-sensitive residues present in their side chains (Winterbourn and Kettle, 2013). Cysteine and methionine are the most frequently affected amino acids given their high reactivity towards hydroxyl radicals (Winterbourn and Kettle, 2013). Although reversible oxidative protein modifications play a physiological role in the regulation of protein function and cell signaling (Lo Conte and Carroll, 2013), proteins may undergo irreversible modifications to their side chains such as carbonylation, thiol overoxidation, or di-tyrosine formation resulting in misfolding or loss of their function (Heinecke *et al.,* 1993; Miki and Funato, 2012).

1.1.6.1. Oxidative protein modifications affecting sulfur-containing amino acids

Methionine and cysteine are the only sulfur-containing amino acids present in proteins. Both amino acids are extremely susceptible to oxidation (Kim, G., Weiss and Levine, 2014). When these amino acids are oxidized in their free form, they become unavailable for cell metabolic processes. However, when their oxidation takes place post-translationally in amino acid residues within a peptide chain of a given protein, it may alter protein function mainly by inactivation (Kim, Weiss and Levine, 2014). The importance of

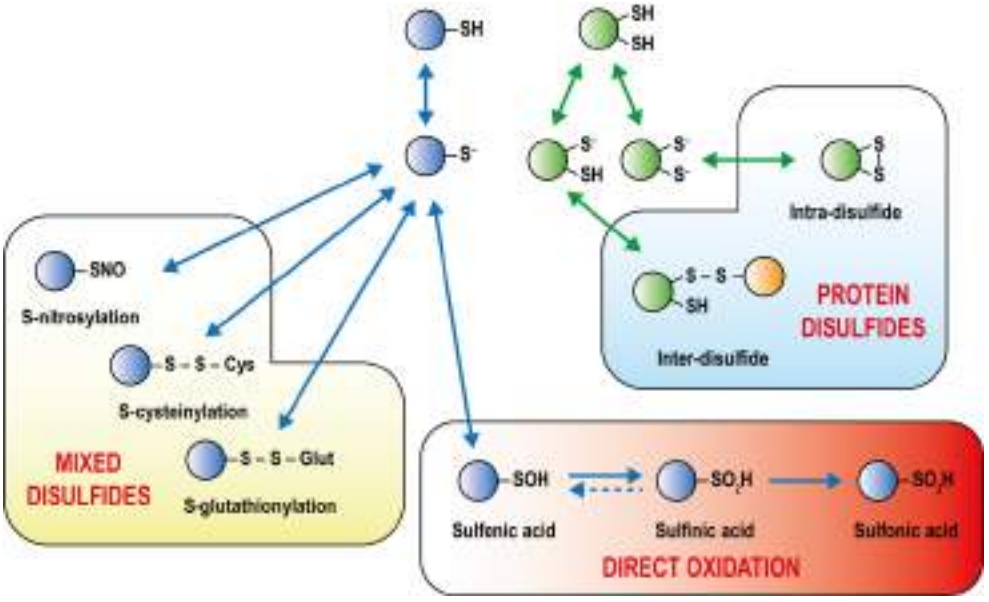

Figure 4. Main protein thiol oxidative modifications.

methionine and cysteine in this regard is due to their involvement in numerous redox-regulated signaling pathways that may be altered upon oxidative stress (Drazic and Winter, 2014). Thus, it is not surprising that both methionine and cysteine residues in proteins can be reversibly oxidized in a controlled manner, either enzymatically or non-enzymatically (Kim, Weiss and Levine, 2014). Methionine oxidation leads to the formation of methionine sulfoxide –as a result of the incorporation of oxygen to its sulfur atom–, which is reduced back to methionine by methionine sulfoxide reductases (Kim, Weiss and Levine, 2014). On the other hand, cysteine oxidation results in the formation of cystine through a disulfide bond that can be reduced back to the free thiol form by different reductases, being thioredoxin the most common one (Arnér and Holmgren, 2000). In this context, TRP14 has also been described to exhibit cystine reductase activity, being able to reduce cystine back to cysteine (Pader *et al.,* 2014), but whether it is able to reduce disulfide bonds between two cysteine residues within a protein or between a

cysteine residue and another cysteine molecule within a protein has not been described yet.

This Thesis is focused on the study of cysteine oxidative modifications within proteins. Cysteinyl thiols are susceptible to different types of oxidation according to the type of oxidant and its concentration, as well as to the thiol exposure within three-dimensional structure of the protein (Lo Conte and Carroll, 2013). The main thiol oxidative modifications are summarized in **Figure 4**.

a) Direct oxidation

The thiol group of cysteine residues within proteins is readily oxidized to the highly-reactive sulfenic acid form (–SOH), which in turn can be further oxidized to sulfinic ($–SO_2H$) and sulfonic acid ($–SO_3H$). Sulfenic and sulfinic forms of the cysteine thiol are reversible oxidation steps, whereas sulfonic acid is widely described as an irreversible form of thiol oxidation (Lo Conte and Carroll, 2013), as showed in **Figure 4**. In this oxidation process, ROS are the main species reacting with thiol groups leading to the formation of sulfenic acid; however, reactive nitrogen and sulfur species also play a role in the formation of protein sulfenic acids (Devarie-Baez, Silva Lopez and Furdui, 2016).

Sulfenic acid formation on protein thiols takes place under the presence of oxidant species such as hydrogen peroxide, although hydroperoxides, as well as peroxynitrite and hypochlorous acid may also play a role in cysteine sulfenic acid formation (Poole, Karplus and Claiborne, 2004). In any case, the reactivity of the cysteine residue towards the oxidant species is determined by the ionization state of

the thiol group, thiolates being more nucleophilic –and thus, more reactive– than their protonated forms (Poole, Karplus and Claiborne, 2004). Sulfenic acids are highly unstable reactive species which are considered to be intermediates in the formation of more stable disulfide bonds (McDonagh, 2017). However, some proteins may harbor stable sulfenate forms, indicating that the stability of sulfenic acid depends on the protein microenvironment, the presence of hydrogen bonds, and the existence of nearby cysteine residues and amines, as well as on solvent accessibility to modified cysteines (McDonagh, 2017).

Besides disulfide bond formation, which is discussed below, sulfenic acids can be further oxidized to sulfinic or sulfonic acids. Sulfonic acid is considered an overoxidized state of the thiol group that is biologically irreversible (Poole, Karplus and Claiborne, 2004). However, despite sulfinic acid being generally considered an irreversible oxidation state of thiol groups, since neither glutathione nor thioredoxin can reduce a sulfinic acid back to the thiolate form, it has been reported that sulfiredoxin exhibits sulfinic acid reduction activity *in vivo* (Woo, H. A. *et al.,* 2003; Chevallet *et al.,* 2003; Akter *et al.,* 2018).

b) <u>Disulfide bond formation</u>

Disulfides in proteins are formed under physiological conditions in the endoplasmic reticulum of the cell by protein disulfide isomerase (PDI) (Tanaka, L. Y., Oliveira and Laurindo, 2020). Under oxidative stress conditions, protein disulfide bonds may be formed in non-physiological positions between two proteins (intermolecular disulfide bonds) or within cysteine residues from the same protein

(intramolecular disulfide bonds) leading to structural changes –mainly conformational changes, protein misfolding and aggregation– or changes in protein activity (Jones, 2008; Cao and Kaufman, 2014). If the affected cysteine residue is part of the active site of an enzyme, the disulfide may act as an 'on/off' switch, whereas when disulfide formation alters protein conformation, it acts as an allosteric regulator of protein activity in a 'rheostat-like' fashion (Jones, 2008). Disulfide formation thus regulates cell signaling pathways either directly –as it is the case of Yap-1 activation in yeast by the formation of a disulfide bond within two of its cysteine residues in presence of hydrogen peroxide (Delaunay, Isnard and Toledano, 2000)– or indirectly through redox switches –as it is the case of Nrf2 activation by Keap1, which is dependent on the intramolecular disulfide bond formation between two cysteine residues of Keap1 upon presence of oxidant species (Dinkova-Kostova *et al.,* 2002). Furthermore, redox relays in which the formation of coupled disulfide bonds between pairs of molecules takes place have also been described (Sobotta *et al.,* 2015). In this regard, peroxiredoxin-2 transfers oxidative equivalents to STAT3 with signaling purposes, leading to STAT3 oxidation with the subsequent formation of covalent dimers or tetramers depending on the cysteine residues involved, attenuating in this way STAT3 transcriptional activity (Sobotta *et al.,* 2015). In all these cases, disulfide bond formation within proteins involved in the control of transcription factor activation importantly seems to have a protective effect against oxidative stress (Shimizu and Hendershot, 2009).

c) Mixed disulfides

Protein thiol groups can react with low-molecular-weight thiols such as free cysteine or GSH, as well as with homocysteine, γ-

glutamylcysteine or cysteinylglycine (Eaton, 2006). The oxidized molecules resulting from these interactions are known as mixed disulfides (Brigelius-Flohé, 2016). Apparently, formation of mixed disulfides upon oxidative stress may have a protective function since it would prevent further oxidative damage by avoiding irreversible protein oxidation (Hochgräfe *et al.,* 2007). These protein modifications can be reverted through reduction of the disulfide bonds formed between proteins and low-molecular-weight thiols by the action of disulfide reductases such as thioredoxin and glutaredoxin systems (Holmgren, 1989). Being GSH and free cysteine the most abundant low-molecular-weight thiols in cells, it is reasonable that the most abundant mixed disulfides formed are *S*-glutathionylated and *S*-cysteinylated proteins (Ghezzi, 2013) .

c) 1. *S*-glutathionylation

Protein glutathionylation is a reversible cysteine modification that takes place when cysteine residues from enzymes, receptors, or transcription factors interact with glutathione forming protein mixed disulfides, both under physiological conditions and, to a greater extent, under oxidative stress (Mailloux, 2020). This process modulates protein function: it is well reported that protein *S*-glutathionylation usually causes an inhibitory effect on protein function; however, some cases of protein activation upon *S*-glutathionylation have been described (Mieyal *et al.,* 2008; Mieyal and Chock, 2012). Taking this into account, protein *S*-glutathionylation can either upregulate or downregulate protein function (Mieyal *et al.,* 2008; Mieyal and Chock, 2012; Mailloux, 2020). Furthermore, protein glutathionylation prevents further protein oxidation, protecting in this way cysteine residues from being further oxidized to sulfinic acid or to

the irreversible sulfonic acid form (Mieyal *et al.,* 2008; Mieyal and Chock, 2012; Mailloux, 2020). *S*-glutathionylation may occur through the following mechanisms:

- **Thiol-disulfide exchange:** The GSH/GSSG ratio governs the glutathionylation degree of a protein (Mailloux, 2020). The ratio is usually around 100/1 under physiological conditions, and in order to achieve protein glutathionylation the ratio should significantly drop (Klatt *et al.,* 1999). Given that fact, thiol-disulfide exchange is not considered a common mechanism, although it plays a key role in the inhibition of the DNA binding capacity of c-Jun since it is prone to undergo glutathionylation at low GSH/GSSG ratios (Klatt *et al.,* 1999).

- **Sulfenic acid intermediates:** Sulfenic acids are extremely unstable and reactive intermediate species that can either be further oxidized to sulfenic and sulfonic acids or react with other thiol groups or GSH giving rise to protein glutathionylation (Klatt and Lamas, 2000; Poole, Karplus and Claiborne, 2004; Forman *et al.,* 2017). Proteins such as NF-κB p50, c-Jun, c-Fos, and caspase-3 have been described to undergo *S*-glutathionylation following sulfenic acid formation (Mieyal *et al.,* 2008; Zamaraev *et al.,* 2017).

- **Sulfenyl amide intermediates:** This special type of intermediate species has been exclusively described as a post-translational modification of PTP1B which can be reduced by GSH leading to glutathionylated PTP1B (Salmeen *et al.,* 2003).

- **Thiyl radical intermediates:** Thiyl radicals ($RS^{\bullet}$) are one of the sulfur radical species with the shortest half-life that readily react with thiolate groups, followed by an oxidation reaction to give rise to glutathionylated proteins (Schöneich, 2017). The same process can also take place through radical recombination reactions (Schöneich, 2017). Furthermore, the glutaredoxin system can catalyze these reactions *in vitro* in presence of glutathione radical (Starke, Chock and Mieyal, 2003).

- **Thiosulfinate intermediates:** Thiosulfinates (GS(O)SG) are extremely reactive species towards thiols, rendering disulfides and water as products of the reaction (Nagy, Lemma and Ashby, 2007). Hence, glutathionyl-thiosulfinate should be able to react with protein cysteine residues leading to their glutathionylation (Huang and Huang, 2002; Gallogly and Mieyal, 2007).

- ***S*-nitrosylated intermediates:** cysteine sulfhydryl groups can become nitrosylated both under physiological and pathological conditions (Fernando *et al.,* 2019). *S*-nitrosoglutathione (GSNO) is the most abundant nitrosothiol in mammalian cells, being readily reactive towards proteins leading within minutes to the formation of glutathionylated proteins (Giustarini *et al.,* 2005; West *et al.,* 2006; Martínez-Ruiz and Lamas, 2007; Forshaw and Conway, 2019).

The disulfide bond resulting from these reactions can be easily reduced; however, if oxidative conditions are maintained, glutathionylated proteins can remain stable indefinitely (Beer *et al.,* 2004; Matsui *et al.,* 2019). A more common situation is transient protein glutathionylation, which takes place when a reducing

environment is recovered after oxidative stress or when a free protein thiol near the glutathionylation site displaces the glutathione moiety leading to the formation of an intramolecular disulfide bond (Matsui *et al.,* 2019).

c) 2. *S*-cysteinylation

If glutathione is the most abundant low-molecular-weight thiol in mammalian cells, cysteine is the most abundant low-molecular-weight thiol in extracellular fluids and plasma, and it is mainly present in its disulfide form: cystine (Jones *et al.,* 2002; Giustarini *et al.,* 2009; Held, 2019). Due to this difference in relative abundance of both species, it seems reasonable that *S*-glutathionylation is the predominant protein modification within the cell in response to oxidative stress, whereas *S*-cysteinylation is more commonly observed in circulating plasmatic proteins (Matsui *et al.,* 2019). Hemoglobin and albumin can be used as a clear example: hemoglobin present in erythrocytes appears glutathionylated in patients suffering from oxidative stress derived from type 2 diabetes (Sampathkumar *et al.,* 2005), while human serum albumin becomes cysteinylated through the formation of a sulfenic acid intermediate when incubated with hydrogen peroxide or peroxynitrite (Carballal *et al.,* 2003; Turell *et al.,* 2008). Increased protein *S*-cysteinylation levels have been found in different pathologies such as Alzheimer's disease (Poulsen *et al.,* 2014; Costa *et al.,* 2019), chronic kidney disease (Regazzoni *et al.,* 2013; Ostrakhovitch and Tabibzadeh, 2015), liver cirrhosis (Domenicali *et al.,* 2014), cardiovascular disease (Belcastro *et al.,* 2017; Wakabayashi *et al.,* 2017), rheumatoid arthritis (Seward *et al.,* 2011), and acute pancreatitis (Moreno *et al.,* 2014).

Contrary to protein *S*-glutathionylation described above in detail, protein *S*-cysteinylation is not that widely studied, and regulation of the cysteinylation process is still unknown. However, this kind of oxidative protein modification may play a key role in the redox regulation of proteins containing thiol groups in the active site and, at the same time, it may be a protection mechanism against protein overoxidation under oxidative stress conditions in addition to glutathionylation or when GSH is not available (Hochgräfe *et al.,* 2007).

1.2. THE THIOREDOXIN SYSTEM

The thioredoxin system is –together with the glutathione system– one of the most important thiol-dependent antioxidant systems in mammalian cells (Lu, J. and Holmgren, 2014). This disulfide reductase system is comprised by thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, which give their electrons to many different acceptor enzymes, being critical for processes such as DNA synthesis or protection against oxidative stress (Lu and Holmgren, 2014).

1.2.1. Thioredoxins

Thioredoxin was identified for the first time as a reducing substrate of ribonucleotide reductase (RNR), an enzyme that is critical for synthesis of deoxyribonucleotides, which are necessary for DNA replication and repair processes (Holmgren, 1985).

Thioredoxins are small reductases (12 kDa) that show a well-conserved CGPC motif as their active site and catalyze the reduction of protein disulfides into the corresponding protein thiols (Lu and Holmgren, 2014). The structure of thioredoxins (**Figure 5**) resembles the fold present in other crucial antioxidant enzymes involved in thiol/disulfide regulation, such as peroxiredoxin, glutaredoxin, and glutathione peroxidase (Wood *et al.,* 2003; Fernandes and Holmgren, 2004; Ladenstein *et al.,* 1979). Trx1 is comprised by five β-strands that form the internal core of the enzyme and four α-helices, and one short stretched helix surrounding the internal β-sheets (Eklund, Gleason and Holmgren, 1991). Trx1 shows a disulfide in its active site

located after the β2-sheet, forming the N-terminal region of the α2-helix (Eklund, Gleason and Holmgren, 1991).

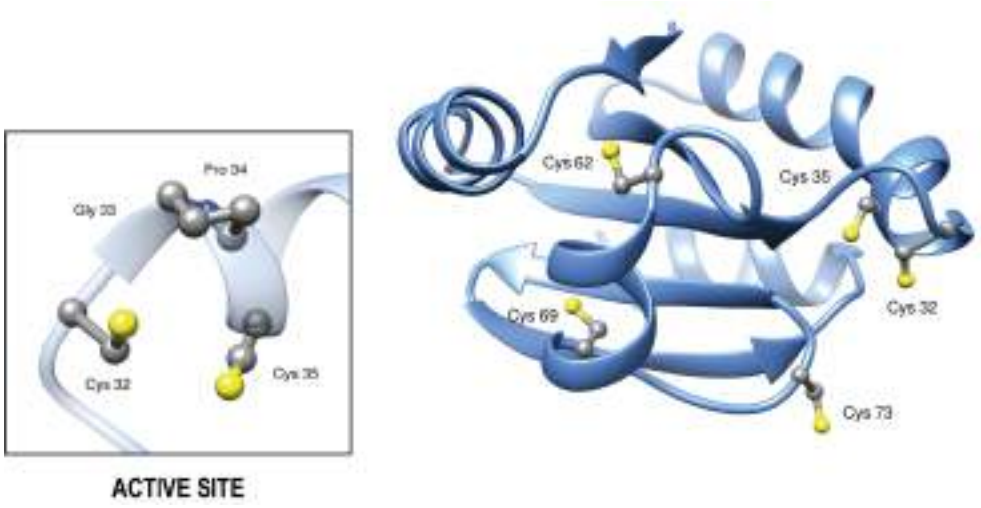

Figure 5. Structure of human Trx1. Trx1 contains five cysteine residues in its structure, two of them being present in its active site (left panel).

1.2.2. Thioredoxin reductases

Thioredoxin reductases (TrxRs) are homodimeric flavoproteins belonging to the pyridine nucleotide-disulfide oxidoreductase family (Lu and Holmgren, 2014). Glutathione reductase (GR), trypanothione reductase (TyrR), alkyl hydroperoxide reductase, lipoamide dehydrogenase, and mercuric reductase are other members of this family (Argyrou and Blanchard, 2004). Among thioredoxin reductases, we can distinguish two different groups: high molecular weight TrxR (55 kDa subunits) present in mammalian cells and higher eukaryotes, and low molecular weight TrxR (35 kDa) found in bacteria (Williams *et al.,* 2000). Three different TrxR isoforms have been described in mammalian cells: TrxR1 (cytosolic), TrxR2 (mitochondrial), and a testis-

specific enzyme called thioredoxin glutathione reductase (TGR), which displays an additional glutaredoxin domain (Lu, J. and Holmgren, 2009; Cheng, Qing *et al.,* 2009). The active site of the different thioredoxin reductases is a well-conserved CVNVGC motif at the N-terminus, as it is the case of the vast majority of pyridine nucleotide reductases (Zhong, Arnér and Holmgren, 2000). Furthermore, mammalian TrxRs show an additional sequence at the C-terminal region containing selenocysteine (Gly-Cys-Sec-Gly), which plays a key role in catalysis, and their two subunits form a so called 'head to tail' pattern (Zhong, Arnér and Holmgren, 2000). The catalytic mechanism involves electrons being transferred from NADPH to FAD, then to the redox-active dithiol motifs in the N-terminal region from where they reach the selenylsulfide residue located in the other subunit, and lastly reaching the disulfide substrates of thioredoxin reductases (Cheng *et al.,* 2009; Zhong, Arnér and Holmgren, 2000; Zhong and Holmgren, 2000). These substrates are very diverse –including enzymes such as thioredoxin, protein disulfide isomerase (PDI), glutaredoxin 2 (Grx2), as well as small molecules like selenite and lipoic acid– due to the fact that mammalian TrxRs present two active sites (Lu, J., Berndt and Holmgren, 2009). It is also interesting to note the ability of mammalian thioredoxin reductases to directly function as antioxidant enzymes by providing electrons to small molecules that are able to react with hydrogen peroxide (Holmgren, 2000; Zhao, Masayasu and Holmgren, 2002).

1.2.3. Antioxidant role of the mammalian thioredoxin system

Mammalian cells possess two different thioredoxin systems: cytosolic Trx1 and mitochondrial Trx2 (**Figure 6**) (Lu and Holmgren, 2014). Besides being expressed at different cellular locations, they also differ structurally:

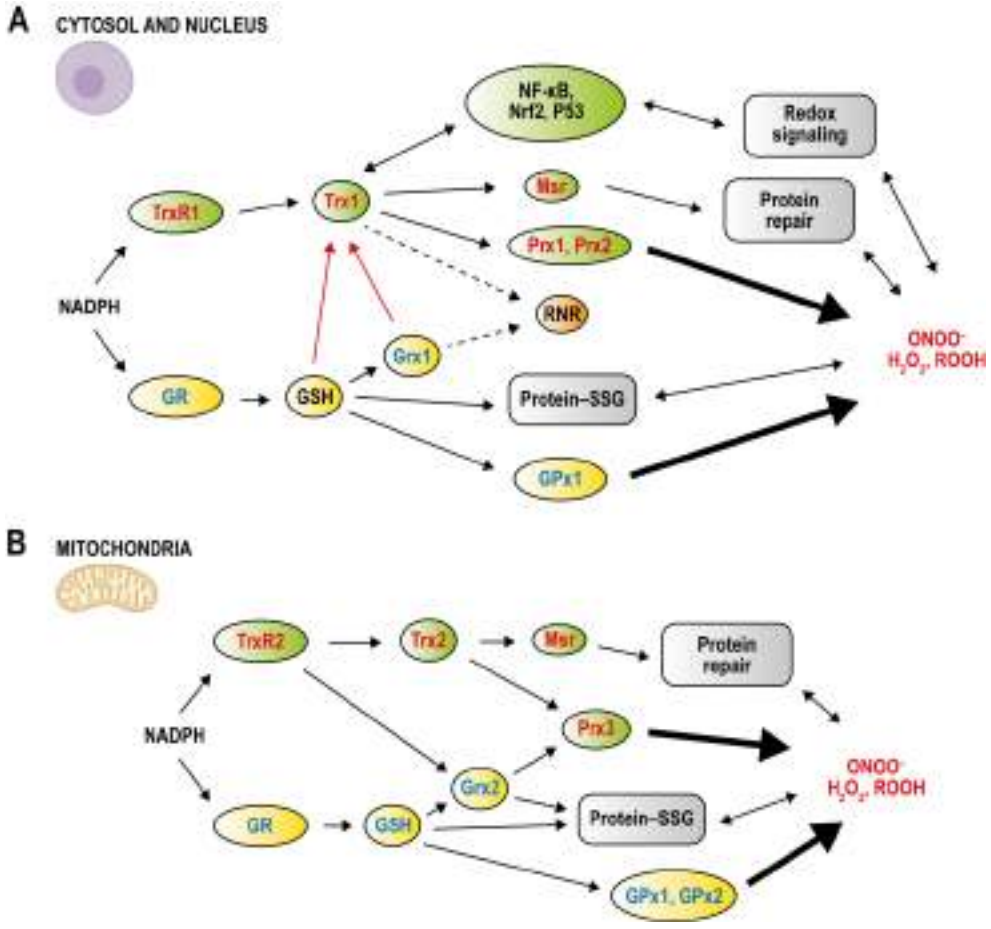

Figure 6. Mammalian thioredoxin system. A. Nuclear and cytosolic thioredoxin system and related enzymes. **B.** Mitochondrial thioredoxin system and related enzymes.

the only cysteine residues present in Trx2 are those from its active site, while Trx1 shows three additional cysteine residues that are essential for the redox regulation of its activity, as well as for nitric oxide signaling due to its denitrosilase activity (Holmgren, 2000).

In order to fulfil its antioxidant activity, the thioredoxin system mainly transfers electrons to peroxiredoxins, methionine sulfoxide reductases, and redox-sensitive transcription factors (Lu and Holmgren, 2014).

1.2.3.1. Peroxiredoxins

Peroxiredoxins are Trx-dependent peroxidases. In humans, six different isoforms present in different subcellular locations have been described (Rhee, Woo and Kang, 2018). According to the number of cysteine residues present in their active sites, peroxiredoxins can be classified as 2-Cys peroxiredoxins (Prx1, Prx2, Prx3 and Prx4), an atypical 2-Cys peroxiredoxin isoform (Prx5), and one 1-Cys peroxiredoxin (Prx6) (Rhee *et al.,* 2012). The so-called 2-Cys peroxiredoxins are the only isoforms that are able to accept electrons from the thioredoxin system in order to support their enzymatic activity clearing hydrogen peroxide, hydroperoxides, and peroxynitrite (Wood *et al.,* 2003; Rhee *et al.,* 2012). They contain two cysteine residues in their active site: the N-terminal cysteine is known as peroxidatic cysteine (Cys_P), which is deprotonated under neutral pH forming a thiolate due to its low pK_a value (around 5-6), while the C-terminal cysteine is called resolving cysteine (Cys_R) (Rhee and Woo, 2020).

The catalytic mechanism of 2-Cys peroxiredoxins (**Figure 7**) takes place in two steps: in a first step, the Cys_P in the thiolate form reacts with hydrogen peroxide forming Cys_P sulfenic acid with the subsequent release of water through a nucleophilic attack; then, in a second step, the Cys_R reacts with the sulfenic acid of Cys_P forming this way an intermolecular disulfide bond in the case of typical 2-Cys peroxiredoxins or an intramolecular bond when the reaction takes place in atypical 2-Cys peroxiredoxins (Rhee and Woo, 2020). Peroxiredoxins are recycled by the thioredoxin system, which is in charge of reducing the disulfide bond that was formed as a consequence of their catalytic mechanism (Wood *et al.,* 2003; Rhee and Woo, 2020). In this way, reduced peroxiredoxins become active again (Wood *et al.,* 2003; Rhee and Woo, 2020). The hydrogen peroxide clearance by peroxiredoxins as

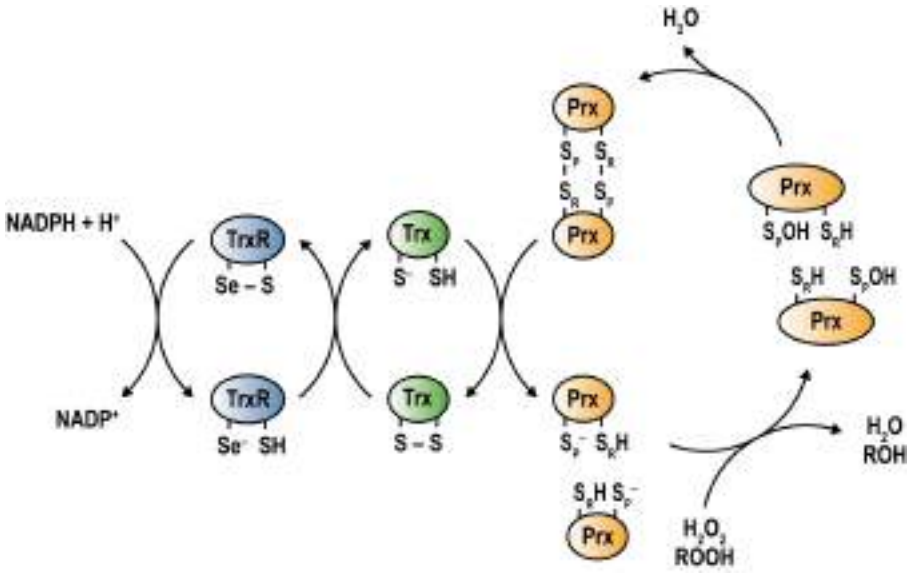

Figure 7. Catalytic mechanism of 2-Cys peroxiredoxins. *TrxR: thioredoxin reductase; Trx: thioredoxin; Prx: peroxiredoxin; S_P: peroxidatic cysteine; S_R: resolving cysteine.*

described above is an extremely efficient reaction, with reaction rates reaching up to 10^7-10^8 M^{-1} s^{-1} (Ogusucu *et al.,* 2007; Manta *et al.,* 2009). However, the normal reaction rate of hydrogen peroxide with protein thiolate groups varies from 0.89 to 500 M^{-1} s^{-1}, even in those proteins with similar pK_a values of the thiol group to that of peroxiredoxins, as it is the case of thioredoxin or protein tyrosine phosphatase 1B (PTP1B) (Winterbourn and Metodiewa, 1999; Winterbourn and Hampton, 2008). The unique peroxiredoxin structure is responsible for their potent catalytic capacity since it allows the formation of stable reaction intermediate species (Hall *et al.,* 2010; Hall *et al.,* 2011). Peroxiredoxins share a conserved PXXXTXXC active site motif (**Figure 8** shows the sequence alignment of all six human peroxiredoxins) as well as a distant arginine residue (Rhee, 2016). The reaction takes place through the thiolate of the peroxidatic cysteine which is in charge of attacking hydrogen peroxide molecules via an S_N2 nucleophilic substitution reaction. At this point, arginine, proline, and threonine residues

Figure 8. Sequence alignment of human peroxiredoxins. Note the conserved active site as well as the peroxidatic and resolving cysteine residues. *Protein sequences were retrieved from UniProt and alignment was performed by Clustal Omega.*

in the neighboring positions stabilize the reaction transition state intermediate through the formation of hydrogen bonds, resulting in an extraordinary efficient catalytic activity of peroxiredoxins (Hall *et al.,* 2010; Hall *et al.,* 2011); their reaction rates being similar to those of glutathione peroxidase (10^8 M^{-1} s^{-1}) and catalase (10^7 M^{-1} s^{-1}) (Marinho, Antunes and Pinto, 1997; Vlasits *et al.,* 2010). Considering that peroxiredoxins are among

the most abundant proteins in mammalian cells, they may be one of the most important mechanisms against oxidative stress given their ability to clear reactive oxygen species from the cellular environment. Furthermore, as it is the case of glutathione peroxidases, they may cooperate clearing hydrogen peroxide locally, since peroxiredoxins are distributed among different organelles within the cell, contributing in this way to signal transduction in a more specific fashion (Kang, Sang Won *et al.,* 2005).

1.2.3.2. Methionine sulfoxide reductases

Methionine sulfoxide reductases (Msrs) are a group of proteins with antioxidant function that accept electrons from the thioredoxin system in order to perform their enzymatic activities on methionine sulfoxide (Martínez *et al.,* 2017). Under oxidative stress conditions, both free and protein methionine residues can be oxidized to methionine sulfoxide, which may lead to impaired protein function (Kaya, Lee and Gladyshev, 2015). Methionine sulfoxide reductases A and B reduce free *S*-methionine sulfoxides and protein *R*-methionine sulfoxides back to methionine, participating in this way in the clearance of reactive oxygen species (Martínez *et al.,* 2017).

1.2.3.3. The glutathione system

The glutathione system is one of the most relevant thiol-dependent antioxidant systems present in mammalian cells thanks to the role of glutathione peroxidases in the removal of different reactive oxygen species that may threaten the cell redox status (Papp *et al.,* 2007; Brigelius-Flohé and Maiorino, 2013). As will be discussed further later on, glutathione

together with glutaredoxins (Grxs) play a key role in regulating protein function under oxidative stress conditions through reversible protein *S*-glutathionylation (Mailloux, 2020). For a long time, both the thioredoxin and the glutathione systems were considered to overlap in their functions by working side by side (Lu and Holmgren, 2014). Nowadays, there are clear evidences of their crosstalk. Although knocking out both *Txnrd1* alleles –coding for TrxR1– was lethal for mouse embryos, which did not survive beyond stage E8.5 (Bondareva *et al.,* 2007), it is interesting that selective knockout of both alleles in the liver resulted in the development of adult mice with no signs of disease (Suvorova *et al.,* 2009; Prigge *et al.,* 2012). In fact, hepatocytes from these mice were viable in the long term and did not show any characteristic sign of chronic oxidative stress (Suvorova *et al.,* 2009; Prigge *et al.,* 2012). Despite the absence of TrxR1 in the liver, both the redox state of Trx and GSH were not altered, nor was an increase in carbonylated protein or peroxide lipid levels observed, confirming the absence of oxidative tissue damage and suggesting that TrxR1 activity could be compensated by some other system (Suvorova *et al.,* 2009). GSH depletion upon buthionine sulfoximine (BSO) treatment led to low hepatocyte replication rates in liver-specific Txnrd1 knockout mice, but no such effect was observed in mice carrying at least one copy of the gene (Prigge *et al.,* 2012). Thus, for normal replication hepatocytes from liver-specific TrxR1 knockout mice critically depend on GSH levels or the presence of at least one *Txnrd1* allele, suggesting that both systems –GSH-dependent and TrxR-dependent systems– are complementary in these cells (Suvorova *et al.,* 2009; Prigge *et al.,* 2012). In this regard, it is worth mentioning that liver-specific Txnrd1 knockout mice exhibited induction of the Nrf2-dependent antioxidant response in the liver and the consequent up-regulation of the Trx system, as well as the GSH system (Suvorova *et al.,* 2009; Prigge *et al.,* 2012). Therefore, these results showed the relationship between the Trx system

and the GSH system as when the activity of the former is compromised, the latter is responsible for preserving the redox state of cellular thiols, as well as for coping with oxidative stress (Bondareva *et al.,* 2007; Suvorova *et al.,* 2009; Prigge *et al.,* 2012). In addition, mitochondrial Grx2 was identified as a substrate of TrxR (Johansson, Lillig and Holmgren, 2004), and mitochondrial Prx3 could be reduced by mitochondrial Trx2 and Grx2 (Hanschmann *et al.,* 2010). The backing-up capacity for thioredoxin reductase activity by the GSH-Grx system was clearly evidenced by its compensatory effects in those situations when TrxR activity was decreased, for instance, when there was selenium deficiency or deficiency in the selenocysteine synthesis machinery, or when TrxR was inhibited or knocked-down or -out (Lu, J. *et al.,* 2009; Lu, J. and Holmgren, 2012; Bondareva *et al.,* 2007).

1.2.3.4. Transcription factors

The thioredoxin system not only transfers its electrons to antioxidant enzymes, but it also plays an important role in signaling, as it is the case of the GSH-Grx system, since the thioredoxin system regulates the activity of many redox-regulated transcription factors involved in health and disease (Lillig and Holmgren, 2007; Holmgren and Lu, 2010; Lu and Holmgren, 2014). Thus, transcription factors Nrf2, Ref-1, NF-κB, p53, and HIF1α, as well as signaling enzymes such as PTP1B and ASK1 among others are regulated by the thioredoxin and glutathione systems (Lee, S. R. *et al.,* 1998; Fourquet *et al.,* 2010; Holmgren and Lu, 2010; Lu and Holmgren, 2014).

1.3. TRP14

Thioredoxin-related protein of 14 kDa (TRP14) is a cytosolic, 123-amino acid protein which is expressed in a wide range of cells and tissues (Jeong, Yoon *et al.,* 2004). Human TRP14 shows a conserved WCPDC motif in the active site, typical of Trx isoforms; however, sequence homology with Trx1 is as low as 20% (Jeong *et al.,* 2004). TRP14 contains 5 cysteine residues in its structure, although only two of them –the ones present in the active WCPDC site– are exposed and redox-sensitive (Jeong *et al.,* 2004). When TRP14 is oxidized, it is readily reduced by cytosolic TrxR1 but not by mitochondrial TrxR2, contrary to both Trx1 and Trx2 which are effectively reduced by both isoforms (Jeong *et al.,* 2004). TRP14 was biochemically characterized as a disulfide reductase like Trx1 (Jeong *et al.,* 2004). Interestingly, TRP14 is unable to reduce typical Trx1 substrates such as insulin, ribonucleotide reductase, peroxiredoxins and methionine sulfoxide reductase, suggesting that TRP14 and Trx1 may have different functions and thus act specifically on different substrates (Jeong *et al.,* 2004). These differences in substrate specificity may be due to an additional α-helix in TRP14 structure, as well as to a different charge distribution of residues compared to Trx1 (Woo, J. R. *et al.,* 2004).

1.3.1. TRP14 structure and active site

Mammalian TRP14 was first discovered in 2000 in rat brains as a result of a search for proteins that contain redox-sensitive cysteine residues in their structure (Kim, J. R. *et al.,* 2000). A few years later, in 2004, Rhee's group identified and characterized for the first time human TRP14 (Jeong *et al.,* 2004). It was initially described as a 14-kDa human thioredoxin-related

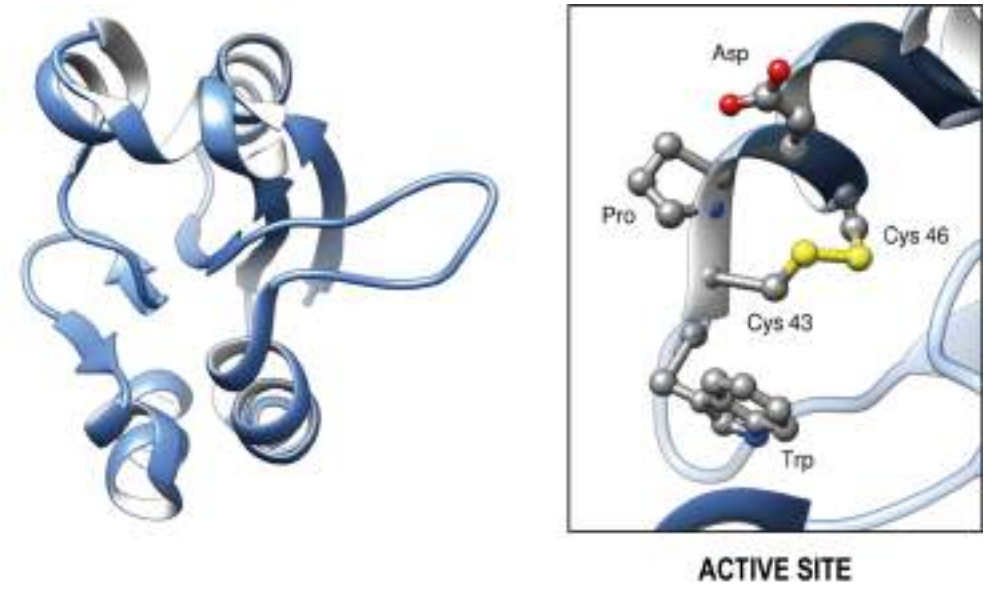

Figure 9. Structure of human TRP14. The two cysteine residues forming a disulfide bond in its active site (right panel) are responsible for TRP14 catalytic activity (Jeong *et al.*, 2004).

protein that seemed to be ubiquitously expressed in a wide variety of tissues and human cell lines, although in smaller amounts than Trx1 (Jeong *et al.,* 2004).

Structurally, TRP14 shows a typical Trx fold (**Figure 9**) (Woo *et al.,* 2004); however, sequence identity between TRP14 and Trx1 is as low as 20% (Weichsel *et al.,* 1996; Jeong *et al.,* 2009). Although TRP14 contains 5 cysteine residues in its structure, only two of them –the ones located within the active site– are exposed and redox-sensitive (Jeong *et al.,* 2004). The active site is a conserved WCPDC motif that is found at the start of the α2 helix, as it is the case in Trx1 (Jeong *et al.,* 2004). The thiolate anion formed in the active site at Cys^{43} is stabilized thanks to the dipeptide Pro-Asp that separates both Cys residues (Cys^{43} and Cys^{46}), rendering a low pK_a value for Cys^{43} (Jeong *et al.,* 2009). In its oxidized form, Cys^{43} and Cys^{46} of TRP14

form an intramolecular disulfide bond, rendering the enzyme inactive (Jeong *et al.,* 2004). TrxR1, but not TrxR2, is able to reduce the disulfide bond between these two cysteine residues, recovering TRP14 activity (Jeong *et al.,* 2004). The structure of the TRP14 active site is depicted in **Figure 9**.

As it has been mentioned above, TRP14 and Trx1 show different substrate specificities despite their structural similarities (Jeong *et al.,* 2004). This fact may be explained at least in part due to the presence of an extra α3a helix near the TRP14 active site, as well as to an extended β2-α2 loop –which cause the structure to protrude– when compared to Trx1 that has relatively flat surfaces in the proximity of its active site (Jeong *et al.,* 2009). Besides, TRP14 and Trx1 also differ in the charge distribution around the active site. In the case of TRP14, there are many positively and negatively charged regions near its active site resulting in a highly charged area, whereas Trx1 basically shows hydrophobic residues between charged areas (Jeong *et al.,* 2009).

1.3.2. L-Cystine reduction by TRP14

TRP14, together with TrxR1, have been shown to reduce cystine by cleaving its disulfide bond in presence of NADPH, thus liberating two cysteine molecules (Pader *et al.,* 2014). TrxR1 supports TRP14 activity by reducing the disulfide in the active site of TRP14 yielding a dithiol by using NADPH (Jeong *et al.,* 2004; Woo *et al.,* 2004). This function is specific to TrxR1 since TRP14 is not a substrate of mitochondrial TrxR2 (Jeong *et al.,* 2004). Pader *et al.* showed that TRP14 was more efficient in reducing cystine than Trx1 (Pader *et al.,* 2014). When using L-cystine as the final electron acceptor, the Michaelis constant (K_m) of TrxR1 for TRP14 was 0.8 μM, whereas for Trx1 it was 4.3 μM (Pader *et al.,* 2014). Although the catalytic

constants (k_{cat}) of TrxR1 for both TRP14 and Trx1 are similar (1.818 min^{-1} and 1.749 min^{-1}, respectively), the catalytic efficiency (k_{cat}/K_m) of TrxR1 is fivefold greater for TRP14 than for Trx1 (2217 $min^{-1}\mu M^{-1}$ for TRP14 compared with 418 $min^{-1}\mu M^{-1}$ for Trx1), indicating that TRP14 is a much better cystine reductase than Trx1 (Pader *et al.,* 2014).

1.3.3. TRP14 in redox signaling

Human TRP14 was discovered in 2004 as an inhibitor of NF-κB signaling (Jeong, Chang *et al.,* 2004). NF-κB is a key transcription factor involved in the regulation of immunity, inflammation, cell proliferation and differentiation, and cell survival (Hayden and Ghosh, 2004; Hayden and Ghosh, 2008; Hayden and Ghosh, 2014). Although Trx1 was described to modulate several transcription factors including NF-κB (Matthews *et al.,* 1992; Mitomo *et al.,* 1994; Watson *et al.,* 2008), despite its lower expression levels, TRP14 is a more potent repressor of inflammatory responses through NF-κB regulation (Jeong *et al.,* 2004). TRP14 may suppress TNF-α-mediated activation of NF-κB through dynein light chain LC8 (Jung *et al.,* 2008). TRP14-mediated reduction of LC8 leads to its activation and further binding to IκB, preventing in this way its phosphorylation and further NF-κB activation (Jung *et al.,* 2008). Furthermore, TRP14 was reported to impair osteoclast differentiation, which is directly related to blockade of NF-κB activation (Hong *et al.,* 2014).

Autophagy –a mechanism of resistance of cancer cells to certain chemotherapeutic agents such as paclitaxel– can be regulated by TRP14. Thus, Beclin-1 (BECN1) –an autophagy inductor that inhibits tumor formation, angiogenesis, and metastasis (Wechman *et al.,* 2018)– is

involved in the mechanism of TRP14-regulated autophagy (Zhang, S. *et al.,* 2015). Paclitaxel treatment led to an increase in the expression of both BECN1 and TRP14 in human colorectal cancer cells (Zhang *et al.,* 2015) . TRP14 siRNA treatment prevented the upregulation of BECN1 upon paclitaxel treatment, evidencing the role of TRP14 in the regulation of autophagy through BECN1 expression (Zhang *et al.,* 2015).

Cysteine nitrosylation is another mechanism of redox signaling controlling cell function (Benhar, Forrester and Stamler, 2009; Wolhuter and Eaton, 2017; Wolhuter *et al.,* 2018). In this regard, Trx1 can regulate cysteine nitrosylation either by reducing directly *S*-nitrosylated groups present in cysteine residues or by taking part in transnitrosylation reactions (Benhar *et al.,* 2008; Hashemy and Holmgren, 2008; Benhar, Forrester and Stamler, 2009; Sengupta and Holmgren, 2013; Benhar, 2015; Benhar, 2016). Interestingly, TRP14 was also reported to efficiently reduce nitrosylated cysteine residues (Pader *et al.,* 2014). However, it has not been shown yet whether Trx1 or TRP14 has a predominant role in the regulation of protein nitrosylation status.

Hydrogen sulfide can react with sulfenic acids within cysteine residues – or might undergo thiol-disulfide exchange with a disulfide– leading to their persulfidation through the addition of either one or several sulfur atoms to the sulfur atom of the cysteine residue through disulfide bonds, being a key player in the control of redox-dependent signaling systems (Kabil, Vitvitsky and Banerjee, 2014; Nagy, 2015; Kasamatsu *et al.,* 2016; Akaike *et al.,* 2017; Álvarez *et al.,* 2017). TRP14 efficiently reduced inorganic polysulfide chains as well as persulfidated cysteine residues in target proteins such as persulfidated human albumin (Dóka *et al.,* 2016). Interestingly, TRP14 was able to reduce oxidized PTP1B (Dagnell *et al.,* 2013), which plays a key role in the activation of tyrosine kinase receptors (Haj *et al.,* 2003) and can be

regulated by persulfidation (Krishnan *et al.,* 2011). Besides, it should be noted that Trx1, TrxR1, and the glutathione system are also able to reduce persulfidated protein motifs (Dóka *et al.,* 2016; Wedmann *et al.,* 2016) and to regulate PTP1B activity (Schwertassek *et al.,* 2014; Dagnell *et al.,* 2017). Interestingly, TrxR1 alone is able to reduce oxidized persulfide species into free thiols, but it showed no effect on the reduction of persulfidated proteins (Dóka *et al.,* 2020). However, when TrxR1 was coupled to TRP14 or Trx1, the enzymatic system was shown to efficiently reduce persulfidated protein motifs (Dóka *et al.,* 2020). Specifically, TRP14, together with TrxR1, reduced both persulfidated PTP1B and Prx2, leading to the recovery of protein function which was lost upon persulfidation (Dóka *et al.,* 2020). Furthermore, protein cysteine persulfidation levels in livers from TRP14-knockout mice were slightly increased when compared to wild-type mice while no significant changes in persulfidated low-molecular-weight thiols were found (Dóka *et al.,* 2020). Taken together, these findings suggest that the GSH system may be the main player in the regulation of general protein persulfide homeostasis whereas TRP14 can act on a very specific subset of persulfidated protein targets, regulating in this way their activity (Dóka *et al.,* 2020).

In a similar fashion, numerous cellular processes are regulated by enzymatic selective oxidation or reduction of target residues present on key signaling proteins that control a wide range of physiological and pathophysiological processes in the cell (Winterbourn and Hampton, 2008; Brigelius-Flohé and Flohé, 2011; Groitl and Jakob, 2014; Sies, 2014; Ye *et al.,* 2015; Zhang, L. *et al.,* 2019). In this context, TRP14 has been described to interact in different steps of several redox-regulated pathways (Espinosa and Arnér, 2019). Some of these pathways are summarized in **Figure 10**.

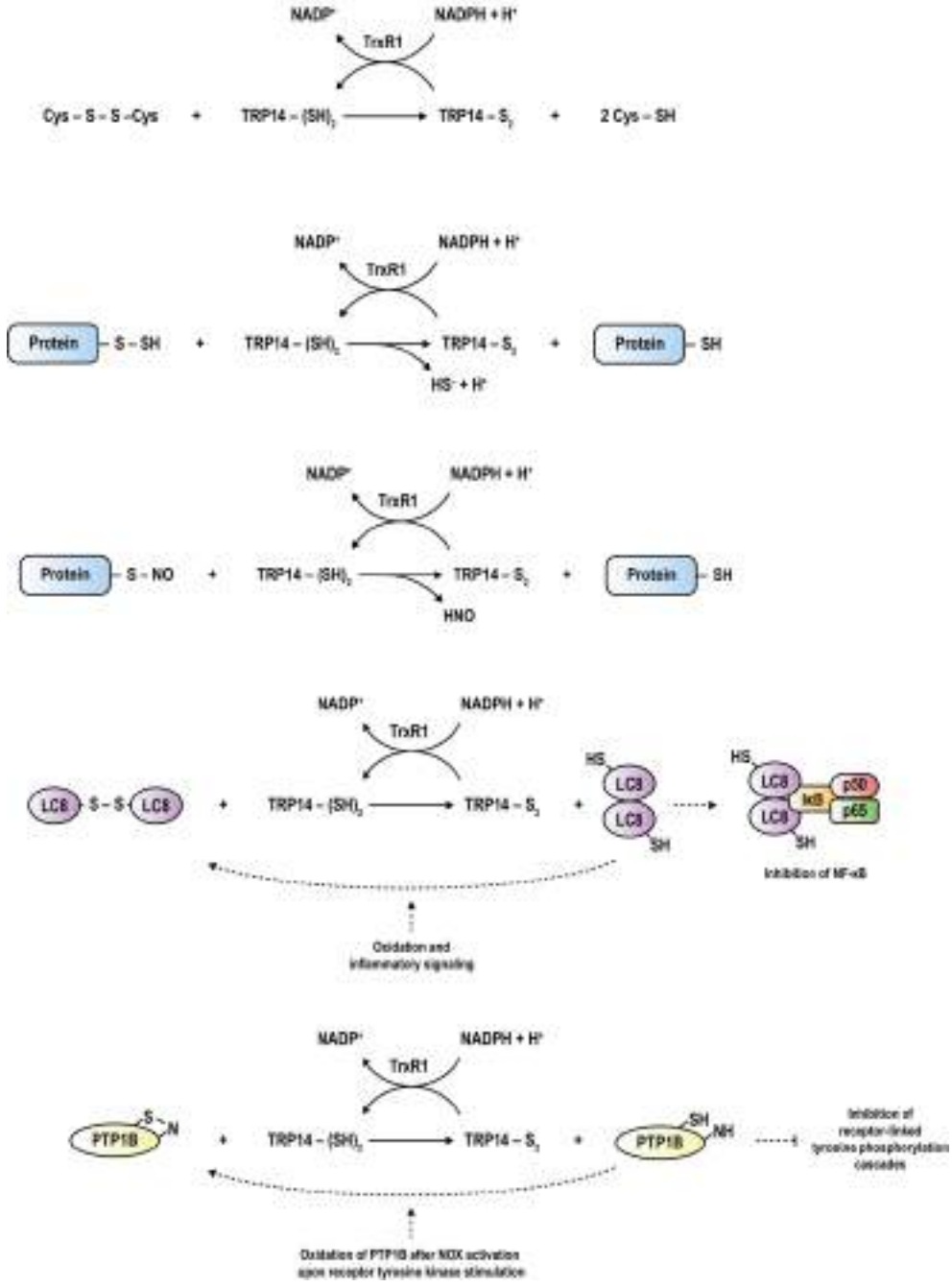

Figure 10. Selected TRP14-catalyzed enzymatic reactions and its role in redox signaling (Modified from Espinosa and Arnér, 2019).

1.4. ACUTE PANCREATITIS

1.4.1. Pancreas anatomy and physiology

The pancreas is a gland whose length and weight varies around 15-23 cm and 70-150 g, respectively, in human adults (Paulsen and Waschke, 2018). It is a retroperitoneal organ in close contact with its surrounding organs such as the duodenum –the head of the pancreas is attached to the duodenal loop–, the stomach –which is in contact with the upper part of the pancreas–, the spleen –attached through the tail of the pancreas–, as well as the transverse colon –in contact with the anterior portion of the pancreas– and the left kidney (Schuenke *et al.,* 2020) (**Figures 11** and **12**).

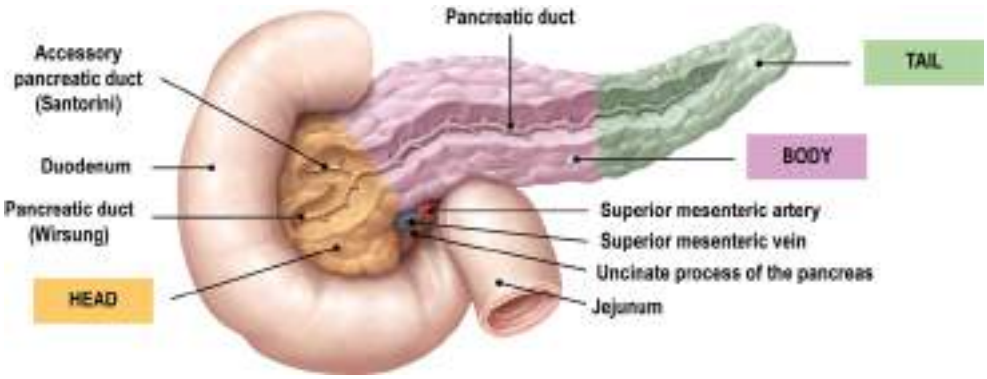

Figure 11. Anatomy of the human pancreas. (Modified from Gilroy *et al.*, 2016).

From the physiological point of view, the pancreas shows a double function. On one hand, its function as an endocrine gland is due to the ability of approximately 1-2% of pancreatic cells to secrete hormones, most of these cells being concentrated in structures called Langerhans islets (Guyton and Hall, 2016; Schuenke *et al.,* 2020). Among these endocrine cells, 60-75% of them are β cells which secrete insulin, and 20-25% are α

cells which secrete glucagon (Guyton and Hall, 2016; Schuenke *et al.,* 2020). The remaining endocrine cells are called γ and PP cells, which secrete somatostatin and pancreatic peptide, respectively (Guyton and Hall, 2016). On the other hand, the vast majority of pancreatic cells, which are acinar cells, play an exocrine role since they are able to synthetize and secrete digestive enzymes: trypsin, chymotrypsin, amylases, lipases, elastases, carboxypeptidases and ribonuclease among others (Guyton and Hall, 2016). Digestive enzymes are usually synthesized and secreted as inactive proenzymes that are then activated by enterokinase in the duodenal lumen where they perform their functions, avoiding in this way pancreatic autodigestion (Guyton and Hall, 2016).

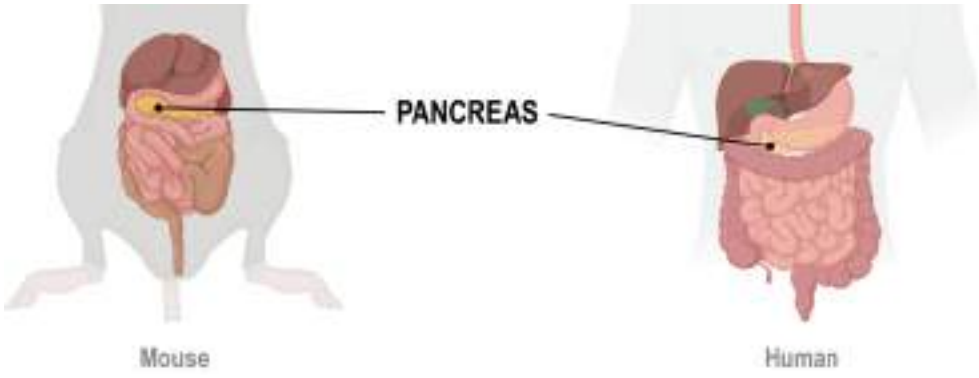

Figure 12. Comparative anatomical location of the mouse and human pancreas. (Elaborated based on Hedrich, 2012 and Gilroy *et al.*, 2016)

1.4.2. Pathology of the pancreas

Histological and functional characteristics of the pancreas allow us to classify its pathology according to the region and function being affected: endocrine or exocrine.

Among the pathological processes affecting the endocrine pancreas, we can highlight the dysfunction of β cells that leads to the development of diabetes mellitus, caused by multiple factors of diverse origin (Jameson *et al.,* 2018). However, in the case of exocrine pancreas pathology, dysfunctions are mainly due to inflammatory processes or tumor development (Jameson *et al.,* 2018) . Among inflammatory diseases of the exocrine pancreas, we highlight acute pancreatitis over chronic pancreatitis due to its frequency and repercussion (Jameson *et al.,* 2018).

1.4.2.1. Acute pancreatitis

Acute pancreatitis (AP) is an inflammatory process of the pancreatic gland of diverse etiology. It courses with a wide clinical spectrum and in most cases it is mild, self-resolving, and improves in a matter of days with therapeutic measures addressing symptomatology. However, acute pancreatitis may suddenly progress into a severe form that does not resolve upon any therapeutic strategy, leading to fatal consequences in around 20-30% of cases. Said variability in the clinical manifestations and development of the disease, as well as its diverse etiology, have not facilitated the study of acute pancreatitis. The first attempt at explaining the disease was made by Reginal H. Fitz, emeritus professor of Pathological Anatomy at Harvard University in 1889 (Fitz, 1889), which was followed by many other attempts to understand and classify the disease according to surgical findings or post-mortem criteria (Navarro, 2018).

Attempts to unify diagnostic, prognostic and severity criteria as well as patient treatment have been made since the early 1970s (Navarro, 2018). Numerous multidisciplinary meetings were held in this regard that included health professionals representing all branches of research, care and

medicine (Navarro, 2018). One of the most fruitful meetings was the International Symposium on Acute Pancreatitis, held in Atlanta in 1992 (Bradley, 1993), where they proposed the definitions, criteria and classifications that are still used –although with certain modifications which will be commented below– nowadays. Since then, terms and definitions have been revised in order to incorporate modern concepts of the disease, to clarify certain areas in which there was debate, to improve the clinical evaluation of the severity of the disease, and to help implementing new treatment strategies for acute pancreatitis (Dervenis, 2000; Uhl *et al.,* 2002; de-Madaria *et al.,* 2010; Banks *et al.,* 2013; Forsmark, Vege and Wilcox, 2016; Slawinski and O'Reilly, 2017; van Dijk *et al.,* 2017; Waller *et al.,* 2018; Hines and Pandol, 2019).

1.4.2.1.1. Definition and classification

According to the revised Atlanta Classification (Banks *et al.,* 2013), the current definition of acute pancreatitis is as follows:

Acute pancreatitis: acute inflammatory process of the pancreas, which may variably affect other surrounding tissues or even other organs and systems. Its manifestations are characterized by the sudden appearance of abdominal pain, frequently accompanied by nausea and vomiting, fever, tachycardia, leukocytosis, as well as elevated pancreatic enzymes in blood and/or urine (Banks *et al.,* 2013).

In most cases there is diffuse enlargement of the pancreatic parenchyma due to inflammatory edema, which is usually accompanied by inflammatory changes in peripancreatic fat, and symptoms tend to wear off during the first

week of the disease (Banks *et al.,* 2013). Peripancreatic fluid accumulation is also common (Banks *et al.,* 2013).

In around 5-10% of cases, necrosis of the pancreatic parenchyma, the peripancreatic tissue or both develop (Banks *et al.,* 2013). Necrosis evolution is variable: it can remain sterile or be infected, it can have a solid or liquid appearance and be persistent or disappear with time (Banks *et al.,* 2013). Patients developing necrotizing pancreatitis show an increase in mortality and intervention rates compared with patients developing edematous pancreatitis (Banks *et al.,* 2013). Mortality rate of acute necrotizing pancreatitis is around 17%, compared to only 2% mortality in those cases of acute interstitial edematous pancreatitis (van Dijk *et al.,* 2017). The mortality rate of necrotizing pancreatitis is much higher among those patients who develop infected necrosis (approximately 30%) compared to those who develop sterile necrosis (around 12%) (van Dijk *et al.,* 2017).

When taking into account the severity of the disease, the classification criteria are the presence of organ failure and its duration, as well as the appearance of local or systemic complications (Banks *et al.,* 2013). According to the revised Atlanta Classification (Banks *et al.,* 2013), there are three clinical types of acute pancreatitis:

a) **Mild acute pancreatitis:** characterized by absence of organ failure and the absence of local or systemic complications. Histologically, mild acute pancreatitis develops as interstitial edema which resolves during the early stages of the disease in most cases. It accounts for approximately 85% of cases, and its mortality is around 2% (Banks *et al.,* 2013; Forsmark, Vege and Wilcox, 2016; Hines and Pandol, 2019).

b) **Moderately severe acute pancreatitis:** characterized by the presence of transient organ failure (the organ failure persists less than 48 hours) or local or systemic complications in absence of persistent organ failure. It may resolve spontaneously, or it may require intervention and specialist care, as it is the case of sterile necrosis without organ failure (Banks *et al.,* 2013). Mortality in this type of pancreatitis is around 5%, much lower than in severe acute pancreatitis (Forsmark, Vege and Wilcox, 2016).

c) **Severe acute pancreatitis:** characterized by the presence of persistent organ failure (the organ failure persists more than 48 hours), which develops during the early phase of the disease as a consequence of persistent systemic inflammatory response syndrome (SIRS). Organ failure could be single or multiple, patients suffering from multiple organ failure being classified as critical (Banks *et al.,* 2013). Local complications such as pancreatic fluid collections, pancreatic pseudocysts, acute necrotic collections, and walled-off necrosis may appear (Hines and Pandol, 2019). Histological analysis shows necrotic areas as well as pancreatic and peripancreatic hemorrhages (Banks *et al.,* 2013). Mortality among patients who develop organ failure a few days after the onset of the disease may be extremely high, around 36% (Padhan *et al.,* 2018). In addition, mortality is further increased up to 50% among those patients presenting persistent organ failure who develop infected necrosis (Padhan *et al.,* 2018).

In the revised Atlanta Classification, organic failure was defined after a thorough analysis of three major organ systems: the cardiovascular system, the respiratory system, and the renal system (Banks *et al.,* 2013). Accordingly, organ failure develops when one of the previous three systems

shows a score of two or more in the modified Marshall's multiple organ disfunction score (**Table 1**) (Marshall *et al.,* 1995). This score system, together with the sepsis-related organ failure assessment (SOFA) system (Vincent *et al.,* 1996), are still useful in the evaluation of dynamic diseases such as acute pancreatitis, which require a daily objective reevaluation of the patient's status (Jameson *et al.,* 2018).

Table 1. Modified Marshall score for the assessment of acute pancreatitis severity.

	Score 0	Score 1	Score 2	Score 3	Score 4
Respiratory status (PaO_2/FiO_2)	> 400	301-400	201-300	101-200	≤ 101
Renal status (Serum creatinine, μmol/L)	< 134	134-169	170-310	311-439	> 439
Cardiovascular status (systolic blood pressure, mmHg)	> 90	< 90 Fluid responsive	< 90 Not fluid responsive	< 90 pH < 7.3	< 90 pH < 7.2

1.4.2.1.2. Epidemiology and etiology

Acute pancreatitis is currently among the main causes of hospital admission regarding gastrointestinal disorders, and its incidence has significantly increased during the last decade (Hines and Pandol, 2019). In the United Estates, the annual incidence of the disease is presently 13-45

cases per 100000 inhabitants (Hines and Pandol, 2019), which doubles the number of hospital admissions due to acute pancreatitis registered in 1988 (Yang *et al.,* 2008).

Although the etiology of acute pancreatitis is quite diverse, gallstones and alcohol misuse are key factors triggering the pathophysiological mechanisms that result in the development of acute pancreatitis (Hines and Pandol, 2019). Around 10% of cases are due to non-common causes such as surgical procedures, infections, drugs or toxics, and ischemia, and in 10-30% of cases the cause remains unknown (idiopathic acute pancreatitis) (Forsmark, Vege and Wilcox, 2016).

Although the probability of developing acute pancreatitis is the same among men and women, age and gender influence the etiology of the disease (Hines and Pandol, 2019). Thus, acute pancreatitis caused by alcohol abuse is more common in men, although sex differences are equaled upon similar alcohol consumption (Lankisch, Apte and Banks, 2015; Hines and Pandol, 2019). In women, acute pancreatitis is generally more related to gallstones and other gallbladder disorders, autoimmune diseases or idiopathic origin (Forsmark, Vege and Wilcox, 2016; Hines and Pandol, 2019). Regarding the age of onset, acute pancreatitis most commonly develops in patients ranging from 30 to 70 years-old, 55-year-old patients showing the highest incidence (Singh *et al.,* 2011; Forsmark, Vege and Wilcox, 2016). In addition, the risk of suffering from acute pancreatitis is three times higher among black population than among Caucasian population (Yang *et al.,* 2008), and further studies are needed to know whether the observed differences are due to diet, genetics, or other factors.

Figure 13 summarizes the epidemiology of acute pancreatitis.

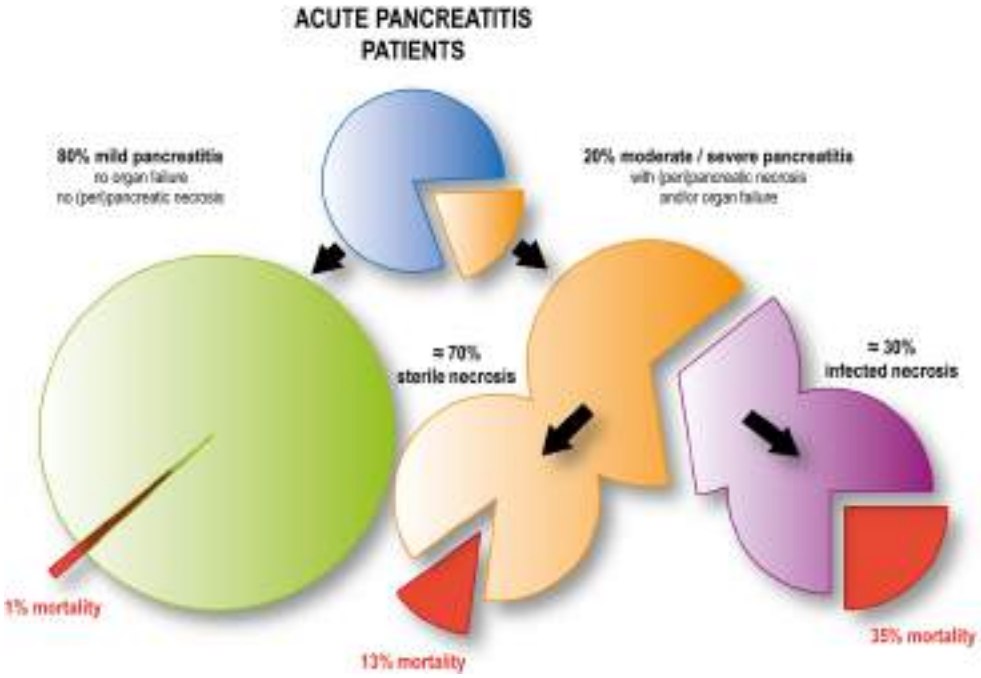

Figure 13. Epidemiology and mortality rates of acute pancreatitis according to the evolution of the disease. (Modified from van Dijk *et al.* 2017).

1.4.2.1.3. Pathophysiology of acute pancreatitis

The mechanisms involved in the onset and development of the disease comprise a complex cascade of events activated in acinar pancreatic cells, generally as a result of an injury of the acini which causes disruption of the cells and the consequent leakage of their contents into the pancreatic tissue (Bhatia *et al.,* 2005). Trypsinogen activation into trypsin and its consequent accumulation into the pancreatic parenchyma initiates an autodigestive process that stimulates an inflammatory response (Lankisch, Apte and Banks, 2015). Pro-inflammatory cytokines, chemokines, reactive oxygen species, and calcium (Ca^{2+}) are the main drivers of the local and systemic

complications such as multiple organ failure and death (Pereda *et al.,* 2006; Pandol *et al.,* 2007; Pérez *et al.,* 2015b).

Therefore, proteolytic enzyme activation and the inflammatory response triggered in acinar cells could be considered as the main molecular events leading to the development of acute pancreatitis.

a) Intracellular zymogen activation

Pancreatic secretion plays a key role in the digestive process since it contains the proteolytic enzymes needed to transform food into simple molecules that can be assimilated and used by the organism. The regulation of the pancreatic secretion process is performed at neural and hormonal level, secretin and cholecystokinin being the most important hormones affecting pancreatic secretion (Guyton and Hall, 2016).

Secretin is released in response to food intake and it triggers secretion of the hydroelectrolytic fraction of the pancreatic juice that is rich in bicarbonate, chloride, sodium and potassium, and which is able to buffer the acidic chyme (Guyton and Hall, 2016). Cholecystokinin (CCK) is released in response to fatty acids and amino acids resulting from the digestion of fat and proteins, leading to the secretion of the enzymatic fraction of the pancreatic juice, which contains enzymes such as lipase, amylase, carboxypeptidase, elastase, trypsin, and chymotrypsin (Guyton and Hall, 2016).

As it has been commented above, due to the highly destructive capacity of the enzymatic fraction of pancreatic juice, the pancreas needs to protect itself against autodigestion. Therefore, proteolytic enzymes are secreted as inactive zymogen granules that are released

into the duodenal lumen where they become activated (Guyton and Hall, 2016). Proenzymes are synthesized in ribosomes of the rough endoplasmic reticulum which, after travelling through the cisternae of the Golgi apparatus, become associated to zymogen granules (**Figure 14**, left) (Guyton and Hall, 2016). Another strategy to prevent autodigestion is the synthesis of inhibitors of pancreatic enzymes, such as trypsin inhibitor α1-antitrypsin (Guyton and Hall, 2016).

Considering that acute pancreatitis starts with autodigestion of the pancreas due to premature zymogen activation, it was discovered at the beginning of the 1980s that zymogen granules colocalize with lysosomal enzymes at early stages of ethionine-induced experimental acute pancreatitis (**Figure 14**, right) (Koike, Steer and Meldolesi, 1982). Other experimental models –induced either by cerulein (an analogue of cholecystokinin) stimulation or by obstruction of the pancreatic duct– confirmed the colocalization phenomenon and

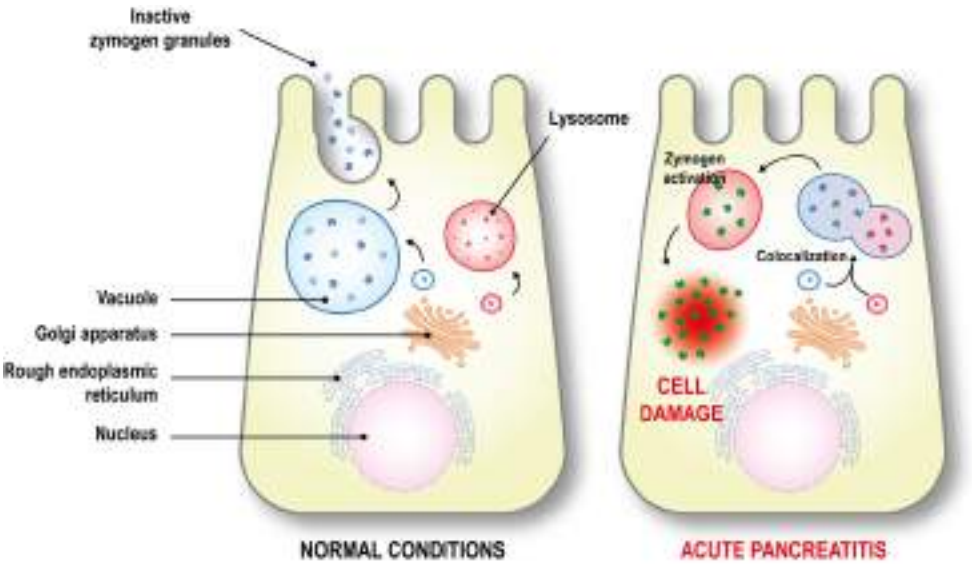

Figure 14. Intracellular zymogen activation. Normal zymogen secretion (left) is altered during acute pancreatitis, leading to intracellular zymogen activation and subsequent cell autodigestion (right).

showed as well endocytosis of secreted enzymes and alterations in zymogen granule transport (Watanabe *et al.,* 1984; Saluja *et al.,* 1989). Later on, additional experiments proved that cathepsin B present in lysosomes is able to produce trypsinogen activation (Halangk *et al.,* 2002). At the same time, cathepsin B release triggers necroptosis of the pancreatic cell (Talukdar *et al.,* 2016), which is regulated through the receptor-interacting protein kinase (RIP) and mixed lineage kinase domain-like (MLKL) pathways (Lee, P. J. and Papachristou, 2019). Necrostatin inhibition of the RIP pathway led to decreased acinar cell damage *in vitro* (Louhimo, Steer and Perides, 2016). Furthermore, cathepsin B knockout mice showed lower intrapancreatic trypsin levels upon acute pancreatitis induction, although no differences were found in the development of the systemic inflammatory response (Halangk *et al.,* 2000).

However, several evidences question this theory, such as the possibility to obtain colocalization without further development of acute pancreatitis (Lüthen *et al.,* 1995). On the other hand, neither the *in vivo* administration of inhibitors of lysosomal enzymes prevented the development of acute pancreatitis, nor their *in vitro* administration decreased trypsinogen activation (Steer, 2002). More recent studies demonstrated that the cellular redistribution of cathepsin B forming proteolytic vacuoles does not cause either intracellular trypsinogen activation or development of acute pancreatitis (Meister, T. *et al.,* 2010).

Thus, other research lines were developed in order to look for different mechanisms that could explain the intracellular activation of proteolytic enzymes, as was the case of those defending NADPH

oxidase from neutrophils as a major cause for zymogen activation (Gukovskaya *et al.,* 2002).

Intracellular calcium can also play an important role in the early activation of digestive enzymes within acinar cells during acute pancreatitis (Lee and Papachristou, 2019). Genetic and pharmacological inhibition of the calcium influx channel TRPC3 in acinar cells showed decreased intracellular activation of zymogens and, as a consequence, ameliorated acute pancreatitis in mice (Kim, M. S. *et al.,* 2011). Extracellular pH is another factor that has been studied in the context of the early development of acute pancreatitis. A decrease of extracellular pH from 7.4 to 7.0 caused activation of acinar cells, leading to development of acute pancreatitis *in vivo* and *in vitro*, probably mediated by enhanced calcium signaling (Bhoomagoud *et al.,* 2009; Reed *et al.,* 2011).

Several theories tried to relate autophagy with the early activation of zymogen granules. Autophagy is a lysosomal degradation mechanism whose main function is maintaining cell homeostasis and assuring cell survival (Mizushima, 2018). However, this process has been associated to different diseases such as cancer, type 2 diabetes, obesity, inflammatory diseases like Chron's disease, infectious diseases, and neurodegenerative disorders among others (Saha *et al.,* 2018). Basal autophagy levels in the exocrine pancreas of mice are elevated in comparison to kidney, liver, heart or endocrine pancreas due to the increased protein synthesis activity found within acinar cells (Gukovskaya *et al.,* 2017). Nowadays, the role of autophagy in acute pancreatitis is under discussion: on the one hand, autophagy can damage acinar cells through activation of trypsinogen and its subsequent transformation into trypsin due to the action of

lysosomes (Hashimoto *et al.,* 2008); on the other hand, a deficiency in autophagic activity is associated with an imbalance between cathepsin L –which degrades trypsinogen and trypsin– and cathepsin B –which converts trypsinogen into active trypsin–, resulting in intracellular accumulation of active trypsin in acinar cells (Mareninova *et al.,* 2009). Therefore, paradoxically, insufficient lysosomal activity may be responsible for the increase in intracellular trypsin during the onset of acute pancreatitis (Mareninova *et al.,* 2009).

More recently, another research group described a specific type of autophagy as a selective process called zymophagy, which is characterized by preventing early activation of zymogens during the early phases of acute pancreatitis preventing cell death (Grasso *et al.,* 2011).

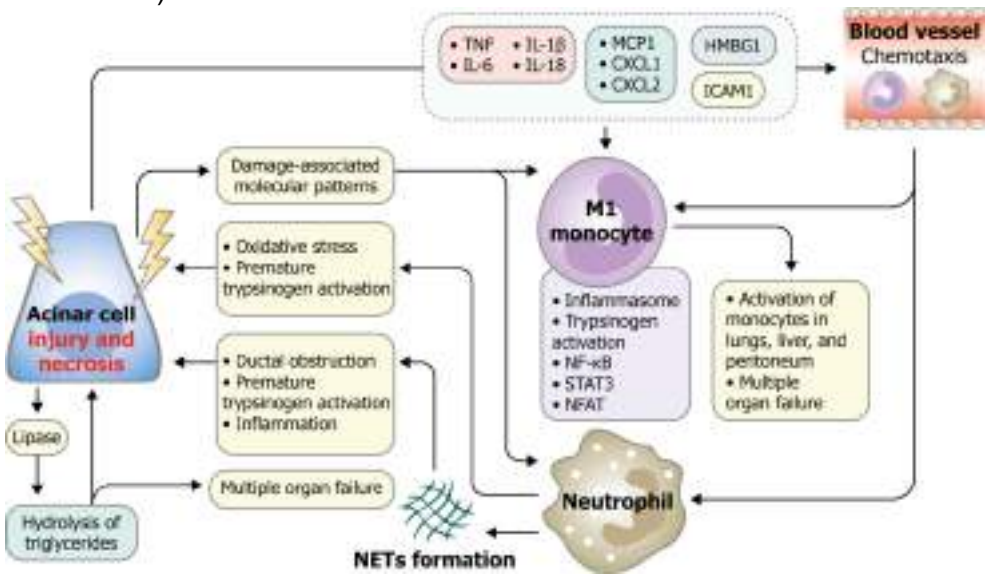

Figure 15. Main events in the pathophysiology of acute pancreatitis. *CXCL1: C-X-C motif chemokine ligand 1; CXCL2: C-X-C motif chemokine ligand 2; HMGB1: high mobility group box 1; ICAM1: intercellular adhesion molecule 1; IL-18: interleukin 18; IL-1β: interleukin 1-beta; IL-6: interleukin 6; MCP1: monocyte chemoattractant protein 1; NETs: neutrophil extracellular traps; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NFAT: nuclear factor of activated T-cells; STAT3: signal transducer and activator of transcription 3; TNF: tumor necrosis factor.*

Currently, several studies have identified alterations in genes that encode mediators of autophagosome formation, such as ATG5 or ATG7, as well as in those involved in the control of lysosomal function like LAMP2, as the cause of pancreatic damage, which includes degeneration and atrophy of the exocrine pancreas, inflammation, and fibrosis (Gukovskaya *et al.,* 2017).

b) Inflammatory response in acute pancreatitis

During the early stages of acute pancreatitis –at the same time that intracellular trypsinogen activation takes place– a series of processes related to cytokine and NF-κB activation occur.

NF-κB is a transcription factor that plays a key role in the development of the inflammatory response in mammals (Zhang, Q., Lenardo and Baltimore, 2017). In the context of acute pancreatitis, NF-κB is tightly involved in the pathogenesis and development of the disease (Baumann *et al.,* 2007). NF-κB is activated during the initial phase of acute pancreatitis both in leukocytes and pancreatic acinar cells (Vaquero *et al.,* 2001). NF-κB activation was shown to be independent of trypsinogen activation *in vitro* (Ji *et al.,* 2009). Overexpression of IKK2 –a mediator in NF-κB activation– is sufficient to induce acute pancreatitis (Baumann *et al.,* 2007). In fact, IKK2 overexpression leads to edema formation, necrosis, and inflammatory infiltrate through the regulation of NF-κB target genes (Baumann *et al.,* 2007). Furthermore, acute pancreatitis induced by cerulein in NF-κB deficient mice showed a decrease in tissue damage, correlated to lower TNF-α mRNA expression (Altavilla *et al.,* 2003). Thus, NF-κB inhibitors are

currently being tested in animal models to assess their therapeutic potential in acute pancreatitis (Lee and Papachristou, 2019).

Cytokines are polypeptides or glycoproteins that are released mainly by immune cells (Abbas, Lichtman and Pillai, 2017). The major role of cytokines is acting as cellular mediators in the modulation of the inflammatory response. Despite being an heterogenous group of proteins, cytokines share some characteristic properties and functions: they are secreted in extremely little amounts –what makes them difficult to be detected– during the effector phases of natural and specific immunity, modulating both immune and inflammatory responses (Abbas, Lichtman and Pillai, 2017). Sometimes, they even regulate cell division and may show chemotactic activity (Abbas, Lichtman and Pillai, 2017).

Cytokine secretion is short, self-limiting, and activated by the expression of their specific genes (Abbas, Lichtman and Pillai, 2017). In some cases, cytokine regulation takes place through posttranslational mechanisms, as it is the case of proteolytic activation for pro-IL-1β (Ahn *et al.,* 2017). Cytokines can develop an autocrine, paracrine or endocrine role, requiring in most cases from mRNA synthesis to produce their responses (Fasshauer and Blüher, 2015).

Proinflammatory cytokines such as TNF-α, IL-6, and IL-8 are elevated in different experimental acute pancreatitis models, as well as in patients suffering from severe acute pancreatitis (Staubli, Oertli and Nebiker, 2015; Sandoval *et al.,* 2016; Pérez *et al.,* 2019). In experimental models, blockade of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 is correlated with decreased systemic damage and improved survival rate (Mayer *et al.,* 2000; Luan *et al.,* 2013;

Pérez *et al.,* 2019). In addition, certain genetic polymorphisms within *TNF-α* and *IL-6* sequences are related to the severity and etiology of acute pancreatitis, respectively (de-Madaria *et al.,* 2008).

IL-6 and IL-8 levels correlate with the severity of the disease (Gunjaca *et al.,* 2012; Fisic *et al.,* 2013; Staubli, Oertli and Nebiker, 2015).

During the course of acute pancreatitis, cytokines are mainly released by activated pancreatic macrophages and neutrophils which infiltrate the pancreatic gland after acute pancreatitis onset, as a response of focal pancreatic damage (Viterbo *et al.,* 2008; Sendler *et al.,* 2013). Furthermore, pancreatic acinar cells can behave like inflammatory cells, synthetizing and releasing cytokines, chemokines and adhesion molecules (De Dios *et al.,* 2005).

In summary, leukocytes, together with acinar cells, trigger the inflammatory cascade in response to the local damage to the pancreas, activated macrophages being mainly responsible for the development of the systemic response (Lee and Papachristou, 2019).

- **Tumor necrosis factor alpha**

Tumor necrosis factor alpha (TNF-α) is a polypeptide that was first described as inducer of tumoral cell death *in vitro* (Old, 1988), although soon it was discovered its ability to induce fever, shock, tumoral necrosis, anorexia, embryogenesis, cell differentiation, and proliferation among others, and it was associated with the pathophysiology of a plethora of diseases, such as acute pancreatitis (Jakkampudi *et al.,* 2016; Abbas, Lichtman and Pillai, 2017; Lee and Papachristou, 2019).

Monocytes produce the highest amounts of TNF-α, although some other cell types such as B and T lymphocytes, natural killer (NK) cells, Kupffer cells, keratinocytes, Paneth cells, enterocytes, smooth muscle cells, pancreatic acinar cells, and certain tumor cell types have been described to secrete TNF-α as well (Ramudo *et al.,* 2005; Chu, 2013; Abbas, Lichtman and Pillai, 2017). The main stimulus leading to TNF-α synthesis and release is lipopolysaccharide (LPS) (Chu, 2013; Abbas, Lichtman and Pillai, 2017). Nevertheless, TNF-α production is increased in response to bacteria, viruses, other cytokines (IL-1, IL-2 or GM-CSF), immunological complexes (major histocompatibility complex), radiation (x rays), urate crystals, complement factor C5a and reactive oxygen species (Chu, 2013; Abbas, Lichtman and Pillai, 2017).

TNF-α may act on two receptors which are coded by different genes: p55/$TNFR_1$ and p75/$TNFR_2$ (Abbas, Lichtman and Pillai, 2017). $TNFR_1$ is expressed in numerous cell types whereas $TNFR_2$ is predominantly expressed in leukocytes and endothelial cells (Abbas, Lichtman and Pillai, 2017). The intracellular portion of $TNFR_1$ contains the 'death domain', which is required for apoptosis and NF-κB activation. Recruitment of certain proteins, such as Fas and caspase-8, lead to apoptosis, whereas recruitment of factor 2, which is associated with the TNF-α receptor and the receptor-interacting protein, leads to JNK and NF-κB activation (Abbas, Lichtman and Pillai, 2017).

Regarding acute pancreatitis, an increase in TNF-α serum levels was observed in patients suffering from this disease (Staubli, Oertli and Nebiker, 2015). TNF-α soluble receptors –the extracellular part of the

TNF-α receptor– are released as well during the early stages of the disease, matching the increase in TNF-α levels (Granell *et al.,* 2004).

During the course of acute pancreatitis, TNF-α is released from different tissues (Lee and Papachristou, 2019). Although, as is has been noted before, acinar cells are able to produce TNF-α, leucocytes forming the inflammatory infiltrate within the pancreatic tissue are considered as the main TNF-α source (Ramudo *et al.,* 2005; Lee and Papachristou, 2019). It is important to note that TNF-α levels in the pancreatic tissue are several times higher than serum levels during the course of the disease, which may be toxic to cells (Lee and Papachristou, 2019). Therefore, TNF-α contributes to the severity of acute pancreatitis through acinar cells apoptosis or necrosis induction (Sendler *et al.,* 2013; Lee and Papachristou, 2019). In this regard, our group observed that inhibition of TNF-α production by pentoxifylline markedly decreased inflammatory infiltrate, edema, and glutathione depletion in pancreas, as well as serum lipase and amylase activity upon cerulein-induced acute pancreatitis in rats (Gómez-Cambronero *et al.,* 2000). Similarly, we observed that pentoxifylline decreased proinflammatory cytokine production *in vitro* in AR42J pancreatic acinar cells through TNF-α inhibition (Escobar, Pereda, Arduini *et al.,* 2012).

- **Interleukin 1**

Interleukin 1 (IL-1) is a potent proinflammatory cytokine that is mainly synthesized in activated macrophages, although epithelial and endothelial cells may synthesize IL-1 as well (Dinarello, 2018). There are two forms of IL-1: IL-1α and IL-1β, both 17 kDa proteins showing

less than 30% identity (Dinarello, 2018). Both types of IL-1 bind the same receptor triggering similar biological effects. IL-1α is more commonly associated to cell membranes whereas IL-1β is generally detected in serum (Mantovani *et al.,* 2019).

Interleukin 1 activity is tightly regulated at several levels. Both IL-1α and IL-1β expression is induced by NF-κB activation, macrophage exposure to TLR4 receptor agonists, LPS, as well as by interleukin 1 levels (Mantovani *et al.,* 2019). IL-1α and IL-1β are initially synthesized as proproteins: proIL-1α is able to directly bind IL-1 receptor I (IL-1RI) and its proteolysis would increase its biological activity; however, proIL-1β cannot interact with IL-1RI and thus requires to undergo a proteolytic process by caspase 1 in order to become active (Mantovani *et al.,* 2019). In turn, caspase 1 activity is regulated by various cytosolic multimolecular complexes called inflammasomes, NLRP3 being the most important one among them. NLRP3 activation, either by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), induces conformational changes that allow the recruitment of adaptor protein ASC, which interacts with inactive procaspase-1 through the CARD domains present in both proteins (Mantovani *et al.,* 2019). Finally, active caspase-1 induces proIL-1β maturation (Mantovani *et al.,* 2019).

In pancreas, macrophages are the main source of IL-1β during the course of experimental acute pancreatitis (Lee and Papachristou, 2019). Said macrophages produce even more proIL-1β than neutrophils, which migrate and infiltrate the pancreatic gland during the onset of acute pancreatitis (Fink and Norman, 1996). Later studies

demonstrated that in absence of IL-1β the maximum degree of inflammatory infiltrate, edema, and necrosis associated to acute pancreatitis was not achieved, mortality being decreased as well (Denham and Norman, 1999). After the discovery of inflammasomes, it was shown that ASC, caspase-1, and NLRP3 are essential for the development of acute pancreatitis through the activation of IL-1β (Hoque and Mehal, 2015).

- **Interleukin 6**

Interleukin 6 (IL-6) is produced by numerous cells such as macrophages, endothelial cells, fibroblasts, and smooth muscle cells (Rose-John, 2018). As a pleiotropic cytokine, it plays a role in immunity, tissue regeneration, and metabolism (Rose-John, 2018). In numerous pathologies, IL-6 signaling dysregulation is involved (Hunter, C. A. and Jones, 2015; Rose-John, 2018). IL-6 is the main cytokine involved in the synthesis of acute phase proteins such as C-reactive protein, fibrinogen, and haptoglobin. Besides, its administration induces pyrexia. IL-6 is synthesized in response to TNF-α, IL-1 and endotoxin stimulation during the acute phase of the inflammatory response, and it is responsible for systemic effects observed in numerous diseases (Mihara *et al.,* 2012; Kang, S. *et al.,* 2019). IL-6 is detected as soon as one hour after induction of the inflammatory response and maintained high up to 10 days later; maximal levels being detected at around 4 to 6 hours after the onset of the inflammatory process. These properties led to successfully correlate serum IL-6 levels with the severity of acute pancreatitis (Gunjaca *et al.,* 2012; Fisic *et al.,* 2013; Staubli, Oertli and Nebiker, 2015; Sternby *et al.,* 2017). Recently, our group demonstrated that

blockade of IL-6 receptor glycoprotein 130 by LMT-28 led to a decrease in pulmonary damage associated to cerulein-induced acute pancreatitis in mice (Perez *et al.,* 2019), evidencing the importance of IL-6 signaling in the development of systemic responses in experimental acute pancreatitis.

- **Interleukin 8**

Interleukin 8 (IL-8) belongs to a family of cytokines known as chemokines. Chemokines are small cytokines ranging from 8 to 10 kDa, which play an essential role in leukocyte activation and migration to damaged zones through a process called chemotaxis (Abbas, Lichtman and Pillai, 2017). Particularly, IL-8 activates neutrophils and T lymphocytes, and it exerts a potent chemoattractant effect on them (Abbas, Lichtman and Pillai, 2017). TNF-α induces IL-8 synthesis in various cell types, macrophages and endothelial cells being the major sources of interleukin 8 (Abbas, Lichtman and Pillai, 2017). In fact, IL-8 is a key mediator in neutrophil activation in response to TNF-α as it induces neutrophil migration, expression of adhesion molecules and neutrophil degranulation, as well as ROS generation (Allen and Kurdowska, 2014). Interestingly, *in vivo* administration of IL-8 did not induce a shock state as TNF-α administration does (Wessely-Szponder, 2008).

In the context of acute pancreatitis, IL-8 plasma levels have been long considered as a marker of severity of experimental acute pancreatitis (Norman, 1998), and more recently in patients suffering from acute pancreatitis (Fisic *et al.,* 2013; Staubli, Oertli and Nebiker, 2015) since it regulates chemotaxis of neutrophils to the pancreas, as well as its

subsequent activation and degranulation (Abbas, Lichtman and Pillai, 2017).

- **Interleukin 10**

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that modulates the expression of proinflammatory cytokines which are produced at the early stages of the inflammatory process by downregulating *TNF-*α, *IL-1*, *IL-6* and *IL-8* (Abbas, Lichtman and Pillai, 2017). Furthermore, it stimulates the production of the antagonist of IL-1 receptor, as well as the release of soluble TNF-α p75 receptor (Ouyang and O'Garra, 2019). When IL-10 or any of its agonists, such as IT9302, were administered in experimental acute pancreatitis models as a therapeutic agent, both severity and mortality of the disease were decreased at least in part as a consequence of *Tnf-*α downregulation (Kotenko *et al.,* 2001).

- **Interleukin 18**

Interleukin 18 (IL-18) is a member of IL-1 cytokine family. As it is the case of IL-1β, IL-18 is synthesized in an inactive form, which needs to be cleaved by caspase-1 in order to become an active signaling peptide (Dinarello, 2018). Mature IL-18 is then released by monocytes and macrophages (Dinarello, 2018). Despite the similarities with IL-1β, IL-18 shows a different biology: differently to IL-1β, the IL-18 precursor can be found in healthy humans, especially in circulating blood monocytes and epithelial cells along the gastrointestinal tract as well as in peritoneal macrophages and spleen from mice (Puren, Fantuzzi and Dinarello, 1999).

In acute pancreatitis, serum IL-18 levels also correlate with the severity of the disease, being significantly higher in those patients suffering from severe acute pancreatitis (Rau *et al.,* 2001). Increased interleukin 18 levels in pancreas led to increased tissue damage after neutrophil activation and T cell differentiation (Li, Z. *et al.,* 2019).

1.4.2.1.4. Intracellular signaling pathways in acute pancreatitis

Intracellular signaling pathways play a key role in the maintenance of gene regulation and expression. In fact, dysregulation of gene expression has often been described as the pathological base of many inflammatory diseases such as pancreatitis, rheumatoid arthritis, hepatitis, psoriasis, and inflammatory bowel disease (Kalliolias and Ivashkiv, 2016). Said mechanisms are essential in the onset of the inflammatory response since the products whose formation they regulate include cytokines, chemokines and adhesion molecules which, in turn, will contribute to cell recruitment and migration to the damaged areas, largely amplifying in this way the initial inflammatory response (Kalliolias and Ivashkiv, 2016).

In eukaryotic cells it is well established that phosphorylation of intracellular protein factors is followed by specific gene transcription (Cramer, 2019). This mechanism is considered to be a universal mechanism of signal transduction, the most important elements in the signaling process being common among humans, plants, and fungi.

a) <u>Mitogen-activated protein kinases</u>

Mitogen-activated protein kinases (MAPKs) are the most ancient and well-conserved signaling pathways (Hotamisligil and Davis, 2016; Yeung *et al.,* 2018). Furthermore, they play a key role in the

development of the immune response. Once activated, MAPKs coordinate the recruitment of the gene transcription machinery, controlling crucial cellular processes such as protein synthesis, cell cycle, apoptosis as well as cell differentiation and proliferation (Kyriakis and Avruch, 2012). Since MAPKs activation is extremely fast in inflammatory processes, they have been considered as potential targets for the treatment of inflammatory disorders, as it is the case of acute pancreatitis (Pereda *et al.,* 2004; Samuel *et al.,* 2008).

There are three major MAPKs families in humans: extracellular signal-regulated kinase (ERK 1/2), c-jun N-terminal kinase (JNK) and p38 (Kim, E. K. and Choi, 2015).

MAPKs are activated upon phosphorylation by MAPKs kinases (MAPKKs) (**Figure 16**). MAPKs phosphorylation is reversible. Dephosphorylation of MAPKs by protein phosphatases leads to their inactivation (**Figure 16**). Since MAPKs regulation directly affects the expression of genes directly involved in immune response, protein phosphatases emerged as important mediators of the negative regulation of the cellular response (Hunter, T., 1995). It is interesting

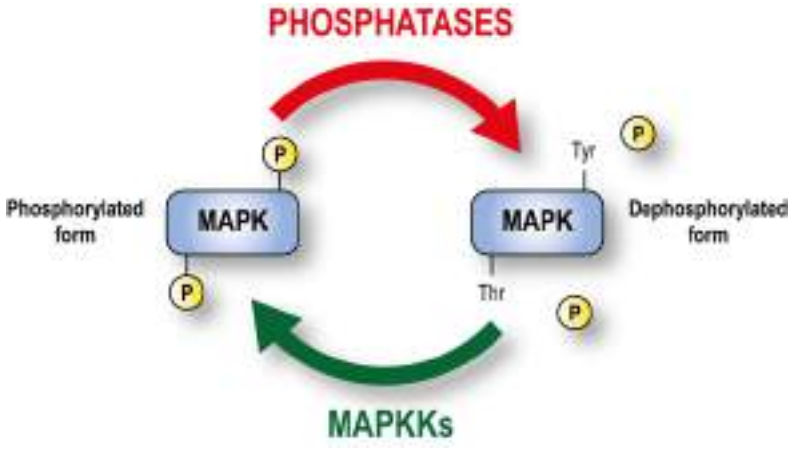

Figure 16. Regulation of MAPKs.

to note that there are around 518 protein kinases compared to only 140 protein phosphatases in mammalian cells (Arena, Benvenuti and Bardelli, 2005). Despite this fact, under normal conditions, dephosphorylation is much more active than phosphorylation (Arena, Benvenuti and Bardelli, 2005).

b) Protein phosphatases

Depending on the residue they dephosphorylate, protein phosphatases can be classified as protein serine/threonine phosphatases (PPPs) –which dephosphorylate serine and threonine residues– or protein tyrosine phosphatases (PTPs) –which dephosphorylate tyrosine residues (Alonso and Pulido, 2016; Meeusen and Janssens, 2018). A special type of protein tyrosine phosphatases known as dual specificity phosphatases (DSP) are able to dephosphorylate both tyrosine and threonine residues (Meeusen and Janssens, 2018). Sodium fluoride specifically inhibits serine/threonine phosphatases, whereas vanadate is the specific inhibitor of protein tyrosine phosphatases (Bertolotti, 2018).

b) 1. Serine/threonine phosphatases (PPPs)

Two different protein families comprise serine/threonine phosphatases: the PPP family that includes PP1, PP2A, and PP2B –also known as calcineurin–, and the PPM family that includes PP2C. PPPs are involved in key processes such as regulation of cell cycle as well as control of cell growth and division (Elgenaidi and Spiers, 2019).

b) 1.1. Type 1 protein phosphatases

Protein phosphatase 1 (PP1) dephosphorylates serine/threonine residues from selected substrates through its association with more than 200 PP1-interacting proteins (PIPs), achieving in this way high specificity and tight regulation of the dephosphorylation process (Ferreira *et al.,* 2019). It is known that PP1 is involved in numerous pathological processes such as cancer, cardiovascular diseases, memory loss, type 2 diabetes and even certain viral infections (Ferreira *et al.,* 2019).

b) 1.2. Type 2 protein phosphatases

Type 2 protein phosphatases (PP2), in contrast to PP1, preferably dephosphorylate the α subunit of phosphorylase kinase and do not respond to Inhibitor-1 and Inhibitor-2 (Bertolotti, 2018). The three different types of PP2 require different divalent cations in order to perform their function.

The PP2A family is comprised by a scaffolding subunit (A), a regulatory subunit (B), and a catalytic subunit (C) (Sangodkar *et al.,* 2016). Different isoforms show variations in the sequence of their subunits (Sangodkar *et al.,* 2016). Subunit A is a member of the Huntington elongation-A-subunit TOR (HEAT) protein family and regulates formation of the enzymatic complex (Sangodkar *et al.,* 2016). When subunit A binds subunit C, the catalytic activity is observed even in absence of subunit B (Sangodkar *et al.,* 2016).

Given the extraordinary amount of potential combinations among the different subunits that conform the protein, PP2A participates in the regulation of a wide range of physiologic and pathophysiologic

processes in mammals (Reynhout and Janssens, 2019). *In vivo* studies with PP2A knockout mice proved PP2A is essential for both male and female fertility since it is involved in the control of the meiotic process which renders gametes (Pan *et al.,* 2015; Tang *et al.,* 2016). Similar studies showed premature death and embryonic lethality in PP2Ac knockout mice due to deficient Wnt signaling and increased cytoplasmic E-cadherin and β-catenin levels, suggesting a possible role of PP2A in the stabilization within cell membranes of the E-cadherin/β-catenin complex, ensuring a correct distribution of these proteins (Götz *et al.,* 2000; Reynhout and Janssens, 2019). In the brain, PP2A was shown to regulate tau kinases CDK5 and GSK-3β, suggesting its role in the development of Alzheimer's disease (Reynhout and Janssens, 2019). Brainstems from mice deficient in PP2Ab showed absence of p35 –an activator of CDK5–, impaired CDK5 activity, and low levels of GSK-3β –which was phosphorylated at Ser 9–, leading to the accumulation of phosphorylated tau in the brainstem and spinal cord (Louis *et al.,* 2011). In the heart, K^+/Ca^{2+} pumps are regulated by PP2A and Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII) (Reynhout and Janssens, 2019). In the liver, specific PP2Ac knockout prevented liver injury, collagen deposition, and increase in serum levels of transaminases in a murine model of liver fibrosis through downregulation of transforming growth factor beta (TGF-β) and Smad pathway (Lu, N. *et al.,* 2015). In the gastrointestinal tract PP2A plays a role in the maintenance of the intestinal barrier function by preserving impermeability of the intestinal epithelium (Reynhout and Janssens, 2019). PP2Ac knockout mice showed undetectable mRNA levels of *Alpha4* –an integrin protein– in intestinal mucosa, as well as decreased levels of epithelial junction proteins –such as claudin-1, claudin-3, and E-cadherin–, leading to

abnormal structure and function of the intestinal tract, which appeared thinner and shorter than in wild type mice (Chung *et al.,* 2018). Furthermore, increased IKKα phosphorylation led to the degradation of HuR, a protein that, when overexpressed, it was shown to recover epithelial permeability (Chung *et al.,* 2018). Recent *in vivo* studies evidenced the tumor-suppressing role of PP2A since its deletion in mice led to an increased susceptibility to spontaneously develop diverse cancer types, such as hematologic disorders –mainly non-Hodgkin lymphoma–, and hepatocellular carcinomas correlated with increased phosphorylation of GSK3-β (Lambrecht *et al.,* 2018). In addition, PP2A generally displays antiapoptotic properties, although it regulates the phosphorylation of antiapoptotic proteins such as Bcl-2, as well as apoptotic ones, like FOXO I (Reynhout and Janssens, 2019). Interestingly, PP2A function may be compromised by ROS due to the oxidation of susceptible thiol groups in the active center of the enzyme (Clark and Ohlmeyer, 2019). In addition, PP2A is involved in the regulation of the inflammatory response through the modulation of NF-κB pathway, as well as MAPKs such as JNK and p38 (Clark and Ohlmeyer, 2019).

b) 2. Protein tyrosine phosphatases

Tyrosine phosphorylation is a key step in the regulation of signal transduction in numerous processes of eukaryote cells, including cell activation and progression, movement, structural changes, apoptosis, cell differentiation, and homeostasis (Alonso *et al.,* 2016). *In vivo*, tyrosine residue phosphorylation is dynamic and reversible, phosphorylation states being regulated by the opposite action of

protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Alonso *et al.,* 2016).

Reactive oxygen species act as second messengers in a plethora of cellular processes such as MAPKs signaling cascades and gene expression, as well as in the regulation of cell proliferation and senescence (Holmström and Finkel, 2014). In this regard, oxidative stress modulates tyrosine phosphorylation too (Chiarugi, 2005). Transient PTP-thiol oxidation leads to inactivation of these enzymes through the formation of either intramolecular disulfide bonds or sulfonamides (Chiarugi, 2005). In turn, PTK-oxidation leads to their activation either directly by thiol modifications or indirectly by PTPs inhibition (Chiarugi, 2005) .

Protein tyrosine phosphatase 1B (PTP1B) has been recently found to be modulated by persulfidation, which in turn might have a protective role against protein overoxidation upon oxidative stress (Dóka *et al.,* 2020). Interestingly, TRP14 is able to reduce persulfidated proteins (Dóka *et al.,* 2016).

Another phosphatase that is worth mentioning due to its importance in the regulation of the immune response is protein tyrosine phosphatase CD45, which is abundantly present in T lymphocytes and susceptible to inactivation by oxidation (Rheinländer, Schraven and Bommhardt, 2018).

c) Protein phosphatases in acute pancreatitis

Protein phosphatases play an extremely complex role in the development of acute pancreatitis. Stimulation of MAPKs phosphatases (MKPs) and PTPs, like SHP-1 and SHP-2, occurs in

the early stages of acute pancreatitis (Höfken *et al.,* 2000). On the other hand, PTP inhibition mediates pancreatic edema formation during the course of AP (Schnekenburger *et al.,* 2005). In this regard, PTP1B knockout mice showed exacerbated inflammatory response together with increased MAPKs activation and endoplasmic reticulum stress in comparison to wild type mice upon cerulein-induced acute pancreatitis (Bettaieb *et al.,* 2016). In a similar fashion, CD45 blockade induced *Tnf-*α expression, enhancing the inflammatory response in acute pancreatitis (De Dios *et al.,* 2005). Pentoxifylline treatment during the early stages of acute pancreatitis prevented the loss of pancreatic PP2A activity, leading to decreased expression of proinflammatory genes (Sandoval *et al.,* 2009) and its administration decreased intensive care unit admission and overall length of the hospital stay (Vege *et al.,* 2015).

Increased Ca^{2+} levels activate PP2B (calcineurin) after exposure to biliary salts, which may contribute to the initial pancreatic damage in acute pancreatitis (Muili *et al.,* 2013). In fact, increased intraductal pressure led to Ca^{2+} dysregulation, causing calcineurin activation which in turn triggered disruption of tight junctions, contributing in this way to pancreatic damage, thus suggesting calcineurin inhibitors may be useful in preventing acute pancreatitis secondary to duct obstruction (Wen *et al.,* 2018).

1.4.2.1.5. Oxidative stress in acute pancreatitis

During last decades, numerous studies related the pancreatic damage observed in acute pancreatitis with oxidative stress. In the 1980s, Sanfey and coworkers showed that antioxidant pre-treatment with superoxide

dismutase or catalase decreased serum amylase levels, edema, and weight increment of the pancreas during acute pancreatitis, indirectly suggesting the involvement of free radicals in the disease (Sanfey, Bulkley and Cameron, 1984; Sanfey, Bulkley and Cameron, 1985).

During the following years, oxidative stress became a possible explanation of both the triggering cause of the disease and the reason why pancreatitis evolves towards the severe form of the disease (Pérez *et al.,* 2015). Xanthine oxidase was set under the spotlight, since it may be one of the most important free radical sources in living organisms. Interestingly, *in vitro* studies showed that trypsin is able to convert xanthine dehydrogenase (XDH) into xanthine oxidase (XO) by proteolysis (Sanfey, Bulkley and Cameron, 1985). Furthermore, free radicals themselves possess chemoattractant properties that favor neutrophil migration to the pancreas, amplifying in this way the inflammatory cascade (Gukovskaya *et al.,* 2002). At the same time, free radicals produced by the infiltrated neutrophils may lead to trypsinogen activation due to the action of NADPH oxidase, closing the loop of events that would lead to destruction of the pancreatic gland (Gukovskaya *et al.,* 2002).

Different research groups have agreed on the key role that xanthine oxidase plays in the initial stages of acute pancreatitis (Sevillano *et al.,* 2003; Escobar *et al.,* 2010) as well as in the development of the systemic inflammatory response associated to the disease (Rau *et al.,* 2000; Que *et al.,* 2010; Zhou, J. *et al.,* 2016).

In situ free radical generation during the early course of acute pancreatitis was demonstrated by Telek *et al.* in rats and in humans using cerium as a free-radical sequestering agent (Telek *et al.,* 1999; Telek *et al.,* 2001). The source of reactive oxygen species may differ according to the experimental

model of acute pancreatitis. In mild acute pancreatitis induced by cerulein, free radicals seem to be mainly generated by the infiltration of activated neutrophils into the pancreatic tissue, whereas in necrotizing acute pancreatitis induced by taurocholate, the main cause of ROS production would be the conversion of XDH into XO (Closa *et al.,* 1994) .

Interestingly, a common feature observed in all experimental models of acute pancreatitis –including mild pancreatitis induced by cerulein or L-arginine, as well as severe pancreatitis induced by taurocholate– is the marked decrease in GSH levels (Pérez *et al.,* 2015). Low GSH levels are maintained during the early course of acute pancreatitis, even before pancreatic edema develops (Pérez *et al.,* 2015). It was suggested that this GSH depletion not only leads to the loss of the antioxidant defense capacity, but also to cytoskeleton and organelle membrane damage, favoring early activation of digestive enzymes within acinar cells, triggering the inflammatory process (Schulz *et al.,* 1999).

GSH depletion can be prevented by pretreating animals with GSH precursors such as *N*-acetylcysteine as well as pentoxifylline, thus decreasing pancreatic damage (Escobar *et al.,* 2012). However, a clinical trial involving 79 patients suffering from acute pancreatitis who were treated with a combination of *S*-adenosylmethionine and *N*-acetylcysteine did not show beneficial effects (Sharer *et al.,* 1995).

Apart from GSH levels, many other parameters reflecting oxidative stress are altered during the course of acute pancreatitis such as antioxidant enzymes, oxidized lipids, carbonylated proteins, or myeloperoxidase (MPO), clearly indicating that AP and oxidative stress are tightly related (Park *et al.,* 2003; Winterbourn *et al.,* 2003; Hernández *et al.,* 2011; Pérez *et al.,* 2015). In fact, the severity of the disease has been long correlated with the

concentration of some of these parameters such as MPO, protein carbonylation, thiobarbituric acid-reactive substances, and ascorbic acid levels (Abu-Zidan, Bonham and Windsor, 2000).

On the other hand, reactive nitrogen species (RNS) can relate as well with the normal functioning of the pancreas and they may be dysregulated during pathological processes affecting the pancreatic gland (Leung and Chan, 2009; Hegyi and Rakonczay, 2011; Lenzen, 2017). In this regard, nitric oxide (NO) participates in the regulation of the normal secretion processes of both endocrine and exocrine pancreas, as well as controlling pancreatic blood flow (Hegyi and Rakonczay, 2011). During inflammatory processes, inducible nitric oxide synthase (iNOS) contributes to the formation of large amounts of nitric oxide, as it is the case of severe AP (Tanjoh *et al.,* 2007). Moderate NO production may be beneficial in experimental edematous AP; however, uncontrolled overproduction of NO may be detrimental (Ozturk *et al.,* 2008). NO synthesis and the different isoforms of nitric oxide synthase in pancreas and lungs correlated with the severity of AP, suggesting a different role for each nitric oxide synthase isoform in the development of the disease (Ang *et al.,* 2009).

During the last decades, oxidative stress emerged as an important player in cell signaling (D'Autréaux and Toledano, 2007). Redox unbalance is not only responsible for oxidative damage, but it also mediates cell signaling involving inflammatory processes such as the regulation of proinflammatory genes (Leung and Chan, 2009). In fact, ROS may act as inflammatory mediators through leucocyte activation, migration, and adhesion, as well as increasing the expression of other well-known mediators like cytokines, chemokines, and adhesion molecules (Pereda *et al.,* 2006). In pancreatic cells, ROS induced NF-κB activation (Algül *et al.,* 2002). In addition, reactive oxygen species generated by xanthine oxidase during AP induced the

expression of P-selectin in the lung, which mediates neutrophilic infiltration (Folch *et al.,* 2000).

In general, intracellular signaling modulation due to redox disbalance is produced by two different mechanisms. On the one hand, oxidant species such a hydrogen peroxide or α and β-unsaturated aldehydes act directly as second messengers in the inflammatory response (Pérez *et al.,* 2015). On the other hand, the redox status of the cell integrates intracellular signal transduction through reversible covalent modifications in redox sensors such as sulfhydryl groups present in proteins (Netto and Antunes, 2016). These thiol groups allow transient oxidation of some proteins, conferring them the ability to transmit intracellular signals (Netto and Antunes, 2016). Antioxidant enzymes would allow these proteins to return to their original reduced state (Netto and Antunes, 2016).

Hydrogen peroxide acts as a second messenger in the activation of NADPH oxidase and NF-κB (Li, Q. *et al.,* 2009). Interestingly, NADPH oxidase expression and activity increased in experimental acute pancreatitis (Chan and Leung, 2007).

Reactive oxygen species produced as a result of mitochondrial respiration take part as well in signaling and transduction of molecules related to proinflammatory cytokines production, this process being dependent or independent of the inflammasome pathway (Bulua *et al.,* 2011; Zhou, R. *et al.,* 2011). Reactive oxygen species also participate in apoptotic processes through the release of cytochrome c and caspase activation, playing a key role in the loss of mitochondrial membrane potential and cell death in pancreatic acinar cells (Odinokova *et al.,* 2009).

Oxidative stress promotes the activation of phosphorylation pathways, such as MAPKs, through the inactivation of protein phosphatases (Lee, K. and Esselman, 2002). Phosphatases act as molecular targets of ROS since their catalytic cysteine residues are more prone to reversible oxidation than other cysteine residues (Tonks, 2005). Under mild oxidative stress conditions, said cysteine residues become oxidized to sulfenic acid (reversible oxidation), whereas harsher oxidative stress conditions would lead to sulfinic and sulfonic acid formation (irreversible oxidation) (Jackson and Denu, 2001).

Consequently, reactive oxygen species and particularly those coming from mitochondrial respiration regulate the balance between MAKPs and some phosphatases, thus regulating the inflammatory response through redox signaling (Escobar *et al.,* 2010; Kim and Choi, 2015). Activation of these phosphorylation pathways, NF-κB, and other transcription factors promote changes in chromatin structure, especially in acetylation of histones that regulate inflammatory genes (Sandoval *et al.,* 2016). Histone acetyltransferase recruitment as well as inactivation of histone deacetylases are a complex processes that are modulated by the redox status of the cell, histone acetylases being the most affected oxidative stress targets within this context (Escobar, Pereda, López-Rodas *et al.,* 2012; Sandoval *et al.,* 2016).

Apart from the previously-mentioned signaling mechanisms triggered by oxidative stress, it is especially interesting to mention the emerging role of disulfide stress in the development of acute pancreatitis. In a model of acute necrotizing pancreatitis induced by sodium taurocholate in rats, increased levels of protein cysteinylation and γ-glutamylcysteinylation were observed in pancreas from rats with pancreatitis, which were accompanied by protein thiol oxidation in certain targets, but without changes in protein

glutathionylation levels (Moreno *et al.,* 2014). The increase in these parameters was explained by the oxidation of cysteine to cystine, as well as γ-glutamylcysteine to γ-glutamylcystine (Moreno *et al.,* 2014). Interestingly, GSH levels dropped over the course of acute pancreatitis, but its depletion was not due to oxidation (Moreno *et al.,* 2014). This type of oxidative stress specifically affected two types of targets: redox buffers, and thiols involved in redox signaling such as Trx1, Keap1, and serine/threonine protein phosphatases among others (Moreno *et al.,* 2014). Thus, disulfide stress plays a role in the regulation of redox signaling associated with acute inflammatory diseases, independently of the glutathione redox status (Moreno *et al.,* 2014).

It is then clear that diverse molecular mechanisms are involved in the development of both local and systemic effects of acute pancreatitis through the expression of inflammatory genes, as well as through the production of proinflammatory cytokines. In this regard, oxidative stress is a key modulator of cell signaling, and it plays a crucial role in the development of the inflammatory cascade. However, the role of oxidative stress is more complex than it seemed, and currently there are numerous ongoing studies aimed at clarifying the precise contribution of oxidative stress to the inflammatory response and tissue damage in acute pancreatitis to be able to design effective therapeutic strategies based on the treatment with antioxidants or redox tone modulators.

II. OBJECTIVES

2. OBJECTIVES

Based on the context described previously about the regulation of mixed disulfides, our aims in this Thesis were the following ones:

1. To determine if thioredoxin-related protein of 14 kDa (TRP14) regulates protein cysteinylation *in vitro* and *in vivo* and to identify its substrates.

2. To study the role of TRP14 and protein cysteinylation in the regulation of the inflammatory response in experimental acute pancreatitis as an *in vivo* model of acute inflammation associated with mixed disulfides.

III. MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Material

3.1.1. Cell lines

HEK-293 cells and TRP14 knockdown cells were used for the development of this Thesis. TRP14 stable knockdown was performed on HEK-293 cells due to their well-reported high-efficient transfection efficiency. TRP14 knockdown cells were developed and kindly provided by Professor Elias Arnér from the Department of Medicinal Biochemistry and Biophysics from the Karolinska Institutet, Stockholm (Sweden).

Cell culture and *in vitro* experiments were performed at the section for Cell Culture and Flow Cytometry from the Central Service for Experimental Research (SCSIE) from the Universitat de València, as well as at Prof. Arnér's laboratory at Biomedicum, Karolinska Institutet.

Both parental HEK-293 and TRP14 knockdown cells were cultured in Eagle's minimum essential medium (EMEM) (Gibco, Massachusetts, USA), supplemented with 10% fetal bovine serum (Gibco, Massachusetts, USA), 100 U/mL penicillin (Gibco, Massachusetts, USA), 100 mg/mL streptomycin (Gibco, Massachusetts, USA), and 50 nM sodium selenite (Sigma-Aldrich, Missouri, USA). Cells were incubated at 37°C in humidified air containing 5% CO_2.

3.1.2. Animals

C57BL/6 *Txndc17* wild type mice encoding TRP14 as well as *Txndc17* null mice were kindly provided by Professor Ed Schmidt from the Department of Microbiology and Immunology from the Montana State University, Bozeman (Montana, USA). **Figure 17** depicts *Txndc17* wild type, floxed, and null alleles. The action of Cre recombinase on loxP sites results in loss of all exons, giving rise to the *Txndc17* null allele (**Figure 17**).

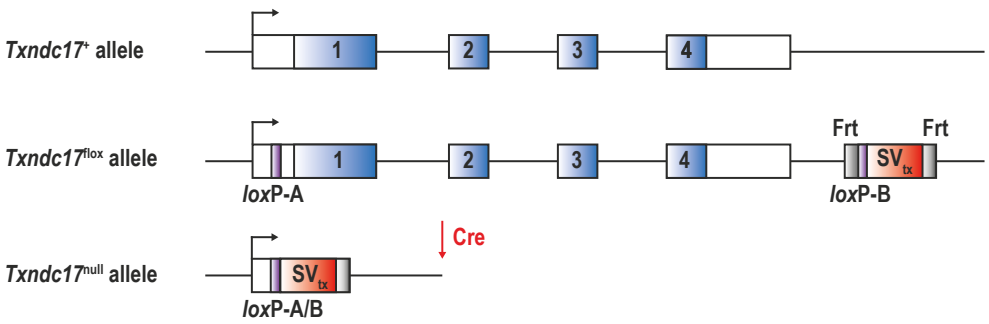

Figure 17. Schematic representation of the Txndc17 conditional-null (flox) allele. Wild type (*Txndc17*$^{+}$), floxed (*Txndc17*^{flox}), and null (*Txndc17*^{null}) *Txndx17* alleles are depicted showing the exons as blue boxes (1-4) and *lox*P sites marked in purple.

Animals were housed at a temperature of 23±1ºC, 60% relative humidity, and constant 12h/12h light/darkness cycles at the animal housing facilities from the Central Service for Experimental Research (SCSIE) located at the Faculty of Pharmacy from in the Universitat de València. Animals were fed a standard chow diet and water *ad libitum*.

All procedures and experiments were performed in compliance with the regulations of the European Parliament and of the Council of 22 September

2010 on the protection of animals used for scientific purposes (Directive 2010/63/EU), incorporated into the Spanish law by the *Real Decreto* 53/2013 (RD 53/2013) from the 1st February 2013, which established the basic guidelines that should be applied for the protection of the animals used in experimental research and with other scientific aims. The experimental protocols were approved by the Ethics Committee for Animal Welfare from the Universitat de València.

3.1.3. Apparatus

Scales:

- An analytical grade *Acculab ATILON* scale from Sartorius (Göttingen, Germany) was used for weighing compounds with a sensitivity of ± 0.0001 g.

- Additionally, a Sartorius *PT 1200* scale (Göttingen, Germany) with a sensitivity of ± 0.1 g was used.

pH-meter:

- The pH of the different buffers and solutions used for the development of this Thesis was adjusted using a Crison *BASIC 20* pH-meter (Barcelona, Spain).

Centrifuges:

- An Eppendorf *5424 R* centrifuge (Hamburg, Germany) was used for spinning samples.

- Additionally, a Bio-Rad *Mini Centrifuge* (California, USA) was used for pulse-spinning samples.

Homogenizer:

- Tissue samples were homogenized using an *RZR 2021* homogenizer from Heidolph Instruments (Schwabach, Germany).

Dry bath incubator:

- An AccuBlock D1100 from LabNet (New Jersey, USA) equipped with an orbital shaking system was used.

Electrophoresis:

- *PowerPac™ Basic Power Supply* from Bio-Rad (California, USA).

- Different electrophoresis chambers were used according to the gel size: *Mini-PROTEAN® Tetra Vertical Electrophoresis Cell* and *Criterion™ Vertical Electrophoresis Cell* from Bio-Rad (California, USA), as well as a *Mini Gel Tank* from Invitrogen (California, USA).

Blotting system:

- Electrotansfer was performed using a *Trans-Blot® Turbo™ Transfer System* from Bio-Rad (California, USA).

Gel Imaging system:

- Western Blot membranes were developed using a *ChemiDoc® XRS+ System* from Bio-Rad (California, USA). Gel fluorescence images were obtained using the same equipment.

Spectrophotometer:

- For end-point measurements, a *Multiskan Spectrum® Microplate Spectrophotometer* from Thermo Fisher (Massachusetts, USA) was used.

- For kinetic measurements, a *TECAN Infinity® 200 PRO* plate reader (Männedorf, Switzerland) was used.

Thermal cycler:

- For reverse transcription and qualitative PCR, a *C-1000 Thermal Cycler* from Bio-Rad (California, USA) was used.

- For real-time quantitative PCR, an *iCycler iQ™ Real-Time PCR Detection System* from Bio-Rad (California, USA) was used.

Cell culture:

- Cell culture was performed under strict sterile conditions in a class II microbiological safety cabinet *Telstar Bio II Advance Plus* (Barcelona, Spain) at the Cell Culture and Flow Cytometry Section from the Central Service for Experimental Research (SCSIE) in the Universitat de València.

- Cell cultures were kept in *Forma™ Steri-Cycle™* CO_2 Incubators (Thermo Fisher, Massachusetts, USA) at the Cell Culture and Flow Cytometry Section from the Central Service for Experimental Research (SCSIE) in the Universitat de València.

Mass spectrometry coupled to high performance liquid chromatography (HPLC-MS/MS):

- Tandem quadrupole (triple quadrupole) mass spectrometer *Xevo TQD* (Waters, Massachusetts, USA) at the Mass Spectrometry Section from the SCSIE in the Universitat de València.

- For proteomic analysis, the nanoESI-qQTOF mass spectrometer *5600 TripleTOF* from ABSCIEX (Massachusetts, USA) at the Proteomics Section from SCSIE in the Universitat de València was used.

Microscope:

- Leica *DM4 B* optical microscope (Leica, Wetzlar, Germany) equipped with a *FLEXACAM C1* microscope camera (Leica, Wetzlar, Germany). High-resolution images were acquired and processed using *Leica Application Suite X* (Leica, Wetzlar, Germany).

3.2. METHODS

3.2.1. *In vitro* methods

3.2.1.1. Generation of stably-transfected HEK-293 cells

TRP14 knockdown cells were developed at Professor Elias Arnér's laboratory at the Karolinska Institutet, Stockholm (Sweden).

HEK293 cells were purchased from the American Type Culture Collection (ATCC, Virginia, USA). Cells were cultured in Eagle's minimum essential medium (EMEM) (ATCC, Virgina, USA), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada), 100 U/mL penicillin and 100 mg/mL streptomycin (Biochrom AG, Berlin, Germany). Cells were incubated at 37°C in humidified air containing 5% CO_2.

TRP14 knockdown was performed in HEK293 cells using shRNA-bearing plasmid KH19389H (human *TXNDC17*) (Qiagen, Hilden, Germany). A negative control shRNA plasmid was included. HEK293 cells were seeded for transfection in six-well plates at a density of 4 x10^5 cells per well and they were incubated for 24 h before transfection. 0.6 mg DNA of each plasmid were transfected using 1.2 mL per well of TurboFect™ (Thermo Scientific, Massachusetts, USA). Selection of transfected cells was performed by adding 0.1 mg/mL Hygromycin B (Invitrogen, California, USA) to the culture medium at 72 h after transfection. Populations of stable transfected cells were generated three weeks after transfection by selecting single resistant cell clones. Freezing stocks were generated at this stage. The knockdown was confirmed in all isolated clones by Western Blot using an antibody against TRP14 (R&D Systems, Minnesota, USA), and GAPDH (Santa Cruz

Biotechnology, Texas, USA) as loading control. After knockdown confirmation, Hygromycin B was removed from the growth medium without affecting the knockdown over several passages.

3.2.1.2. Recombinant protein expression

Recombinant protein expression and purification was carried out in the laboratory of Professor Elias Arnér at the Karolinska Institutet, Stockholm (Sweden) by Dr. Qing Cheng. The protocols for the expression and purification of the recombinant proteins used in this Thesis are detailed below (Pader *et al.,* 2014; Dóka *et al.,* 2016; Cheng, Q. and Arner, 2017; Cheng, Q. and Arner, 2018; Dóka *et al.,* 2020).

a) Human TRP14 expression and purification

The open reading frame (ORF) of a cDNA clone (imaGenes) encoding human TRP14 was amplified by PCR using the forward primer (5'GC**CATATG**GCCCGCTATGAGGAGGTGAGC-3') containing a NdeI site (underlined) and the start codon (red) in combination with the reverse primer (5'CGC**GGATCCTTA**ATCTTCAGAGAACA ACATTTCC-3'), which contains a BamHI site (underlined) and a stop codon (red). The PCR product was ligated into a pET20b expression vector (Invitrogen, California, USA) and an *N*-terminal His-tag was introduced.

The plasmid containing the final sequence was transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) competent cells (Invitrogen, California, USA), and the ORF was confirmed by DNA sequencing (GATC Biotech, Solna, Sweden).

Transformed *E. coli* BL21 (DE3) cells were cultured at 37°C in Lisogeny Broth (LB) medium supplemented with 0.1 mg/mL ampicillin and grown until the optical density at 600 nm (OD_{600}) was around 0.6. At that point, 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) was used to induce protein expression, and cells were cultured for 12 h at 18°C. Cells were harvested and lysed in purification buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, pH 7.5) containing 25 mM imidazole. Nucleic acids present in the lysate were removed with DNase (Roche, Basel, Switzerland) while cell debris were removed by centrifugation at 34500 x *g* for 30 min. The resulting lysates were filtered through 0.2 µM pore diameter filters and His-tagged proteins were purified using Ni Sepharose® Fast Flow beads or HisTrap™ columns (GE Healthcare, Illinois, USA) following the manufacturer's instructions. During the purification process, absorbance was followed at 280 nm in order to estimate protein concentration, and the eluted fractions were analyzed by SDS/PAGE electrophoresis (Invitrogen, California, USA) to check for purity upon Coomassie staining. If needed, protein fractions were further purified by size-exclusion chromatography (Superdex® G200, GE Healthcare, Illinois, USA). Since the activity of TRP14 was not affected by the His-tag, it was not removed.

b) <u>Human Trx1 expression and purification</u>

Human Trx1 was expressed in *E. coli* Stellar strain (Clontech, Shiga, Japan). The coding region of Trx1 was codon-optimized for protein expression in *E. coli* and the resulting sequence was synthesized (DNA2.0, California, USA). To facilitate the purification process, a His-tag was introduced at the *N*-terminal end of Trx1, followed by a tobacco etch virus (TEV) protease recognition site.

After bacterial transformation, the protein was expressed as described above. Trx1 was purified by immobilized metal affinity chromatography (IMAC). The His-tag was removed using TEV protease. In order to separate the nontagged protein from the rest of the mixture, a counter-IMAC was performed. The resulting purified protein was concentrated using a Centriprep™ Centrifugal Filter Unit with Ultracel® 3 kDa membrane (Millipore, Massachusetts, USA) and resuspended in TE buffer (50 mM Tris, 1 mM EDTA). Final protein concentration was calculated using UV absorbance at 280 nm and the molar extinction coefficient of Trx1 (ε = 6970 M^{-1} cm^{-1}).

c) <u>Human TrxR1 expression and purification</u>

Human TrxR1 was expressed in *E. coli* BL21 (DE3) gor^- strains cotransformed with $pETTRS_{TER}$ and pSUABC plasmids. After checking for proper transformation, 25 mL of LB medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol was inoculated with the transformed bacteria and let grow overnight at 37ºC. Then, a 500-mL culture was inoculated with the previous overnight culture and incubated at 37ºC. OD_{600} was followed with time and when the late exponential phase was reached, the culture was cooled down to room temperature and protein expression was induced by addition of 1µM L-cysteine, 5 nM selenite and 0.5 mM IPTG. Subsequently, the culture was incubated overnight at room temperature under shaking (around 150 turns/min). Cells were harvested, resuspended, treated with lysozyme (0.5 mg/mL), and centrifuged again to remove cell debris.

Affinity chromatography was used to purify TrxR1 from 2'5'-ADP Sepharose® (GE Healthcare, Illinois, USA). The purification process was followed by size exclusion chromatography using a Superdex®

G200 column equipped on an ÄKTAexplorer 100 workstation (GE Healthcare, Illinois, USA). The resulting purified TrxR1 was concentrated using a Centriprep™ Centrifugal Filter Unit with Ultracel® 30 kDa membrane (Millipore, Massachusetts, USA). Final protein concentration was calculated by measuring flavin adenine dinucleotide absorbance at 463 nm (ε = 11,300 M^{-1} cm^{-1}).

d) <u>Human Prx2 expression and purification</u>

To induce human Prx2 (hPrx2) expression in *E. coli*, the plasmid pET53-hPrx2 (*N*-terminal His tag and *C*-terminal StrepII tag) was constructed using Gateway cloning technology (Thermo Fisher, Massachusetts, USA) with the following primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCTCCGGTAAC GCGCGCATCGGAA-3' and 5'-GGGGACCACTTTGTACAAGA AAGCTGGGTGATTGTGTTTGGAGAAATATTCCTTG-3'), pET53-DEST (Millipore, Massachusetts, USA), and a cDNA library generated from HEK293 cells.

To obtain and purify recombinant hPrx2, the *E. coli* strain T7 Express (New England Biolabs, Massachusetts, USA) transformed with pET53-hPrx2 was grown at 37°C in LB medium containing 100 µg/mL carbenicillin. When the OD_{600} value was 0.6, protein synthesis was induced by adding 0.2 mM IPTG to the bacterial culture. Cells were harvested after 18 hours of incubation and resuspended in ice-cold buffer A containing 50 mM phosphate, 300 mM NaCl, 10 mM imidazole, 1 mM dithiotreitol (DTT), and protease inhibitor (pH 8.0). Cell suspension was sonicated on ice to lyse the cells. After centrifugation, the supernatant was loaded onto Ni-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) in order to purify the protein.

Recombinant hPrx2 was eluted from the column with ice-cold buffer A containing this time a higher imidazole concentration (250 mM). After diluting the sample three times with ice-cold buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT (pH 8.0), the sample was loaded onto a Strep-Tactin® Sepharose® column (IBA Lifesciences, Goettingen, Germany), and Prx2 was eluted from the column with ice-cold buffer B containing 2.5 mM desthiobiotin (IBA Lifesciences, Goettingen, Germany). Purified Prx2 was desalted by using a Sephadex® G-25 PD-10 column (GE Healthcare, Illinois, USA) and then resuspended in 50 mM tris-HCl buffer.

3.2.1.3. Identification and quantification of cysteinylated proteins in TRP14 knockdown cells

The identification and quantification of protein cysteinylation targets was performed *in vitro* in TRP14 knockdown cells incubated with biotinylated cysteine followed by streptavidin affinity purification and MS/MS identification and quantitation.

a) Cell culture

Cells ($4x10^6$) were seeded in T-75 Falcon® culture flasks (Corning, Massachusetts, USA) containing 10 mL of fresh medium (EMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 50 nM sodium selenite).

Two different cell lines were used:

- Control cells (NC-2): HEK-293 parental cells.

- TRP14 knockdown cells (TRP14-20): HEK-293 with stable TRP14 knockdown.

Experiments were performed at 24 hours after seeding.

b) Biotinylated cysteine incubation and lysate obtention

Biotinylated cysteine (Cys-BIO) was kindly provided by Dr. Antonio Martínez-Ruíz, from the Unidad de Investigación of the Hospital Universitario La Princesa, Instituto de Investigación Sanitaria Princesa, Madrid (Spain).

1. Cells were incubated with 250 μM Cys-BIO for 1 h.

2. The incubation medium was removed.

3. Cells were washed with ice-cold phosphate-buffered saline (PBS) and collected in 300 μL of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 1% Triton, 10 mM N-ethylmaleimide (NEM), and a protease inhibitor cocktail (Sigma-Aldrich, Missouri, USA).

4. In order to lyse the cells, three freezing-thawing cycles were performed.

5. Cell lysates were centrifugated at 3500 rpm for 10 min and supernatants were collected for analysis.

c) Streptavidin pulldown

Pierce™ Monomeric Avidin Agarose Kit (Thermo Scientific, Massachusetts, USA) was used to purify cysteinylated proteins tagged with biotin. Different columns were used to purify samples from

different experimental groups (NC-2 + Cys-BIO, TRP14-20 + Cys-BIO, and TRP14-20 + Cys) to avoid potential interferences among samples. The following protocol was used:

1. After column stabilization, samples (2 mL) were loaded into the column and incubated at room temperature for 1 hour.

2. Columns were washed six times with PBS (6x2 mL).

3. To elute bound biotinylated molecules, 0.1M glycine at pH 2.8 was loaded into the column. A total of 6 fractions (6x2 mL) were eluted from the columns.

4. Western Blot was performed at non-reducing conditions using streptavidin-horseradish peroxidase (streptavidin-HRP, Cell Signaling Technology, Massachusetts, USA) to detect cysteinylated proteins, and subsequently fraction #2 of each sample was selected for proteomic analysis due to the highest content in cysteinylated proteins of these fractions in all groups.

Performing the whole process under non-reducing conditions was required for preserving cysteinylated proteins in these samples. The presence of reducing agents such as DTT would lead to the reduction of the mixed disulfides present in the samples, resulting in loss of the biotin tag.

d) <u>Proteomic analysis</u>

The proteomic analysis was carried out at the Proteomics Section of the Central Service for Experimental Research from the Universitat de València.

d) 1. Sample preparation

14 µg of every sample obtained by the streptavidin pulldown were dried in a rotatory evaporator and loaded in a 1D PAGE gel in order to clean and concentrate the samples.

d) 2. In-gel processing

Samples were in-gel reduced with DTT and *S*-alkylated with iodoacetamide (Sigma-Aldrich, Missouri, USA) as previously described (Shevchenko *et al.,* 2006). Then, they were digested with 100 ng sequencing-grade trypsin (Promega, Madison, USA). The digestion mixture was dried in a vacuum centrifuge and re-suspended in 20 µL of 2% acetonitrile, 0.1% trifluoroacetic acid.

d) 3. Liquid chromatography and tandem mass spectrometry

1. 5 µL of sample were loaded onto a trap column (NanoLC Column, 3µ C18-CL, 350 µm x 0.5 mm; Eksigent, Massachusetts, USA) and desalted with 0.1% trifluoroacetic acid at 2 µL/min during 10 min.

2. Desalted samples were then loaded into an analytical column (LC Column, 3C18-CL-120, 3 µ, 120 Å 75 µm x 15 cm, Eksigent, Massachusetts, USA) equilibrated with 5% acetonitrile 0.1% formic acid. Elution was carried out with a linear gradient of 5 to

35% B in A for 60 min (A: 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min.

3. Eluted samples were analyzed in a mass spectrometer (5600 TripleTOF, ABSCIEX, Massachusetts, USA). Samples were ionized applying 2.8 kV to the spray emitter. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350-1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired at 100-1500 m/z for 50 ms in the high sensitivity mode. The following switch criteria were used: charge: 2+ to 5+; minimum intensity: 70 counts per second. Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled using 2 fmol of 6 different proteins (LC Packings, California, USA).

d) 4. ProteinPilot v5.0 search engine

ProteinPilot default parameters were used to generate a peak list directly from 5600 TripleTof wiff files. The Paragon algorithm was used to search the SwisProt database with the following parameters: trypsin specificity and cys-alkylation with taxonomy restricted to human, and false discovery rate (FDR) correction for proteins.

Protein grouping was performed using the Pro Group algorithm, which considers protein sets sharing physical evidence. Unlike sequence alignment analyses were performed when full length theoretical sequences were available, the formation of protein groups in Pro Group was guided only and entirely by observed peptides. Since the

observed peptides were actually determined from experimentally acquired spectra, the grouping could be considered to be guided by the spectra.

d) 5. Differential expression analysis

All eluted samples from all experiments were combined to create a spectra library for differential expression analysis. A total of 1441 proteins were identified with 95% confidence and FDR lower than 1%.

Peak View 1.1. (Sciex, Massachusetts, USA) was used to quantify the areas for all the peptides assigned in the library and the computed areas for proteins.

Dimensionality reduction, principal component analysis and discriminant analysis (both with Pareto scaling) were carried out for the different experimental groups.

A multiple T-test statistical analysis was performed to determine differentially-expressed proteins with P-value < 0.05.

3.2.1.4. Cysteinylation of recombinant Prx2

We developed an *in vitro* method in the laboratory of Professor Elias Arnér at the Karolinska Institutet, Stockholm (Sweden), for cysteinylating recombinant human Prx2. Protein cysteinylation was achieved *in vitro* upon incubation of purified hPrx2 with BODIPY® FL L-Cystine (Thermo Fisher, Massachusetts, USA).

a) Principle

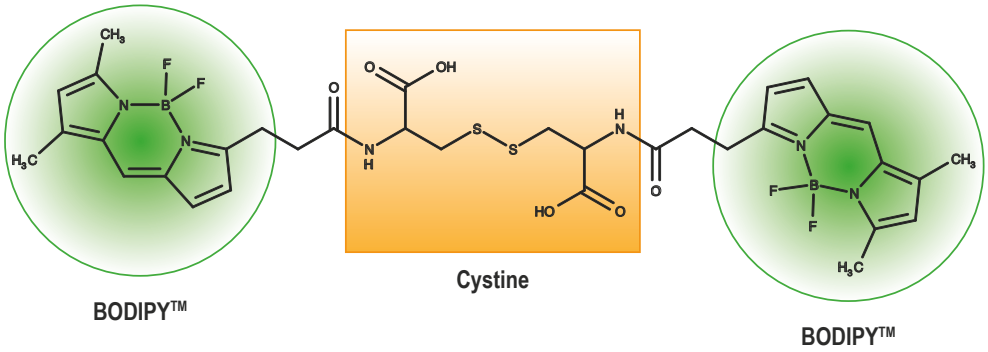

Figure 18. Chemical structure of BODIPY® FL L-Cystine.

BODIPY® FL L-Cystine consists on a L-cystine molecule with two BODIPY® FL fluorophores attached to the amino groups of the amino acid (**Figure 18**). In this form, BODIPY® FL L-Cystine is non-fluorescent due to the proximity of both fluorophores. However, due to the presence of a disulfide bond in the L-cystine structure, it can react with free thiols in order to form a mixed disulfide. In that case, the resulting mixed disulfide becomes fluorescently labelled since the BODIPY® FL moiety would be no longer quenched.

b) Sample preparation

hPrx2 samples were pre-reduced with 1 mM DTT for 30 min at room temperature and then desalted using a Zeba™ Spin Desalting Column, 40 K MWKO, 0.5 mL (Thermo Fisher, Massachusetts, USA).

c) Procedure

Pre-reduced, desalted protein samples were incubated with 100 µM BODIPY® FL L-Cystine overnight at 4ºC and protected from light. After

incubation, the remaining non-bound BODIPY® FL L-Cystine was removed by desalting the samples with Zeba™ Spin Desalting Columns, 40 K MWKO, 0.5 mL.

Protein samples were loaded into a gel (Novex™ 4-20% Tris-Glycine, Invitrogen, California, USA) using non-reducing loading buffer (NuPAGE™LDS Sample Buffer, Invitrogen, California, USA) in order to conserve the mixed disulfide formed in the previous step. Electrophoretic separation was performed at a constant voltage of 200 V for 20-25 min while the gel was protected from light.

The gel containing cysteinylated proteins was developed in a UV transilluminator (ChemiDoc™ XRS+ Imaging System, Bio-Rad, California, USA) and Coomassie staining was later performed as loading control.

3.2.1.5. Protein de-cysteinylation assays

Different approaches developed in the laboratory of Professor Elias Arnér at the Karolinska Institutet, Stockholm (Sweden), were used in this Thesis to study the ability of Trx1 and TRP14 to de-cysteinylate cysteinylated proteins by reduction of the disulfide bond of this mixed disulfide. According to the substrate used in the de-cysteinylation reaction, we can divide these assays into two groups: on the one hand, assays using cell lysates from TRP14 knockdown cells incubated with biotinylated cysteine, and on the other hand assays using cysteinylated purified hPrx2 that was obtained *in vitro* upon BODIPY® FL L-Cystine incubation.

a) De-cysteinylation assays using cell lysates from cultures incubated with biotinylated cysteine as substrate

Cell lysates containing cysteinylated proteins were incubated at room temperature with 20 µM TRP14, 1 µM TrxR1, and 1 mM NADPH in TE buffer (50 mM Tris, 1 mM EDTA). Different combinations of the components of the enzymatic system were tested, as well as different incubation times (0-120 min). After the different incubation times, non-reducing loading buffer (NuPAGE™ LDS Sample Buffer, Invitrogen, California, USA) was added to the samples and they were heated at 95ºC for 5 min to perform a Western Blot.

b) De-cysteinylation assays using recombinant hPrx2 incubated with BODIPY® FL L-Cystine as substrate

Cysteinylated Prx2 was incubated at room temperature with the Trx1 enzymatic system (10 µM Trx1, 30 nM TrxR1, and 300 mM NADPH) or the TRP14 enzymatic system (10 µM TRP14, 30 nM TrxR1, and 300 mM NADPH) in TE buffer (50 mM Tris, 1 mM EDTA). Different Trx1 and TRP14 concentrations, incubation times, and combinations of the components of each reaction system were tested. After the incubation, non-reducing loading buffer (NuPAGE™LDS Sample Buffer, Invitrogen, California, USA) was added to samples and they were heated at 95 ºC for 5 min before loading them into a gel (Novex™ 4-20% Tris-Glycine, Invitrogen, California, USA) to perform electrophoresis. Electrophoretic separation was performed at a constant voltage of 200 V for 20-25 min while the gel was protected from light. The gel was developed in a UV transilluminator

(ChemiDoc™ XRS+ Imaging System, Bio-Rad, California, USA) and Coomassie staining was later performed as a loading control.

3.2.1.6. Prx2 activity assay

Prx2 activity was assessed monitoring H_2O_2 clearance from the reaction medium in the presence of the thioredoxin system using the ferrous oxidation-xylenol orange (FOX) assay.

The FOX assay (Jiang, Hunt and Wolff, 1992) is based on the rapid oxidation of Fe^{2+} to Fe^{3+} in acidic media in presence of peroxides. Fe^{3+} produced in the previous step reacts with xylenol orange forming a complex that can be spectrophotometrically measured at 560 nm. Thus, absorbance at 560 nm can be correlated with peroxide concentration in the sample.

The FOX reagent has the following composition:

- 100 µM xylenol orange
- 250 µM ferrous ammonium sulfate
- 100 mM sorbitol
- 25 mM sulfuric acid

a) <u>Sample preparation</u>

Two different reaction mixtures were prepared for each sample in order to assess hPrx2 and cysteinylated hPrx2 activity: 1 µM Prx2 or

1 µM Prx2-S-SCys (cysteinylated Prx2), 1 µM Trx1, 2 nM TrxR1, 300 nM NADPH, and 100 mM H_2O_2 in TE buffer (50 mM Tris, 1 mM EDTA).

b) Assay procedure

At different time points (0-1.5 h), 10 µL of sample were withdrawn from the reaction mixture and added to 190 µL of FOX reagent in a microtiter plate. After 30 min, absorbance was measured at 560 nm.

c) Calculations

H_2O_2 concentration in the reaction medium was calculated by interpolating the absorbance measurements of the sample in a calibration curve containing different concentrations of H_2O_2 (0-200 µM). Both the calibration curve and the sample measurements were run in parallel for each experiment.

3.2.2. *In vivo* methods

3.2.2.1. Acute pancreatitis induction

AP was induced in 12 weeks-old mice by seven intraperitoneal injections of cerulein (Merck, New Jersey, USA) (50 µg/kg body weight) at 1 h intervals as previously described (Niederau, Ferrell and Grendell, 1985; Pérez *et al.,* 2019). 1 h after the last injection, animals were euthanized under anesthesia with 3% isoflurane, exsanguinated, and the pancreas was immediately

removed and processed according to the technique to be used in the different determinations that will be detailed later in this section. Death was confirmed by cervical dislocation. 0.9% saline (B. Braun, Melsungen, Germany) was administered to the control group through seven intraperitoneal injections at hourly intervals. **Figure 19** summarizes the protocol for AP induction.

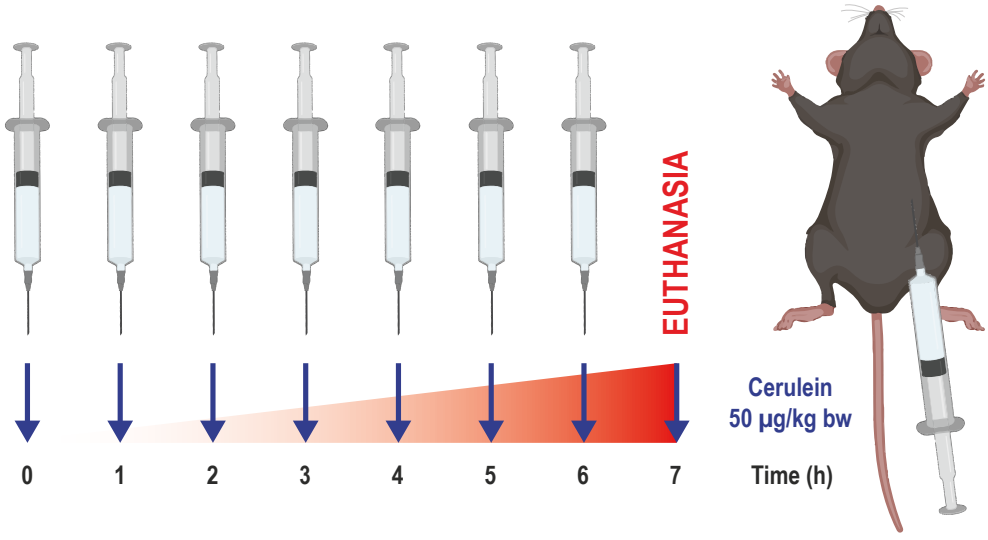

Figure 19. Acute pancreatitis induction by cerulein.

3.2.2.2. Determination of free and protein-bound low-molecular-weight thiols from pancreatic samples

Low molecular weight thiols and their oxidized disulfide form as well as mixed disulfides between low molecular weight thiols and proteins through disulfide bonds were quantified from pancreatic samples by HPLC-MS/MS as previously described by our group (Moreno *et al.,* 2014).

a) Sample preparation

Different conditions were required to analyze accurately the different thiol-containing analytes in tissues, and samples were processed as indicated in the following items.

a) 1. Sample preparation for determination of free low-molecular-weight thiols

1. Fresh tissue samples were homogenized in PBS with 10 mM NEM (400 µL of buffer / 100 mg of tissue).
2. In order to deproteinize the samples, perchloric acid (PCA) was added to the samples 1 min after homogenization to achieve a final concentration of 4%.
3. Samples were centrifuged at 15000 x *g* for 15 minutes at 4ºC.
4. Supernatants were collected and stored at -80ºC untill measurement of low molecular weight thiols.

a) 2. Sample preparation for the determination of mixed disulfides between low-molecular-weight thiols and proteins

1. Fresh tissue samples were homogenized in 10% trichloroacetic acid (TCA) (100 µL of 10% TCA / 100 mg of tissue).
2. Homogenates were centrifuged at 10000 x *g* for 2 min.
3. Pellets containing thiolated proteins were washed twice with 500 µL of 10% TCA.

4. After washing, pellets were dissolved in 50 mM Hepes buffer, pH 8, containing 2% sodium dodecyl sulfate (SDS). SDS was added after adjusting the pH of the buffer when preparing it.

5. Sodium bicarbonate (powder) was added to saturation.

6. Once the pellet was completely dissolved, an aliquot was taken to measure total protein content –as described further on in item 2.3. from this section– and to check that the pH was not higher than 9.

7. Another 100 µL aliquot was taken, and DTT was added to a final concentration of 2.5 mM.

8. Samples were incubated for 1 h at 40ºC under gentle shaking. Subsequently, pH was measured again.

9. After checking that pH was not extremely alkaline –what could lead to alkaline hydrolysis of disulfides–, 10 µL of 100 mM NEM were added to each sample.

10. Immediately after step 9, 12 µL of 40% PCA were added to samples, rendering final concentrations of 10 mM NEM and 3.93% PCA.

11. Samples were centrifuged for 10 min at 10000 x *g*, 4ºC. Supernatants were collected and stored at -80ºC until determination of the low-molecular-weight thiols that were bound to proteins.

b) High-performance liquid chromatography coupled to mass spectrometry

HPLC-MS/MS analysis to measure low-molecular-weight thiols was performed at the Central Service for Experimental Research of the Universitat de València.

Analytical separation was performed using a UPLC BEH C18 Acquity column (2.1 x 50 mm, 1.7 µm, Waters) with an injection volume of 5 µL. Elution was performed using a mix of solvent A: water-formic acid (100:0.5 v/v) and solvent B: isopropanol-acetonitrile-formic acid (50:50:0.1 v/v/v). Elution gradient was performed as follows: from 0 to 2.52 min, 0% B; from 2.52 to 4.4 min, from 0 to 65% B; from 4.4 to 6 min, 65% B; from 6 to 6.1 min, from 65 to 0% B, from 6.1 to 11 min, 0% B. The flow rate of the mobile phase was set at 0.35 mL/min and column separation was performed at room temperature (23±3 ºC). Sample ionization and nebulization were performed under the following conditions: capillary voltage: 3.5 kV; source temperature: 120 ºC; desolvation temperature: 350 ºC. Nebulization of cone gases were set at 690 and 25 L/h, respectively. Cone and collision voltages were optimized for each analyte as shown in **Table 2**.

A linear calibration curve was prepared with a concentration range from 185.5 to 190000 nmol/L for GSH, from 9.31 to 9500 nmol/L for GSSG, and from 1.9 to 1900 nmol/L for the remaining analytes, all of them corrected by the internal standard signal. The internal standards used were cystine-D4, cystine-D2, phenylalanine-D5, methionine-D3, GSH-C13N15, and GSSG-C13N15.

Table 2. Transitions for analytes determined by HPLC-MS/MS.

Analyte	Precursor ion (m/z)	Cone (V)	Fragmented ions (m/z)			
			Quantification	CE (eV)	Confirmation	CE (eV)
GSH	433.2	30	304.3	15	201.2	15
GSSG	613.2	30	355.3	25	231.1	25
Cysteine	247.1	25	158.1	20	184.2	20
Cystine	241.2	15	152.1	15	120.1	15
Homocysteine	261.3	30	126	25	98	25
Homocystine	269.1	35	136.1	11	90	11
γ-glutamylcysteine	376.1	40	230.1	20	201.3	20
γ-glutamylcystine	499.1	20	241.1	18	370.1	18
Methionine	150.2	20	104.2	15	133.2	15
Cystathionine	223.2	20	88.1	15	134.1	15
Cystine-D4	245	25	154	15	-	-
Cysteine-D2	249	25	232	15	-	-
Phenylalanine-D5	171.1	35	125	10	-	-
Methionine-D3	153	25	107	15	-	-
GSH-C13N15	436.1	30	201.25	20	-	-
GSSG-C13N15	618.1	30	361.14	15	-	-

The concentration range in samples was determined using the area of each peak. Samples were analyzed twice, in the obtained supernatants as indicated in previous items and also in diluted forms. Supernatants were used for detecting all analytes except GSH. GSH was measured through dilution of supernatants (1:20 v/v) in order to fit GSH levels within the calibration range.

Data processing was performed using MassLynx 4.1 software (Waters, Manchester, United Kingdom). Results were expressed either by individual analytes –in nmoL/mg protein or μmoL/mg protein– or as ratios (GSSG/GSH, Cystine/Cysteine, γ-glutamylcystine/γ-glutamylcysteine, and homocystine/homocysteine).

3.2.2.3. Histological analysis

a) Sample preparation

To perform the histological study of the pancreas of wild type and TRP14 knockout mice, both under basal conditions and after the induction of acute pancreatitis, the pancreas was removed and fixed in freshly prepared 4% paraformaldehyde (PFA). After 24 hours of fixation in PFA, the samples were sent to the Microscopy Section from the Central Service for Experimental Research (SCSIE) from the Universitat de València for paraffin inclusion. 5 μm thick tissue sections were then cut and stained with hematoxylin-eosin.

b) Procedure

At least 20 fields per tissue sample were observed under the brightfield microscope.

c) Histological score

The extent and severity of tissue edema and inflammatory infiltrate were assessed blindly using a scale ranging from 0 to 3 as previously described by Van Laethem *et al.*, observing pancreas sections stained with hematoxylin-eosin (Van Laethem *et al.,* 1998).

Table 3. Criteria for the evaluation of tissue edema and inflammatory infiltrate in pancreatic tissue sections stained with hematoxylin-eosin (Van Laethem *et al.*, 1998).

Condition	Score	Observations
Edema	0	Absent
	1	Focally increased between lobules
	2	Diffusely increased between lobules
	3	Disrupted and separated acini
Inflammatory infiltrate	0	Absent
	1	In ducts (around ductal margins)
	2	In the parenchyma (in < 50% of the lobules)
	3	In the parenchyma (in > 50% of the lobules)

3.2.2.4. Determination of α-amylase levels in serum

Serum α-amylase levels were determined using the *Amylase-LQ* detection kit from Spinreact (Barcelona, Spain).

a) Principle

Amylase serum levels were measured spectrophotometrically due to the ability of α-amylase to hydrolyze 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) to release 2-chloro-4-nitrophenol (CNP) and to form 2-chloro-4-nitrophenyl-α-D-maltoside (CNPG2), maltotriose, and glucose, according to the following reaction:

$$10\ \text{CNPG3} \longrightarrow 9\ \text{CNP} + 1\ \text{CNPG2} + \text{maltotriose} + \text{glucose}$$

b) Sample preparation

Blood samples were carefully withdrawn with a heparinized syringe from mice under inhalation anesthesia (3% isoflurane) and centrifuged at 400 x *g* for 15 min at room temperature. Serum samples were immediately frozen at -80ºC until analysis.

c) Procedure

20 µL of each sample (1:100 dilution) were added to 1 mL of the reagent medium (2.25 mM 2-chloro-4-nitrophenyl-α-D-maltotrioside, 350 mM sodium chloride, 6 mM calcium acetate, and 900 mM potassium thiocyanate in 100 mM MES buffer, pH 6.0) in a spectrophotometric cuvette. The increase in absorbance at 405 nm was followed over 3 min.

d) Calculations

The difference between absorbances was used to calculate the average increase of absorbance per minute (ΔABS/min) in each of the experimental groups. To calculate α-amylase levels in serum expressed in international units per liter of sample (IU/L), the following equation was used

$$\text{Amylase (IU/L)} = \Delta\text{ABS/min} \times 3954 \times \text{sample dilution}$$

where an international unit corresponds to the amount of enzyme that is able to transform 1 µmol of substrate per minute under standard conditions.

3.2.2.5. Determination of pancreatic lipase levels in serum

Serum pancreatic lipase levels were determined using the *Lipase-HQ* kit from Spinreact (Barcelona, Spain).

a) Principle

Pancreatic lipase is able to hydrolyze 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methyl resorufin)-ester in the presence of colipase, desoxycholate, and calcium ions, according to the following reactions:

1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester ⟶

⟶ 1-2-O-dilauryl-rac-glycerol + Glutaric-6'-methyl resorufin-ester ⟶

⟶ glutaric acid + methyl resorufin

Methyl resorufin formation can be spectrophotometrically measured at 580 nm, and it is proportional to the concentration of lipase in the sample.

b) Sample preparation

Blood samples were carefully withdrawn with a heparinized syringe from mice under inhalation anesthesia (3% isoflurane) and centrifuged at 400 x *g* for 15 min at room temperature. Serum samples were immediately frozen at -80ºC until analysis.

c) Procedure

The reaction mixture was prepared by adding 1 mL of *Reagent 1* (1 mg/L colipase, 1.8 mM deoxycholate, and 7.2 mM taurodeoxycholate in 40 mM TRIS buffer, pH 8.3) and 200 µL of *Reagent 2* (0.7 mM lipase substrate and 0.1 mM calcium chloride in 15 mM tartrate buffer, pH 4.0) in a spectrophotometric cuvette. Either 10 µL of sample, calibrator, or distilled water used as blank were added to the reaction mixture, which was incubated for 1 min at 37ºC. The increase in absorbance at 580 nm was then followed for 2 min.

d) Calculations

The difference between absorbances was used to calculate the average increase of absorbance per minute (∆ABS/min) in each of the experimental groups. The ∆ABS/min of the blank was calculated as well and subtracted from each of the experimental groups, including the calibrator. To calculate pancreatic lipase levels in serum expressed in international units per liter of sample (IU/L), the following equation was used

$$\text{Lipase (IU/L)} = \frac{\Delta ABS/\min sample}{\Delta ABS/\min calibrator} \text{ x calibrator activity}$$

where an international unit corresponds to the amount of enzyme that is able to transform 1 µmol of substrate per minute under standard conditions.

3.2.2.6. Determination of myeloperoxidase activity in pancreatic tissue samples

Myeloperoxidase activity determination in tissue samples was performed as previously described (Schierwagen, Bylund-Fellenius and Lundberg, 1990).

Myeloperoxidase (MPO) is an enzyme found in polymorphonuclear neutrophil granules, representing up to 5% of the cell's weight, and can also be found in other cell types such as monocytes. The function of this enzyme is to destroy the microorganisms phagocytized by the neutrophil, or to be released to the exterior to create an oxidizing and cytotoxic environment in the focus of the infection. Neutrophils generate large amounts of superoxide radicals that are rapidly converted into hydrogen peroxide by superoxide dismutase. MPO then catalyzes the reaction between hydrogen peroxide and chlorine anion to yield hypochlorous acid, which has strong oxidizing and cytotoxic power.

$$H_2O_2 + Cl^- \longrightarrow HOCl + H_2O$$

a) Principle

The assay is based on the MPO-catalyzed oxidation of tetramethylbenzidine (TMB). The oxidized form of TMB shows a blue color that can be measured spectrophotometrically at 650 nm.

For the determination to be specific, it is crucial to perform a proper enzyme extraction. Homogenization of the sample in a non-detergent buffer allows the precipitation of MPO-containing granules, leaving non-specific peroxidase in the supernatant. Resuspension of the pellets with detergent buffer allows for the release of the MPO, and

subsequent incubation at 60°C inactivates any possible enzyme inhibitors present in the sample.

b) <u>Sample preparation</u>

1. Pancreatic tissue samples were homogenized in monopotassium phosphate (KH_2PO_4) buffer (pH 6.0) following a ratio of 100 mg tissue/mL buffer.

2. Tissue homogenates were centrifuged at 20000 x g for 20 minutes at 4 ºC to allow the separation of MPO, which will precipitate, from the rest of non-specific peroxidases, which will remain in the supernatant.

3. Supernatants, which were kept to measure total proteins, were separated from pellets, which were resuspended in 1 mL of monopotassium phosphate buffer (pH 6.0) to which HTMAB, a detergent that facilitates the breakdown of neutrophil granules and thus the release of MPO, was added.

4. To facilitate the complete breakdown of the granules, samples were subjected to three sonication and freeze-thaw cycles.

5. To inactivate any possible MPO inhibitors present in the samples, they were incubated for two hours at 60°C.

6. Samples were centrifuged at 15000 x g for 15 minutes at room temperature, and supernatants were stored for the determination of MPO activity.

c) Procedure

The determination of myeloperoxidase activity was performed in a 96-well plate.

1. Samples, blanks, and the calibration curve were pipetted into the plate.
2. TMB was added to all the wells.
3. The reaction was triggered by the addition of hydrogen peroxide.
4. The reaction kinetics was followed at 620 nm for 3 min.

d) Calculations

Myeloperoxidase activity was calculated by comparison with a horseradish peroxidase curve of known activity. The results were expressed in mU/mg, where U is the amount of oxidized TMB per minute.

3.2.3. Determination of protein concentration

Two different methods for determining protein concentrations in biological samples were used in this Thesis: bicinchoninic acid (BCA) method and Bradford's method.

3.2.3.1. BCA method

Total protein concentration was measured using *Pierce® BCA Protein Assay Kit* (Thermo Scientific, Massachusetts, USA) according to the manufacturer's instructions. This method combines the reduction of Cu^{2+} to Cu^{1+} by proteins under alkaline conditions together with the high-sensitive colorimetric detection of Cu^{1+} in presence of a reagent that contains bicinchoninic acid (BCA). The product of the reaction, which was purple, was formed when one Cu^{1+} was chelated by two molecules of BCA. The complex is water-soluble and shows a strong absorbance at 562 nm, which was lineal in the range of 2-20 mg/mL of protein in the samples.

a) <u>Procedure</u>

1. In a 96-well plate, 3 µL of sample and 260 µL of reagent solution were added in triplicate.

2. In order to perform a correct protein concentration determination, a standard curve was prepared with bovine serum albumin (BSA) in the range of 0.3 to 20 mg/mL. As in the case of samples, 3 µL of standard solution and 260 µL of reagent solution were added per well.

3. A blank was prepared by adding 3 µL of distilled water and 260 µL of the reagent solution.

4. The plate was incubated for 30 minutes at 37ºC, and then the absorbance was measured at 565 nm.

b) Calculations

First, the absorbance of the blank was subtracted from the absorbance values of standards and samples. Then, a calibration curve was plotted representing absorbance versus protein concentration of the different concentrations of the standard. Since the system follows Lambert-Beer's law, a straight line was obtained. After obtaining the equation of the line by linear regression, the absorbance of each sample was interpolated in order to calculate the total protein concentration in samples.

3.2.3.2. Bradford's method

Bradford Reagent (Bio-Rad, California, USA) was used according to the manufacturer's instructions for determination of total protein in samples expected to contain low protein concentration, as is the case with samples from *in vitro* experiments. This method is more sensitive than BCA. When proteins in the sample bound Bradford's reagent, a color change –and thus a change in absorbance– took place. Bradford's reagent contains phosphoric acid, methanol and Coomassie blue, which binds to amino acid residues in proteins.

a) Procedure

1. In a 96-well plate, 3 µL of sample and 260 µL of Bradford's reagent were added in triplicate to each well.

2. A standard curve was prepared with BSA in the range of 0.015 to 2 mg/mL. 3 µL of standard solution and 260 µL of Bradford's reagent were added per well.

3. A blank was prepared by adding 3 µL of distilled water and 260 µL of the reagent solution.

4. Absorbance was measured at 595 nm within 5 min.

b) Calculations

The absorbance of the blank was subtracted from the absorbance values of standards and samples. The resulting absorbance values were interpolated in the standard curve and the obtained values were expressed as mg of protein per mL of sample.

3.2.4. Gel electrophoresis

Vertical electrophoresis in polyacrylamide gels were used for protein separation. When SDS polyacrylamide gel electrophoresis (SDS-PAGE) is performed under denaturing conditions, protein separation is then performed according to the molecular weight.

10.5 µL of protein samples were mixed with 3.5 µL of NuPAGE™ LDS Sample Buffer (Thermo Fisher, Massachusetts, USA) and loaded into the gel. When electrophoresis was performed under reducing conditions, DTT was added to the loading buffer to give a final concentration of 100 nM. Together with samples, a marker of protein molecular weight (Thermo Fisher, Massachusetts, USA) was also loaded into the gel.

Gradient gels ranging from 4-20% acrylamide were used. These gels allow better protein separation according to their molecular weight, and they do not require a stacking gel.

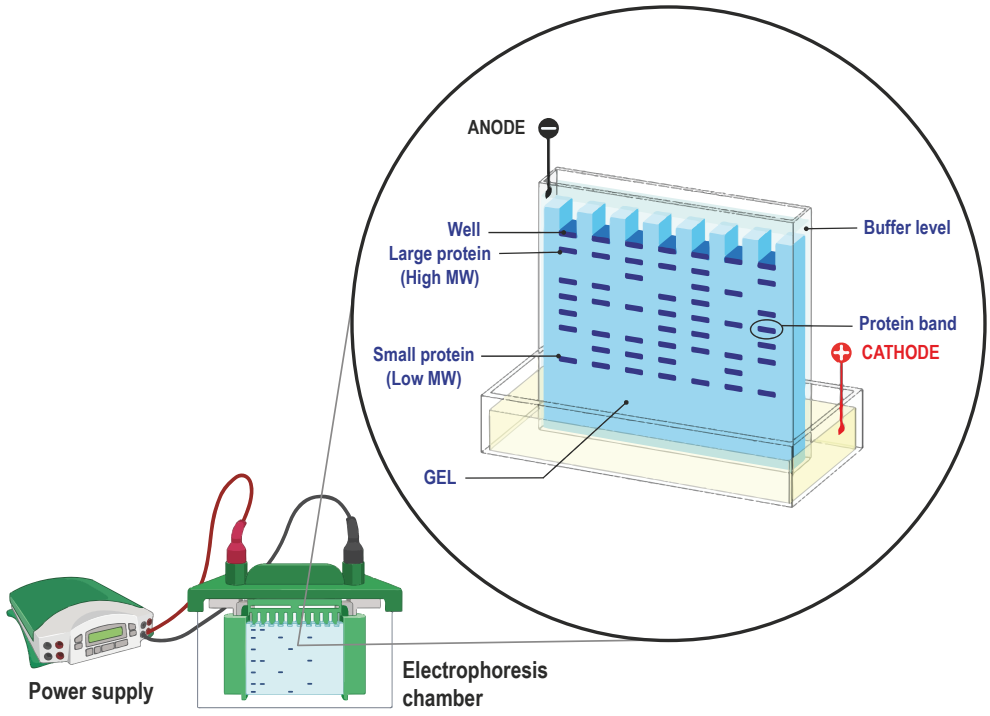

Figure 20. Vertical gel electrophoresis setup.

A constant-voltage electric field was applied to the gel (120 volts) through a Tris-glycine running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS, pH 8.3).

Figure 20 shows a typical vertical gel electrophoresis setup.

3.2.5. Western Blot

Western Blot is a complex Molecular Biology technique that combines several different steps to determine in a semiquantitative way the amount of a given protein in a sample. The different procedures used for this purpose are protein gel electrophoresis (see above), its electrotransference from the

gel to a nitrocellulose or polyvinylidene fluoride membrane (according to the procedures developed throughout this Thesis, only nitrocellulose membranes were used), blocking, antibody incubation and its detection by chemiluminescence.

a) Sample preparation

Frozen tissue samples were homogenized on ice in lysis buffer (100 mg tissue/1 mL buffer). The lysis buffer contained 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Igepal, 30 mM sodium pyrophosphate, 50 mM sodium fluoride and 50 µM sodium vanadate. A protease inhibitor cocktail (Sigma-Aldrich, Missouri, USA) was added at a concentration of 10 µL/mL before homogenization.

b) Procedure

1. 25-50 µg of proteins from tissue samples or cell lysates were loaded into each well of discontinuous Criterion® acrylamide gels (Bio-Rad, California, USA). As explained before, a constant voltage electric field was applied to gels in Tris-glycine buffer.

2. Once proteins were separated according to their molecular weight, the gel was transferred to a Trans-Blot® Turbo™ Midi Nitrocellulose transfer pack (Bio-Rad, California, USA). Semi-dry electrotransference was performed using the Trans-Blot® Turbo™ transfer system (Bio-Rad, California, USA).

3. Once the proteins had been transferred onto the nitrocellulose membrane, the membrane was incubated for 1 h in blocking buffer to avoid unspecific antibody binding. Blocking buffer consisted in 5% BSA in t-TBS (0.1% Tween-20, 20 nM Tris, 137 mM NaCl, pH 7.6).

4. After blocking, the membrane was washed three times for 5 min in t-TBS.

5. Membranes were incubated with primary antibody at the concentration specified by the manufacturer. Working dilution and incubation time for each of the antibodies used in this Thesis are stated in **Table 4**.

6. After incubation with the primary antibody, the membrane was washed three times for 5 min in t-TBS.

7. The membrane was incubated for 1 h with the corresponding secondary antibody. Secondary antibodies (Cell Signaling Technology, Massachusetts, USA) used in this Thesis were linked to HRP and showed reactivity towards rabbit, mouse or goat IgGs. The working dilution was 1:5000 in antibody buffer (1% BSA in t-TBS).

8. After secondary antibody incubation, the membrane was washed three times for 5 min in t-TBS.

9. To develop the membrane, a chemiluminescent substrate was used. The membrane was incubated with SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific, Waltham,

USA) for 5 min before being developed in the ChemiDoc® XRS+ Imaging System® (Bio-Rad, California, USA).

Table 4. Antibodies used for Western Blot.

Reference	Manufacturer	Species	Dilution
Streptavidin-HRP (#3999)	Cell Signaling	-	1:2000
Anti-CBS (ab140600)	Abcam	Rabbit	1:1000
Anti-NRF2 (#12721)	Cell Signaling	Rabbit	1:1000
Anti-rabbit IgG-HRP (#7074)	Cell Signaling	Goat	1:5000

c) Quantification

The images corresponding to the membranes were analyzed with the ImageLab™ software (Bio-Rad, California, USA) to perform a densitometric quantification.

3.2.6. RNA isolation

In order to extract and isolate RNA from the pancreas, the tissue was stored in RNAlater™ solution (Invitrogen, California, USA) and processed within 24 h to prevent RNA degradation by RNAses present in the pancreas. 25-30 mg of pancreatic tissue were homogenized in 500 µL of TRI Reagent® (Sigma-Aldrich, Missouri, USA). The reagent contains guanidine thiocyanate –which denaturalizes RNAse, contributes to the rupture of the plasma membrane, and separates rRNA– and phenol –an organic solvent saturated with water or Tris buffer. Both components dissolve DNA, RNA, and proteins.

a) Procedure

1. After homogenization, 200 µL of chloroform per mL of TRI Reagent® were added to samples to favor the extraction process. Chloroform is able to separate phenol from the rest of the components of the reagent, and after centrifuging the samples for 15 min at 12000 x *g* at 4ºC, three phases were observed: a reddish organic phase which contained proteins at the bottom, a white intermediate phase which corresponded to DNA, and an aqueous phase on top which contained RNA.

2. After collecting the aqueous phase, 500 µL of 2-propanol were added per mL of TRI Reagent®. 2-propnanol is a very water-miscible alcohol which helps dehydrating RNA. After incubating the mix for 10 min at room temperature, the samples were centrifuged for 10 min at 10000 x *g* at 4ºC to achieve RNA precipitation.

3. The supernatant was discarded and the precipitate was washed with ice-cold 75% ethanol (1 mL 75% ethanol/1 mL TRI Reagent®) to get rid of any remaining 2-propanol. Samples were centrifuged at 7500 x *g* for 5 min at 4ºC. After discarding the ethanol, precipitates were dried by air-drying.

4. Precipitates were dissolved in nuclease-free water and an aliquot was taken to measure RNA concentration and check for RNA integrity.

3.2.7. Reverse transcription and cDNA amplification

In order to study gene expression, once the RNA was extracted, a reverse transcription was performed to obtain the complimentary DNA (cDNA) sequences. Then, using the polymerase chain reaction (PCR), cDNA was amplified using specific primers for a given gene.

The PCR technique can be divided into different steps:

- **Initiation:** thermal shock at high temperature which allows polymerase activation.
- **Denaturation of the DNA double helix:** 95ºC (30 s).
- **Annealing:** hybridization of the primer to the DNA strand. This temperature was 60ºC (30 s).
- **Elongation:** the complimentary DNA strand was synthesized from the 5' end to the 3' end at a speed of 1000 bp/min. The optimum temperature for this step was 72ºC.

a) <u>RNA reverse transcription</u>

Reverse transcription was performed using PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan).

A master mix containing retrotranscriptase, random primers and nucleotides was added to the RNA aliquots (100 ng/µL). Reverse transcription was performed in a C1000 Thermal Cycler (Bio-Rad,

California, USA) at 37ºC for 15 min followed by 5 s at 85ºC to inactivate the enzyme.

b) Analysis of gene expression using quantitative real-time polymerase chain reaction

Quantitative real-time PCR was carried out using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, California, USA) thermal cycler coupled to fluorescence detection. Every reaction was performed in triplicate and melt curves were analyzed with iQ™ Real Time Detection System Software (Bio-Rad, California, USA) to check that only one PCR product per sample was formed.

Specific primers were designed for each of the studied genes. Oligonucleotide sequences were synthetized by Sigma-Aldrich (Missouri, USA), and they are shown in **Table 5**. The master mix used for PCR was TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Shiga, Japan) which contains polymerase, nucleotides and flurorescent dye, which were needed to perform the reaction. TATA-box-binding protein (*Tbp*) was used as the housekeeping gene. The thermal cycler was set according to the following amplification scheme: 10 min at 95ºC, 40 cycles of 15 s at 95ºC followed by 30 s at 60-64ºC for annealing (according to the optimum hybridization temperature for each primer set) and 30 s at 72ºC for elongation.

Table 5. Oligos used for qRT-PCR.

Target Gene	Forward/reverse Oligonucleotide Sequence
Trx1	5′- CCAATGTGGTGTTCCTTGAA - 3′ 5′- AGGCTTCAAGCTTTTCCTTG - 3′
Trxr1	5′- AAGACGATGAACGTGTCGTG - 3′ 5′- GCCAGACTGGAGGATGTCTC -3′
Gr	5′- TGGTGGAGAGTCACAAGCTG -3′ 5′- CATAGGCATCCCTTTTCTGC -3′
Catalase	5′- GGAGCAGGTGCTTTTGGATA - 3′ 5′- GAGGGTCACGAACTGTGTCA - 3′
Sod1	5′- TTTTTGCGCGGTCCTTTC - 3′ 5′- CCATACTGATGGACGTGGAA - 3′
Sod2	5′- GGCCAAGGGAGATGTTACAA -3′ 5′- GAACCTTGGACTCCCACAGA - 3′
Nqo-1	5′- TAGCCTGTAGCCAGCCCTAA - 3′ 5′- AATGGGCCAGTACAATCAGG - 3′
Gclc	5′- CCATCACTTCATTCCCCAGA - 3′ 5′- GATGCCGGATGTTTCTTGTT - 3′
Ho-1	5' - CCTTCCCGAACATCGACAGCC 3' 5' - GCAGCTCCTCAAACAGCTCAA 3'
Tbp	5′- CAGCCTTCCACCTTATGCTC - 3′ 5′- CCGTAAGGCATCATTGGACT -3′

The expression of several other genes was carried out using commercially available TaqMan® probes (Applied Biosystems, California, USA) (**Table 6**). In this case, Premix Ex Taq™ (Probe qPCR) (Takara, Shiga, Japan) was used as the master mix containing all the components which were needed to perform the reaction. The

amplification scheme used was the same as the previous one, but annealing was performed at 60 ºC in all cases.

Table 6. **TaqMan® probes used for qRT-PCR.**

Target Gene	TaqMan® Probe Reference
Tnf-a	Mm00443258_g1
Il-1b	Mm00434228_m1
Il-6	Mm00446190_m1
Cxcl1	Mm04207460_m1
Il-18	Mm00434226_m1
Tbp	Mm01277042_m1

c) Calculations

Relative mRNA expression was calculated taking in to account the threshold cycle from each gene according to the following formula:

$$\textbf{Relative expression} = 2^{-\Delta(\Delta CT)}$$

where

- ΔCT = Target gene CT - Housekeeping gene CT

- $\Delta(\Delta CT)$ = Target gene ΔCT - Average of control group ΔCT

3.2.8. Statistical analysis

The results were expressed as a mean ± standard deviation. For statistical analysis of the results, a two-way ANOVA was first performed to compare the mean of the different groups. When significant differences were

observed, the difference between the individual groups was determined using Scheffé's test. Those analyses in which a $P < 0.05$ was obtained were considered significant.

IV. RESULTS

4. RESULTS

4.1. *In vitro* STUDIES

4.1.1. TRP14 knockdown leads to increased protein cysteinylation *in vitro*

In order to study the role of TRP14 in the regulation of protein cysteinylation, we first incubated both parental HEK-293 cells and TRP14 knockdown HEK-293 cells with 250 µM biotinylated cysteine (Cys-BIO) for 1 hour. The biotin molecule incorporated into the cysteine structure allowed us to assess by western blot cysteine incorporation into proteins after a short incubation. Streptavidin-HRP was used to detect the biotin moieties present in samples.

As it is shown in **Figure 21**, protein cysteinylation levels increased in TRP14 knockdown cells (TRP14-20) when compared to cysteinylation levels

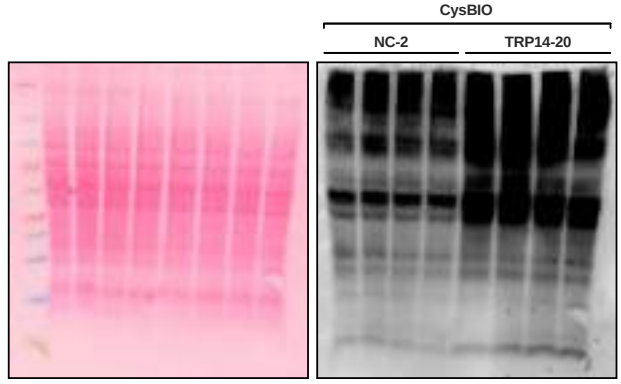

Figure 21. Effect of TRP14 knockdown on protein cysteinylation *in vitro* in HEK cells. Representative western blot image showing increased protein cysteinylation levels in TRP14 knockdown (TRP14-20) cells incubated with biotinylated cysteine (CysBIO) compared with parental HEK cells (NC-2) incubated with biotinylated cysteine (CysBIO) (n=4).

from parental HEK-293 cells (NC-2), suggesting a role of TRP14 in protein cysteinylation.

4.1.2. The TRP14 system reduces cysteinylated proteins *in vitro*

Since TRP14 knockdown resulted in increased protein cysteinylation when HEK-293 cells were incubated with biotinylated cysteine, we hypothesized that TRP14 should be able to reduce cysteinylated proteins.

In order to test whether TRP14 in presence of thioredoxin reductase and NADPH is able to reduce cysteinylated proteins, cell lysates obtained from TRP14 knockdown cells incubated with 250 µM Cys-BIO for 1 hour were used as substrates and they were incubated with the TRP14 system (20 µM TRP14, 1 µM TrxR1 and 1 mM NADPH) at room temperature. Samples were

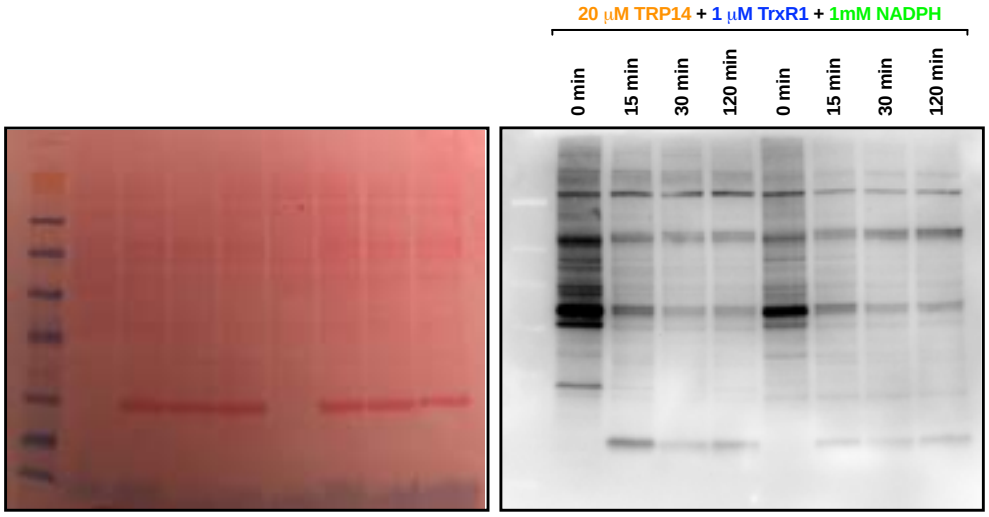

Figure 22. De-cysteinylating effect of the TRP14 enzymatic system on cysteinylated proteins from cell lysates. Representative western blot image (right) showing the resulting streptavidin-HRP signal after incubating cysteinylated proteins from cell lysates with the TRP14 enzymatic system (20 µM TRP14, 1 µM TrxR1, and 1mM NADPH) for different time periods (15-120 min) at room temperature. Ponceau staining of the membrane (left) is shown as loading control (n=4).

taken at different time points in order to analyze protein cysteinylation by western blot as in the previous cases.

Figure 22 shows how incubation of cysteinylated proteins from cell lysates with the TRP14 enzymatic system results in a time-dependent decrease of protein cysteinylation as shown by western blotting with streptavidin-HRP, suggesting that TRP14 together with TrxR1 and NADPH are able to reduce cysteinylated proteins.

4.1.3. Optimal TRP14 de-cysteinylation activity requires all components of the enzymatic system

Given the de-cysteinylating activity of the TRP14 enzymatic system on cysteinylated proteins from cell lysates, the effects of the individual components of the system were then studied.

Thus, cysteinylated proteins from cell lysates were incubated with 20 μM TRP14, 1 μM TrxR1, 1 mM NADPH and different combinations of the components. DTT was used as a positive control since it is able to reduce the disulfide bond of mixed disulfides. Incubations were performed at room temperature for 15 min, and samples were analyzed by western blotting using streptavidin-HRP.

Although a slight de-cysteinylating effect was observed in all conditions (**Figure 23**), the strongest effect was observed when cysteinylated proteins from lysates were incubated with all components of the TRP14 system (TRP14, TrxR1 and NADPH) (**Figure 23**).

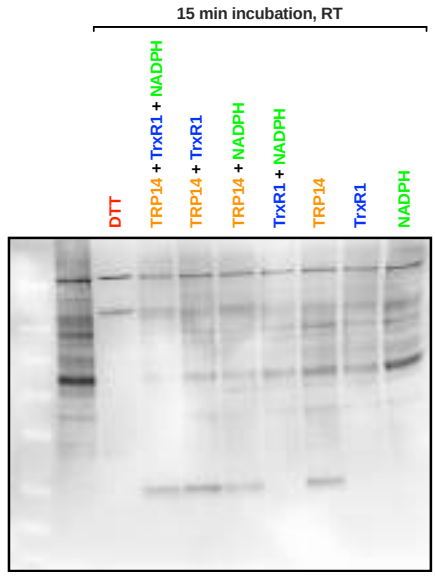

Figure 23. De-cysteinylating effects of individual components of the TRP14 system on cysteinylated proteins from cell lysates *in vitro*. Representative western blot image showing cysteinylation levels after incubating cysteinylated proteins from cell lysates with different combinations of individual components of the TRP14 enzymatic system (20 µM TRP14, 1 µM TrxR1, and 1mM NADPH) for 15 min at room temperature. Dithiothreitol (DTT) was used as a positive de-cysteinylation (reduction) control (n=4).

4.1.4. Enrichment of cysteinylated proteins by streptavidin pulldown

Cysteinylated proteins present in cell lysates coming from cell cultures incubated with biotinylated cysteine were enriched by streptavidin pulldown since they were labelled with biotin.

Figure 24 shows a representative streptavidin-HRP western blot of the different fractions eluted from a streptavidin column containing cell lysates incubated with biotinylated cysteine. TRP14 knockdown cells (TRP14-20) incubated with biotinylated cysteine showed a higher signal in the first eluted fractions compared to parental HEK-293 cells (NC-2). TRP14 knockdown

cells (TRP14-20) incubated with non-labelled cysteine were used as a negative control, and thus showed no signal.

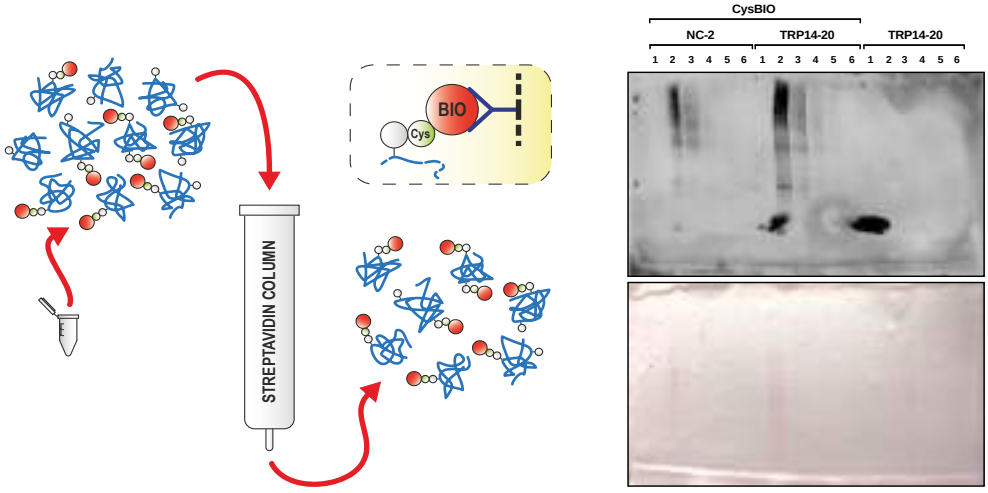

Figure 24. Cysteinylated proteins enrichment by streptavidin pulldown. Left: schematic representation of the streptavidin pulldown enrichment process. **Right:** representative western blot image and Ponceau staining of the membrane showing protein cysteinylation levels in the different fractions eluted from the streptavidin columns (n=4).

4.1.5. Identification of cysteinylated proteins by proteomic analysis in TRP14 knockdown cells incubated with biotinylated cysteine

Once we had purified cysteinylated proteins from our cell culture samples by streptavidin pulldown, we devised a proteomic approach to identify and quantify those cysteinylated proteins from TRP14 knockdown cells or parental cells to look for differences among groups.

Figure 25 shows a heatmap comprised by 42 proteins whose cysteinylation degree was significantly increased in TRP14 knockdown cells (TRP14-20) incubated with biotinylated cysteine when compared to parental

HEK-293 cells (NC-2) incubated with biotinylated cysteine. Common protein names and UniProt identifiers for each of the proteins are listed in **Table 6**.

A STRING analysis of the cysteinylated proteins (**Figure 26**) showed a network of closely interacting proteins. Most of the cysteinylated proteins found in TRP14 knockdown cells were involved in translation (**Figure 27**) since they are mostly constituents of the ribosome. Additionally, two proteins were involved in peroxide removal: peroxiredoxins 1 and 2 (Prx1 and Prx2) (**Figure 28**).

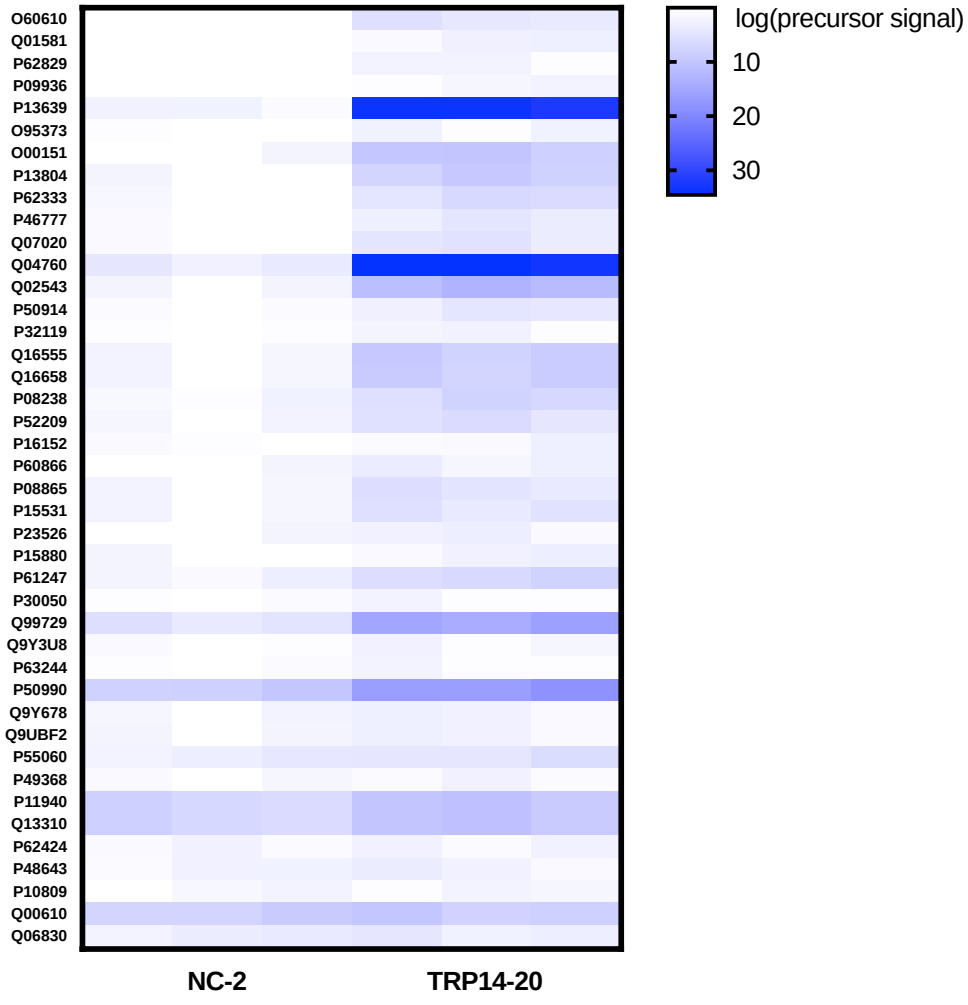

Figure 25. Differential analysis of cysteinylated proteins from wild type or TRP14 knockdown cells incubated with biotinylated cysteine. The heatmap shows 42 proteins which were identified in cell lysates incubated with biotinylated cysteine and purified by streptavidin pulldown. The logarithm of the precursor signal intensity for the total of cysteinylated peptides corresponding to each protein identified by MS/MS is represented. Each row corresponds to one protein (identified by the standard UniProt code), whereas each column represents a different experiment. *NC-2: parental HEK-293 cells; TRP14-20: TRP14 knockdown HEK-293 cells* (n=3).

Table 7. Cysteinylated proteins identified from wild type or TRP14 knockdown cells incubated with biotinylated cysteine. Protein list showing 42 proteins whose cysteinylation degree was significantly higher in TRP14 knockdown cells incubated with biotinylated cysteine.

UniProt ID	PROTEIN NAME
P62333	26S proteasome regulatory subunit 10B (26S proteasome AAA-ATPase subunit RPT4)
P15880	40S ribosomal protein S2 (40S ribosomal protein S4)
P60866	40S ribosomal protein S20 (Small ribosomal subunit protein uS10)
P61247	40S ribosomal protein S3a (Small ribosomal subunit protein eS1) (Fte-1)
P08865	40S ribosomal protein SA (37 kDa laminin receptor precursor) (37LRP)
P52209	6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)
P10809	60 kDa heat shock protein, mitochondrial (EC 5.6.1.7) (HSP-60)
P30050	60S ribosomal protein L12 (Large ribosomal subunit protein uL11)
P50914	60S ribosomal protein L14 (CAG-ISL 7)
Q07020	60S ribosomal protein L18 (Large ribosomal subunit protein eL18)
Q02543	60S ribosomal protein L18a (Large ribosomal subunit protein eL20)
P62829	60S ribosomal protein L23 (60S ribosomal protein L17)
Q9Y3U8	60S ribosomal protein L36 (Large ribosomal subunit protein eL36)
P46777	60S ribosomal protein L5 (Large ribosomal subunit protein uL18)
P62424	60S ribosomal protein L7a (Large ribosomal subunit protein eL8)
P23526	Adenosylhomocysteinase (AdoHcyase) (EC 3.3.1.1) (S-adenosyl-L-homocysteine hydrolase)
P16152	Carbonyl reductase [NADPH] 1 (EC 1.1.1.184)
Q00610	Clathrin heavy chain 1 (Clathrin heavy chain on chromosome 17) (CLH-17)
Q9Y678	Coatomer subunit gamma-1 (Gamma-1-COP)
Q9UBF2	Coatomer subunit gamma-2 (Gamma-2-COP)
Q16555	Dihydropyrimidinase-related protein 2 (DRP-2)
P13804	Electron transfer flavoprotein subunit alpha, mitochondrial (Alpha-ETF)
P13639	Elongation factor 2 (EF-2)
P55060	Exportin-2 (Exp2)
Q16658	Fascin (55 kDa actin-bundling protein) (p55)
P08238	Heat shock protein HSP 90-beta (HSP 90)
Q99729	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)
Q01581	Hydroxymethylglutaryl-CoA synthase, cytoplasmic (HMG-CoA synthase) (EC 2.3.3.10)
O95373	Importin-7 (Imp7)
Q04760	Lactoylglutathione lyase (Glyoxalase I)
P15531	Nucleoside diphosphate kinase A (NDK A) (NDP kinase A) (EC 2.7.4.6)
O00151	PDZ and LIM domain protein 1 (C-terminal LIM domain protein 1)
Q06830	Peroxiredoxin-1 (EC 1.11.1.15)
P32119	Peroxiredoxin-2 (EC 1.11.1.15)
P11940	Polyadenylate-binding protein 1 (PABP-1)
Q13310	Polyadenylate-binding protein 4 (PABP-4)
O60610	Protein diaphanous homolog 1 (DRF1)
P63244	Receptor of activated protein C kinase 1 (Cell proliferation-inducing gene 21 protein)
P48643	T-complex protein 1 subunit epsilon (TCP-1-epsilon)
P49368	T-complex protein 1 subunit gamma (TCP-1-gamma)
P50990	T-complex protein 1 subunit theta (TCP-1-theta)
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) (EC 3.4.19.12)

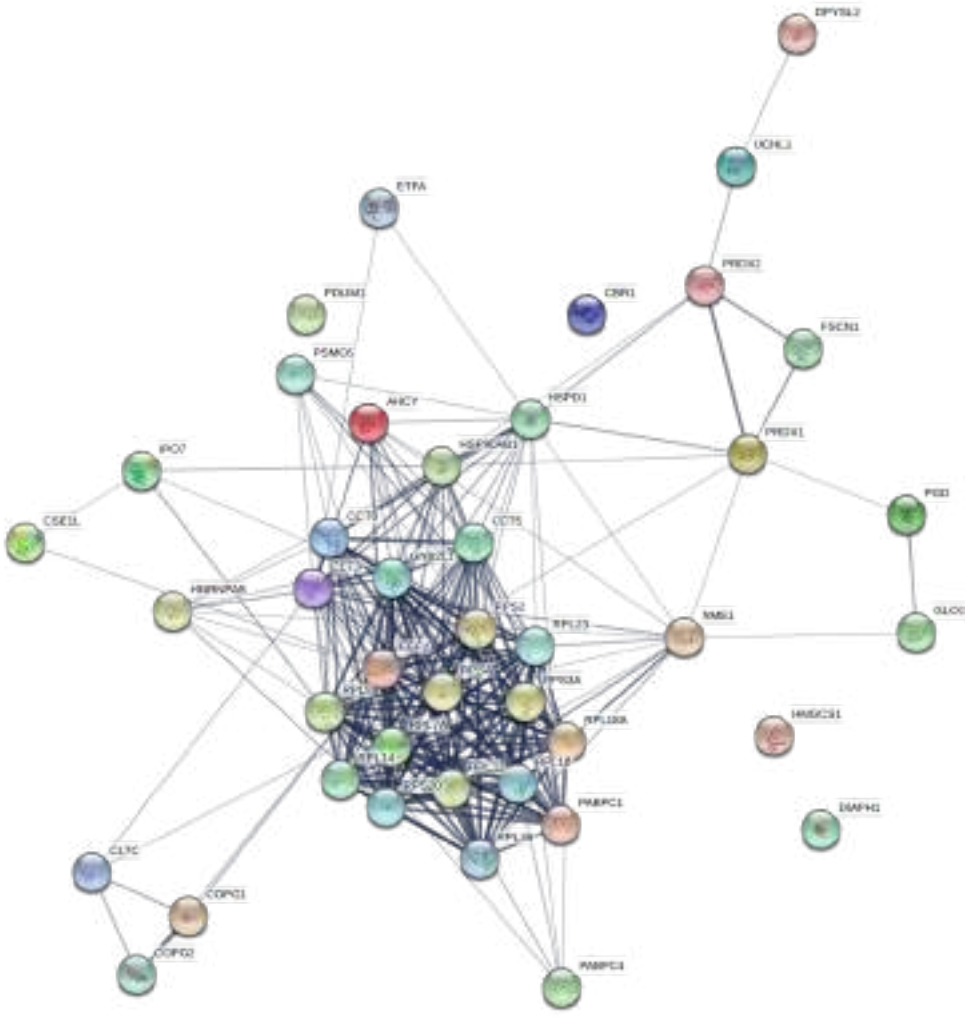

Figure 26. STRING analysis of cysteinylated proteins *in vitro*. STRING network showing 42 proteins whose cysteinylation degree was significantly higher in TRP14 knockdown cells incubated with biotinylated cysteine.

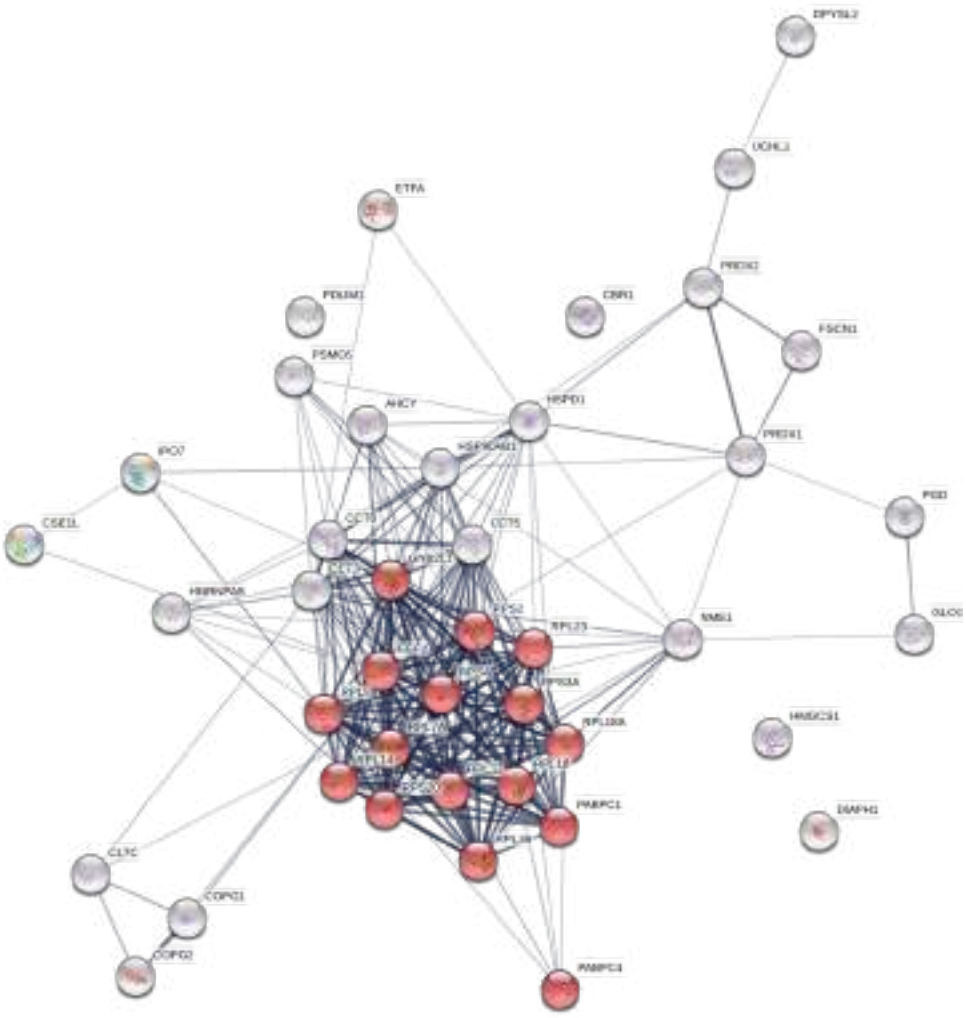

Figure 27. STRING analysis of cysteinylated proteins involved in translation. STRING interaction network showing 16 proteins involved in translation (shown in red). *Elongation factor 2 (EF-2), guanine nucleotide binding protein beta polypeptide 2-like 1, polyadenylate-binding protein 1 (PABP-1), polyadenylate-binding protein 4 (PABP-4), 60S ribosomal protein L12, 60S ribosomal protein L14 (CAG-ISL 7), 60S ribosomal protein L18, 60S ribosomal protein L18a, 60S ribosomal protein L23, 60S ribosomal protein L36, 60S ribosomal protein L5, 60S ribosomal protein L7a, 40S ribosomal protein S2, 40S ribosomal protein S20, 40S ribosomal protein S3a, 40S ribosomal protein SA.*

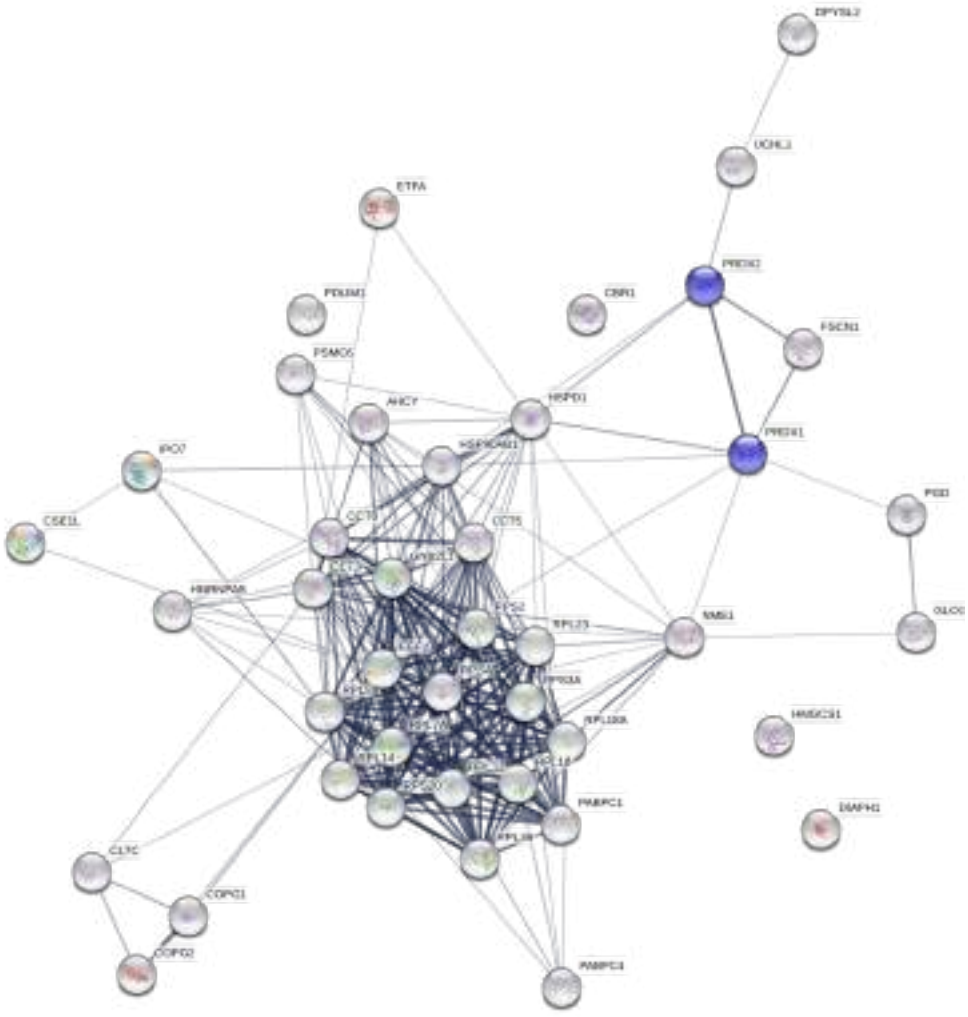

Figure 28. STRING analysis of cysteinylated proteins involved in peroxide removal. STRING interaction network showing two proteins involved in peroxide removal processes (shown in blue). *Peroxiredoxin 1 (Prx1), peroxiredoxin 2 (Prx2)*.

4.1.6. Cysteinylated peroxiredoxin 2 was reduced by both TRP14 and Trx1 systems in presence of thioredoxin reductase 1 and NADPH

Peroxiredoxin 2 (Prx2) was selected as a candidate to study whether it could be cysteinylated *in vitro* and later de-cysteinylated by the TRP14 system. These experiments were performed in the laboratory of Prof. Elias Arnér at the Karolinska Institutet, Stockholm (Sweden). After overnight incubation of recombinant human Prx2 with BODIPY™ FL L-Cystine, gel electrophoresis using non-reducing conditions was performed. The resulting cysteinylated peroxiredoxin 2 was labelled with a fluorescent BODIPY™ moiety and thus could be detected in a UV transilluminator. The band corresponding to Prx2 was digested in gel with sequencing grade trypsin and later alkylated with NEM to avoid thiol scrambling. MS analysis revealed that cysteine 172 was cysteinylated and labelled with BODIPY™, whereas cysteines 51 and 70 kept their reduced thiol form and hence were alkylated with NEM (**Figure 29**).

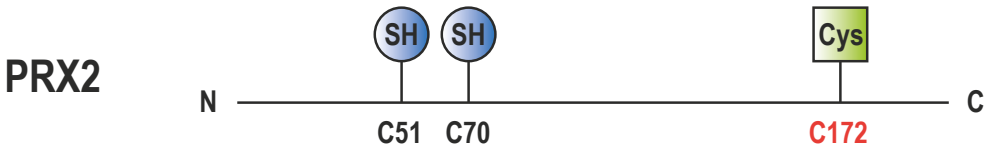

Figure 29. Schematic representation of cysteine residue modifications in recombinant human peroxiredoxin 2 incubated with BODIPY™ FL L-Cystine. Only C172 was cysteinylated following detection by mass spectrometry. *N: N-terminal end; C: C-terminal end; C51: cysteine 51; C70: cysteine 70; C172: cysteine 172; SH: free thiol at the cysteine residue; Cys: cysteinylated cysteine residue.*

DTT was used as a positive control for reduction of cysteinylated peroxiredoxin 2. Reduction of this disulfide bond led to loss of BODIPY™-labelled cysteine and thus, to a loss of fluorescence, so no signal was seen upon UV transillumination (**Figure 30**, second lane). When cysteinylated

peroxiredoxin 2 was incubated with either Trx1 system or TRP14 system (10 μM Trx1 or 10 μM TRP14, 30 nM TrxR1, and 300 mM NADPH), the fluorescence signal was lost (**Figure 30**, third and fourth lanes), indicating that both Trx1 and TRP14 were able to reduce cysteinylated peroxiredoxin 2. When cysteinylated peroxiredoxin 2 was incubated with the different individual components of the enzymatic system, no effect on fluorescence signal was observed (**Figure 30**, lanes 5-7). Coomassie staining of the gel is shown as loading control (**Figure 30**, left panel).

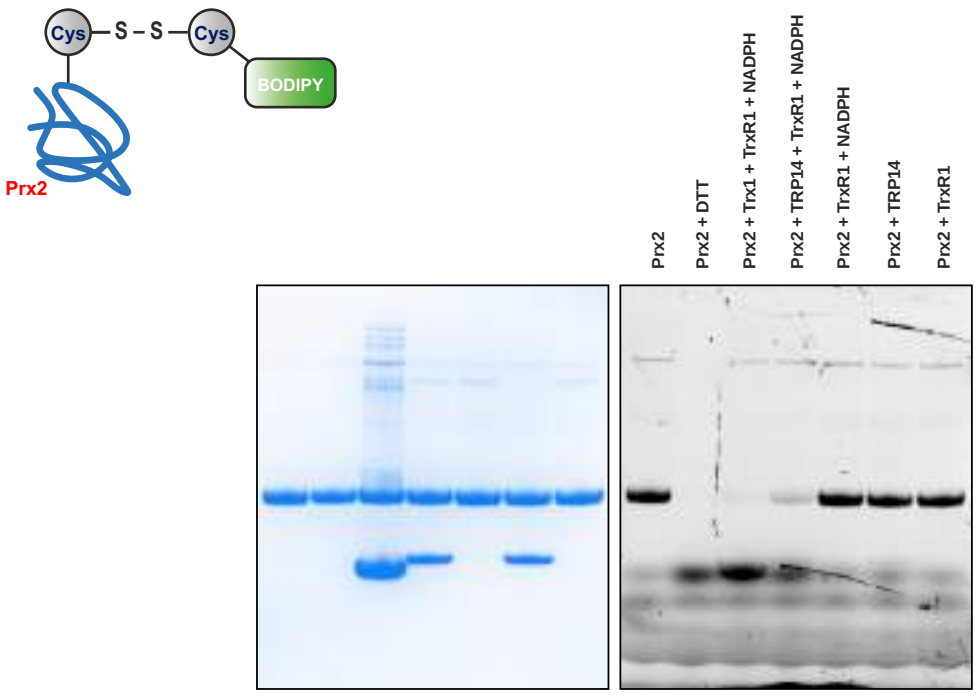

Figure 30. Cysteinylated peroxiredoxin 2 was reduced by the Trx1 and TRP14 enzymatic systems. Representative fluorescence image (right) showing that cysteinylated peroxiredoxin 2 was reduced by both Trx1 and TRP14 systems (10 μM Trx1 or 10 μM TRP14, 30 nM TrxR1 and 300 mM NADPH). Incubation time was 1 hour. Coomassie staining of the gel is shown as a loading control (left). *Prx2: peroxiredoxin 2; DTT: dithiothreitol; Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

4.1.7. Trx1 exhibits higher activity than TRP14 reducing cysteinylated Prx2

We performed a set of incubations with different Trx1 and TRP14 concentrations ranging from 0.1 to 10 µM in presence of the rest of the components of the enzymatic system (30 nM TrxR1 and 300 mM NADPH). **Figure 31** shows that Trx1 de-cysteinylated peroxiredoxin 2 even at the lowest concentrations tested (**Figure 31**, right), whereas some BODIPY™ signal was still detected at the lowest concentrations of TRP14 (**Figure 31**, left).

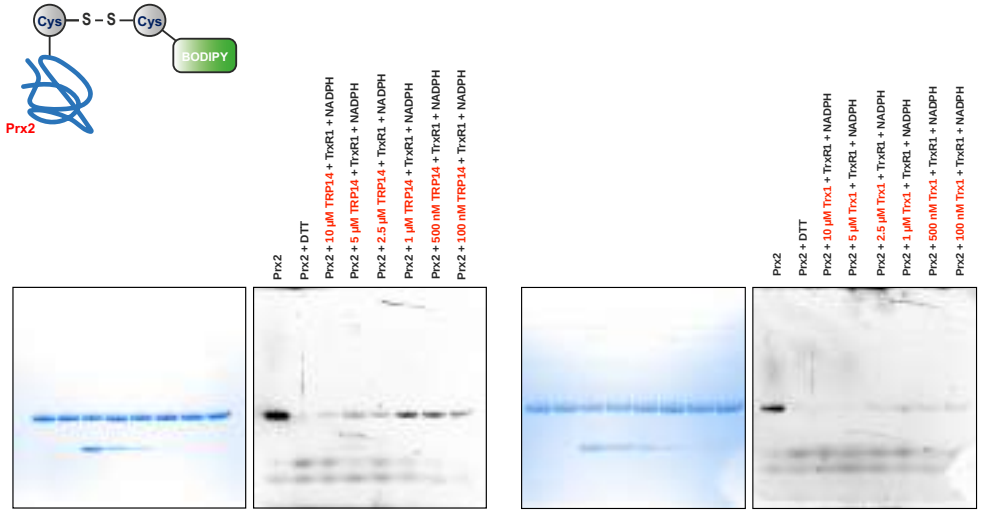

Figure 31. Reduction of cysteinylated peroxiredoxin 2 by Trx1 and TRP14 enzymatic systems was concentration dependent. Representative fluorescence images corresponding to incubation of cysteinylated peroxiredoxin 2 with 0.1-10 µM TRP14 or 0.1-10 µM Trx1, 30 nM TrxR1 and 300 mM NADPH. Incubation time was 1 hour. Coomassie staining of both gels is shown as a loading control. *Prx2: peroxiredoxin 2; DTT: dithiothreitol; Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

Given these results, we decided to perform a time-course study in order to compare the de-cysteinylating activities of Trx1 and TRP14. Cysteinylated peroxiredoxin 2 was incubated with 10 µM TRP14 or 10 µM Trx1 and the

rest of the components of the enzymatic system (30 nM TrxR1 and 300 mM NADPH) for 20 min. Incubation times as short as 5 minutes led to marked de-cysteinylation in the case of Trx1, and almost no signal was observed after 20 min (**Figure 32**, right). However, in the case of TRP14, a decrease in fluorescence was not evident until 10 min incubation, the signal being still detectable after 20 min incubation (**Figure 32**, left). We performed a longer incubation with the TRP14 enzymatic system and we observed that the fluorescent BODIPY™ signal was extremely faint after 1 hour (**Figure 33**).

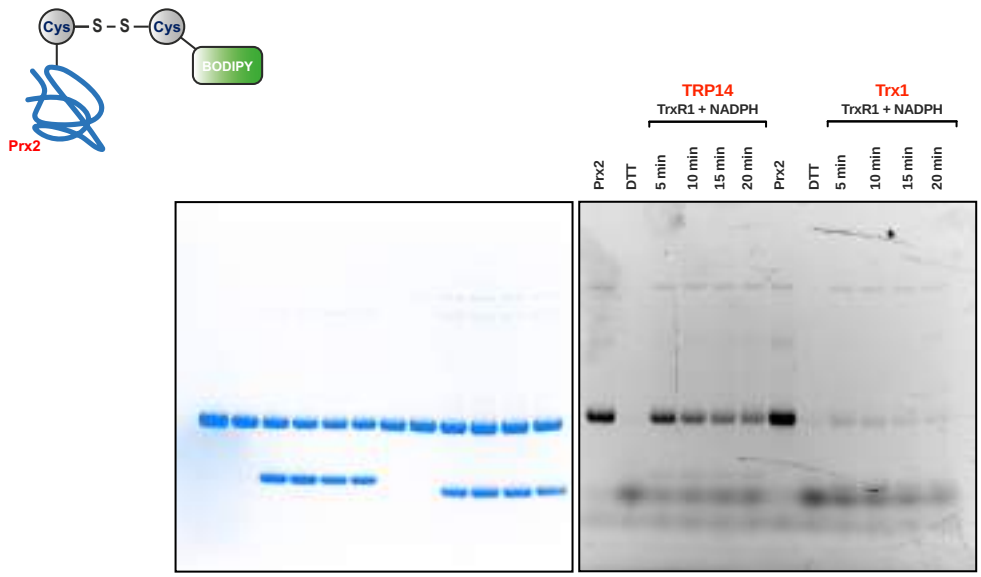

Figure 32. Comparative de-cysteinylation time-course of cysteinylated peroxiredoxin 2 by TRP14 and Trx1 enzymatic systems. Representative fluorescence image (right) of cysteinylated peroxiredoxin 2 incubated with 10 µM TRP14 or 10 µM Trx1 in presence of 30 nM TrxR1 and 300 mM NADPH for 5-20 min. Coomassie staining of the gel is shown as a loading control (left). *Prx2: peroxiredoxin 2; DTT: dithiothreitol; Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

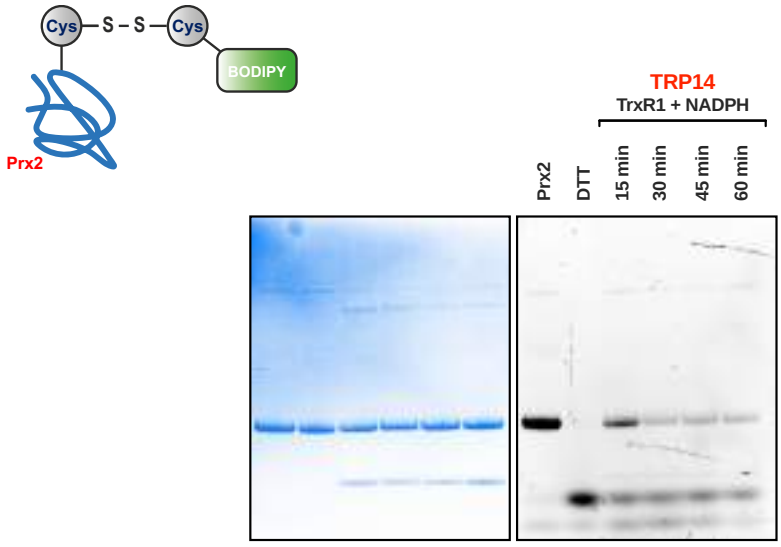

Figure 33. Long time-course of de-cysteinylation of cysteinylated peroxiredoxin 2 by the TRP14 enzymatic system. Representative fluorescence image (right) of cysteinylated peroxiredoxin 2 incubated with 10 µM TRP14 in presence of 30 nM TrxR1 and 300 mM NADPH for 15-60 min. Coomassie staining of the gel is shown as a loading control (left). *Prx2: peroxiredoxin 2; DTT: dithiothreitol; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

4.1.8. Cysteinylated Prx2 lacks peroxidase activity towards H_2O_2 but its activity is recovered upon de-cysteinylation by TRP14

Since protein cysteinylation could be a mechanism involved in the regulation of enzyme activity, in the laboratory of Prof. Elias Arnér at the Karolinska Institutet we devised an experiment in order to check whether cysteinylated peroxiredoxin 2 was functional or not. The ferrous oxidation-xylenol orange (Fox) assay (Jiang, Hunt and Wolff, 1992) was performed in order to assess the ability of peroxiredoxin 2 to reduce hydrogen peroxide in the reaction medium. For this purpose, peroxiredoxin 2 (1 µM) and cysteinylated peroxiredoxin 2 (1µM) were incubated in presence of the

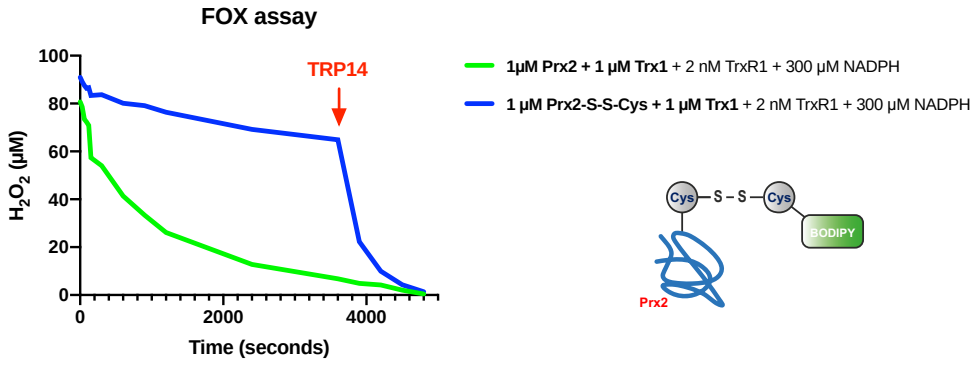

Figure 34. Peroxiredoxin 2 activity was lost upon cysteinylation. Representative Fox assay showing that 1 μM cysteinylated peroxiredoxin 2 (blue line) was not able to clear H_2O_2 from the reaction medium in presence of 1 μM Trx1, 2 nM TrxR1 and 300 μM NADPH. However, upon 10 μM TRP14 addition (red arrow), cysteinylated peroxiredoxin 2 recovered its ability to clear H_2O_2 from the medium. 1 μM peroxiredoxin 2 was used as a control (green line). *Prx2: peroxiredoxin 2; Prx2-S-S-Cys: cysteinylated peroxiredoxin 2; Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

thioredoxin system (1 μM Trx1, 2 nM TrxR1, and 300 μM NADPH) and 100 μM H_2O_2. Peroxiredoxin 2 was able to rapidly reduce hydrogen peroxide in the reaction medium (**Figure 34**, green line), whereas cysteinylated peroxiredoxin 2 did not show ability to reduce hydrogen peroxide in the medium (**Figure 34**, blue line). However, when 10 μM TRP14 was added into the reaction mixture containing cysteinylated Prx2 (red arrow in **Figure 34**), peroxiredoxin 2 could rapidly reduce the hydrogen peroxide present in the medium, suggesting that upon de-cysteinylation of the mixed disulfide by TRP14, peroxiredoxin 2 recovered its function.

In order to prove that TRP14 does not support peroxiredoxin 2 activity but only de-cysteinylates cysteinylated peroxiredoxin 2, both peroxiredoxin 2 (1 μM) and cysteinylated peroxiredoxin 2 (1 μM) were incubated with 1 μM TRP14 and the components of the enzymatic system (2 nM TrxR1 and 300 μM NADPH). As expected, reduction of hydrogen peroxide was observed in

the medium when TRP14 was used instead of Trx1 for supporting Prx2 function (**Figure 35**). However, upon addition of 10 µM Trx1 to the reaction medium, marked H_2O_2 reduction was observed in both peroxiredoxin 2 (green line) and cysteinylated peroxiredoxin 2 (blue line) (**Figure 35**), consistent with the recovery of Prx2 peroxidase activity after de-cysteinylation, which took place during TRP14 pre-incubation (plateau in **Figure 35**).

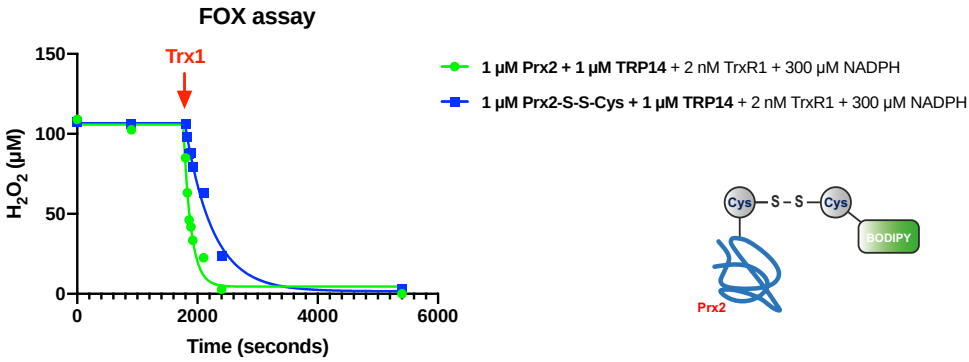

Figure 35. Peroxiredoxin 2 activity was not supported by TRP14. Representative Fox assay showing 1 µM peroxiredoxin 2 (green line) and 1 µM cysteinylated peroxiredoxin 2 (blue line) ability to reduce H_2O_2 in the reaction medium in presence of 1 µM TRP14, 2 nM TrxR1, and 300 µM NADPH. After the addition of 10 µM Trx1 to the reaction mixture (red arrow), both peroxiredoxin and cysteinylated peroxiredoxin were able to clear H_2O_2 from the reaction medium. *Prx2: peroxiredoxin 2; Prx2-S-S-Cys: cysteinylated peroxiredoxin 2; Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; TRP14: 14 kDa thioredoxin-related protein* (n=3).

4.2. *In vivo* studies

4.2.1. Protein cysteinylation and protein γ-glutamylcysteinylation increased in pancreas from TRP14 knockout mice upon acute pancreatitis

Given the changes in protein cysteinylation observed *in vitro* upon deficiency of TRP14, we decided to test whether these effects could be mimicked *in vivo* in TRP14 knockout mice under basal conditions and also in acute pancreatitis as a model of acute inflammation that may be associated with protein cysteinylation (Moreno *et al.,* 2014).

Surprisingly, the levels of protein cysteinylation observed by UHPLC-MS/MS in pancreas from TRP14 knockout mice under basal conditions were much lower than those observed in wild type mice (**Figure 36**). After acute pancreatitis induction, protein cysteinylation levels decreased in wild type mice (**Figure 36**); however, they markedly increased in TRP14 knockout mice with pancreatitis, differences being significant compared to both knockout controls under basal conditions, and wild type mice with pancreatitis (**Figure 36**).

A different profile was observed for protein γ-glutamylcysteinylation. Although no differences were observed at the basal level between wild type and TRP14 knockout mice, a remarkable increase in protein γ-glutamylcysteinylation was detected in pancreas of TRP14 knockout mice with pancreatitis, compared to both knockout controls under basal conditions and wild type mice with pancreatitis (**Figure 37**). Interestingly, no significant changes in protein glutathionylation were observed between wild type mice

and TRP14 knockout mice under basal conditions nor in acute pancreatitis (**Figure 38**).

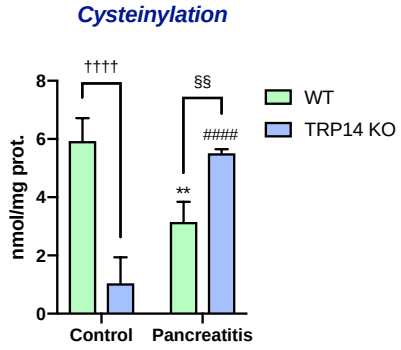

Figure 36. Protein cysteinylation levels in pancreas from wild type and TRP14 knockout mice under basal conditions and in acute pancreatitis. *†††† P < 0.00005 vs. WT control; ** P < 0.005 vs. WT control; #### P < 0.00005 vs. KO control; §§ P < 0.005 vs. WT pancreatitis* (n=6).

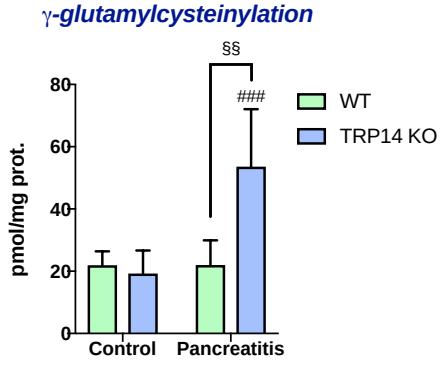

Figure 37. Protein gamma-glutamylcysteinylation levels in pancreas from wild type and TRP14 knockout mice under basal conditions and in acute pancreatitis. *### P < 0.0005 vs. KO control; §§ P < 0.005 vs. WT pancreatitis* (n=6).

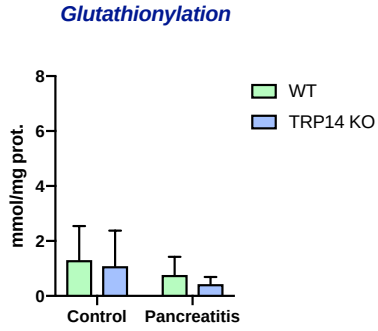

Figure 38. Protein glutathionylation levels in pancreas from wild type and TRP14 knockout mice under basal conditions and in acute pancreatitis. *No significant changes in protein glutathionylation levels were observed* (n=6).

4.2.2. Cystine accumulation was observed in pancreas from TRP14 knockout mice with acute pancreatitis

Since TRP14 shows cystine reductase activity (Pader *et al.*, 2014), it is not surprising that cystine levels in pancreas rose dramatically after the induction of acute pancreatitis only in TRP14 knockout mice (**Figure 39**). Although a significant increase in cysteine levels was also observed in pancreas from TRP14 knockout mice with pancreatitis, the cystine/cysteine ratio showed that this redox pair was shifted to the oxidized form in TRP14 knockout mice with pancreatitis (**Figure 39**). It is worth noting that in wild type mice both cysteine and cystine levels were markedly lower upon pancreatitis than under basal conditions, whereas the cysteine/cysteine ratio did not change significantly.

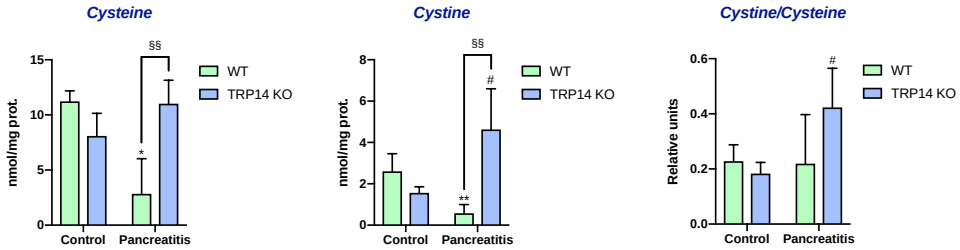

Figure 39. Cysteine and cystine levels, and cystine/cysteine ratio in pancreas from wild type and TRP14 knockout mice under basal conditions and upon cerulein-induced acute pancreatitis. ** P < 0.05 vs. WT control; ** P < 0.005 vs. WT control; # P < 0.05 vs. KO control; § P < 0.05 vs. WT pancreatitis; §§ P < 0.005 vs. WT pancreatitis* (n=6).

4.2.3. The GSSG/GSH ratio did not change during acute pancreatitis

Due to its importance in the maintenance of intracellular redox status and thiol redox status in proteins, we decided to analyze GSH and GSSG levels in pancreas from wild type mice and TRP14 knockout mice (**Figure 40**). Pancreatic GSH levels were not significantly different between wild type and TRP14 knockout mice under basal conditions (**Figure 40**). GSH levels in pancreas from wild type mice decreased in acute pancreatitis; however, surprisingly, in TRP14 knockout mice with pancreatitis, GSH levels were about fivefold higher compared to wild type mice with pancreatitis (**Figure 40**). Interestingly, upon pancreatitis induction, an increase in GSSG levels was measured in TRP14 knockout mice with pancreatitis (**Figure 40**). Interestingly, upon pancreatitis, an increase in pancreatic GSSG levels was also found in TRP14 knockout mice, but no significant increase occurred in GSSG levels in wild type mice (**Figure 40**). However, the GSSG/GSH ratio showed no significant changes among experimental groups, indicating that no imbalance of this redox pair occurred in the course of acute pancreatitis nor upon deficiency of TRP14 (**Figure 40**).

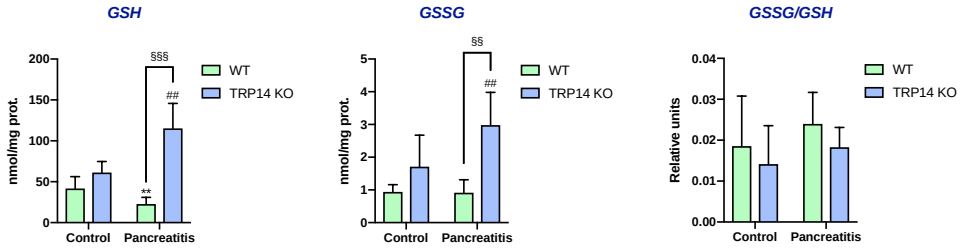

Figure 40. GSH and GSSG levels, and GSSG/GSH ratio in pancreas from wild type and TRP14 knockout mice under basal conditions (control) and upon acute pancreatitis. ** *P < 0.005 vs. WT control;* ## *P < 0.005 vs. KO control;* §§ *P < 0.005 vs. WT pancreatitis;* §§§ *P < 0.0005 vs. WT pancreatitis* (n=6).

4.2.4. TRP14 deficiency triggered an increase in γ-glutamylcysteine and γ-glutamylcystine levels in pancreas upon pancreatitis

Continuing with the study of redox pairs, we analyzed the levels and redox status of another low-molecular-weight thiol: γ-glutamylcysteine. Although TRP14 deficiency did not change γ-glutamylcysteine levels in pancreas under basal conditions, upon acute pancreatitis γ-glutamylcysteine levels decreased significantly in pancreas from wild type mice but increased by sevenfold in pancreas from TRP14 knockout mice (**Figure 41**). Its oxidized form, γ-glutamylcystine, also increased markedly in TRP14 knockout mice with pancreatitis without affecting the γ-glutamylcystine/γ-glutamylcysteine ratio (**Figure 41**). Strikingly, the γ-glutamylcystine/γ-glutamylcysteine ratio showed an increase in pancreas from wild type mice with pancreatitis due to a marked decrease in γ-glutamylcysteine levels in wild type mice following acute pancreatitis induction (**Figure 41**).

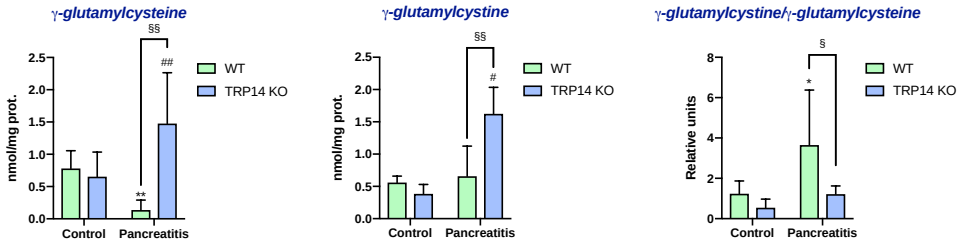

Figure 41. γ-glutamylcysteine and γ-glutamylcystine levels, and γ-glutamylcystine/γ-glutamylcysteine ratio in pancreas from wild type and TRP14 knockout mice under basal conditions and upon cerulein-induced acute pancreatitis. ** P < 0.05 vs. WT control; ** P < 0.005 vs. WT control; # P < 0.05 vs. KO control; ## P < 0.005 vs. KO control; § P < 0.05 vs. WT pancreatitis; §§ P < 0.005 vs. WT pancreatitis* (n=6).

4.2.5. TRP14 deficiency caused homocysteine accumulation in pancreas

We also measured the levels and redox status of homocysteine in pancreatic samples from wild type and TRP14 knockout mice (**Figure 42**). Homocysteine levels in TRP14 knockout mice were significantly higher than in wild type mice under basal conditions, and the difference remained after acute pancreatitis induction (**Figure 42**). No significant changes in homocystine levels were observed among the four groups (**Figure 42**). The homocystine/homocysteine ratio slightly increased in the case of wild type mice with pancreatitis which was not observed in TRP14 knockout mice with pancreatitis (**Figure 42**).

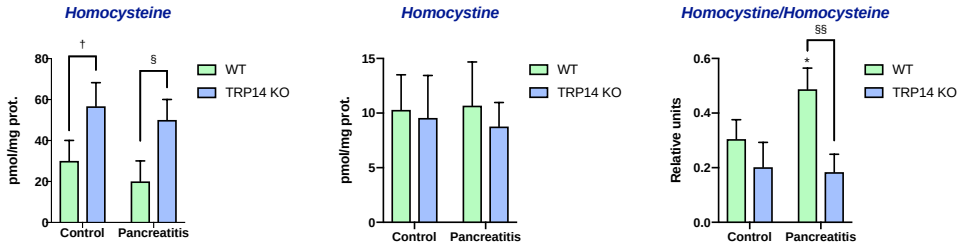

Figure 42. Homocysteine and homocystine levels, and homocystine/homocysteine ratio in pancreas from wild type and TRP14 knockout mice under basal conditions and upon cerulein-induced acute pancreatitis. ** P < 0.05 vs. WT control; † P < 0.05 vs. WT control; §§ P < 0.005 vs. WT pancreatitis* (n=6).

4.2.6. TRP14 deficiency triggered upregulation of the transsulfuration pathway in pancreas upon pancreatitis

As we found marked changes in the previously-mentioned redox pairs that are metabolites of the transsulfuration pathway, we also considered it interesting to measure the levels of other key metabolites of this pathway in pancreas of wild type and TRP14 knockout mice under basal conditions and upon acute pancreatitis induction.

It is noteworthy that methionine levels were lower in pancreas from knockout mice when compared with pancreas from wild type mice (**Figure 43**). They markedly dropped upon acute pancreatitis in both wild type and TRP14 knockout mice, especially in the latter group where methionine levels were even lower (**Figure 43**).

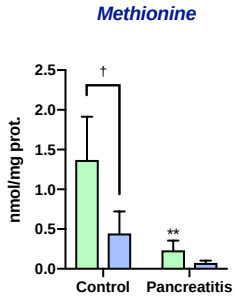

Figure 43. Methionine levels in pancreas from wild type and TRP14 knockout mice under basal conditions and upon cerulein-induced acute pancreatitis. *** P < 0.005 vs. WT control; † P < 0.05 vs. WT control* (n=6).

Cystathionine is another key metabolite of the transsulfuration pathway. No significant changes were found in cystathionine levels under basal conditions and TRP14 deficiency, but a marked increase in cystathionine levels was observed in TRP14 knockout mice with pancreatitis (**Figure 44**, left). This increase correlated with a rise in protein levels of cystathionine β-synthase, as shown by western blot (**Figure 44**, right), which is the enzyme responsible for the synthesis of cystathionine from homocysteine and serine.

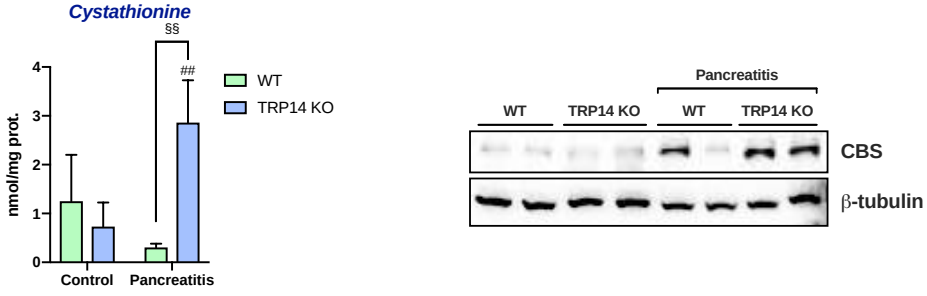

Figure 44. Cystathionine and CBS levels in pancreas from wild type and TRP14 knockout mice under basal conditions and upon cerulein-induced acute pancreatitis. Cystathionine levels determined by HPLC-MS/MS and representative western blot image showing CBS levels. β-tubulin is shown as loading control. *WT: wild type mice; TRP14 KO: TRP14 knockout mice; CBS: cystathionine beta-synthase. ## P < 0.005 vs. KO control; §§ P < 0.005 vs. WT pancreatitis* (n=6).

4.2.7. TRP14 deficiency triggered upregulation of the enzymatic antioxidant defense in pancreas upon pancreatitis

While no significant differences in mRNA expression of *Trx1* and *Trxr1* were observed between wild type and TRP14 knockout mice under basal conditions (**Figure 45**), the mRNA levels of both enzymes were markedly increased upon acute pancreatitis induction, this increase being significantly higher in TRP14 knockout mice than in wild type mice (**Figure 45**). Interestingly, a similar pattern was observed for glutathione reductase mRNA levels (**Figure 45**).

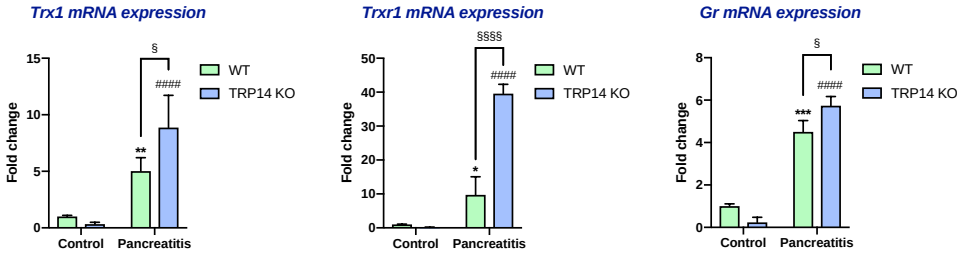

Figure 45. *Trx1*, *Trxr1*, and *Gr* mRNA expression in pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. *Trx1: thioredoxin 1; TrxR1: thioredoxin reductase 1; Gr: glutathione reductase. * P < 0.05 vs. WT control; ** P < 0.005 vs. WT control; *** P < 0.0005 vs. WT control; #### P < 0.00005 vs. TRP14 KO control; § P < 0.05 vs. WT pancreatitis; §§§§ P < 0.00005 vs. WT pancreatitis* (n=6)

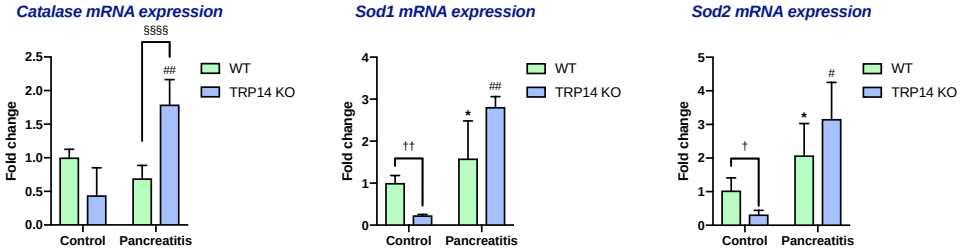

Figure 46. Expression of catalase as well as superoxide dismutase 1 and 2 in pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. *Sod1: superoxide dismutase 1; Sod2: superoxide dismutase 2. * P < 0.05 vs. WT control; # P < 0.05 vs. TRP14 KO control; ## P < 0.005; † P < 0.05 vs. WT control; †† P < 0.005 vs. WT control §§§§ P < 0.00005 vs. WT pancreatitis* (n=6).

Although under basal conditions the mRNA expression of *Sod1* and *Sod2* was lower in TRP14 knockout mice when compared to wild type mice (**Figure 46**), their mRNA expression increased with pancreatitis. In the case of catalase, its mRNA expression was significantly increased upon acute pancreatitis induction only in TRP14 knockout mice (**Figure 46**).

4.2.8. TRP14 deficiency triggered Nrf2 activation in pancreas upon pancreatitis

To elucidate if there was induction of genes involved in the antioxidant defense in pancreas of TRP14 knockout mice with pancreatitis, we analyzed the mRNA expression of typical Nrf2 target genes in both wild type and knockout mice under basal conditions and after acute pancreatitis induction. No significant changes were observed in the mRNA expression of any of the studied target genes under basal conditions between wild type and TRP14 knockout mice (**Figure 47**). However, the induction of acute pancreatitis caused a remarkable increase in the mRNA expression of *Nqo-1*, *Gclc* and *Ho-1* in TRP14 knockout mice which, in addition, was significantly higher

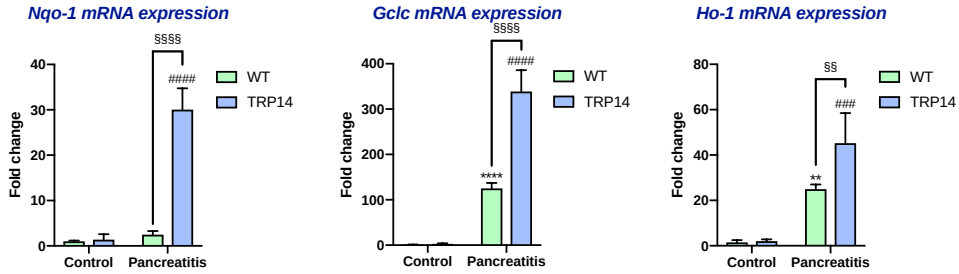

Figure 47. mRNA expression of Nr2 target in pancreas from wild type and TRP14 knockout mice under basal conditions, and in cerulein-induced acute pancreatitis. *Nqo-1: NAD(P)H dehydrogenase [quinone] 1; Gclc: glutamate-cysteine ligase catalytic subunit; Ho-1: heme oxygenase 1. ** P < 0.005 vs. WT pancreatitis; **** P < 0.00005 vs. WT pancreatitis; ### P < 0.0005 vs. KO control; §§ P < 0.005 vs. WT pancreatitis; §§§§ P < 0.00005 vs. WT pancreatitis* (n=6).

than the increase observed in wild type mice with pancreatitis, in the case of *Gclc* and *Ho-1* (**Figure 47**). No significant increase was found in the mRNA of *Nqo-1* in wild type mice with acute pancreatitis (**Figure 47**). After isolating nuclei from pancreatic tissue to study the nuclear translocation of NRF2 in the different experimental groups, we observed that the levels of NRF2 in nuclei from pancreas of wild type mice under basal conditions were much higher than those found in TRP14 knockout mice (**Figure 48**). However, after acute pancreatitis induction, NRF2 levels in the nuclei of wild type mice decreased, while NRF2 levels in knockout mice with pancreatitis markedly increased, thus inverting the pattern observed in basal conditions (**Figure 48**).

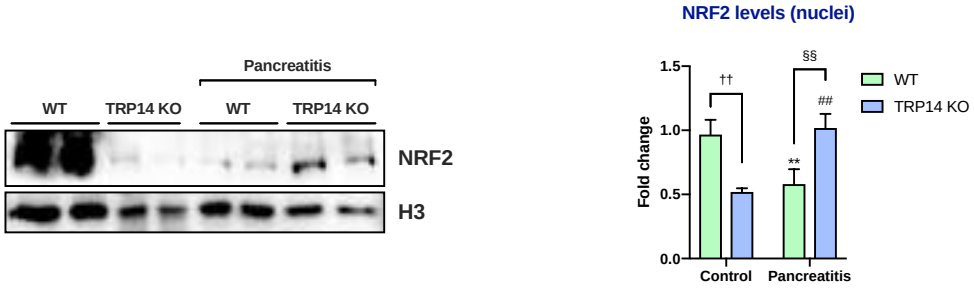

Figure 48. Nuclear Nrf2 levels in pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. Representative western blot image (left) showing nuclear NRF2 levels from pancreas upon acute pancreatitis induction. Histone 3 is shown as loading control. A densitometric analysis (right) of the blots is shown as well. *WT: wild type mice; KO: TRP14 knockout mice; Nrf2: nuclear factor erythroid 2-related factor 2; H3: histone 3.* ** *P < 0.005 vs. WT pancreatitis;* ## *P < 0.005 vs. KO control;* †† *P < 0.005 vs. WT control;* §§ *P < 0.005 vs. WT pancreatitis* (n=6).

4.2.9. mRNA expression of pro-inflammatory cytokines in acute pancreatitis was not affected by TRP14 deficiency

Figure 49 shows the mRNA levels of a panel of pro-inflammatory cytokines to characterize the inflammatory cascade in our acute pancreatitis model. No significant differences were observed under basal conditions in pancreas when comparing wild type mice with TRP14 knockout mice (**Figure 49**). Acute pancreatitis caused an increase in the mRNA levels of *Tnf-α*, *Il-6*, *Cxcl-1*, *Il-1β*, and *Il-18* in the pancreas from both, wild type and TRP14 knockout mice, with no significant difference between the two groups (**Figure 49**).

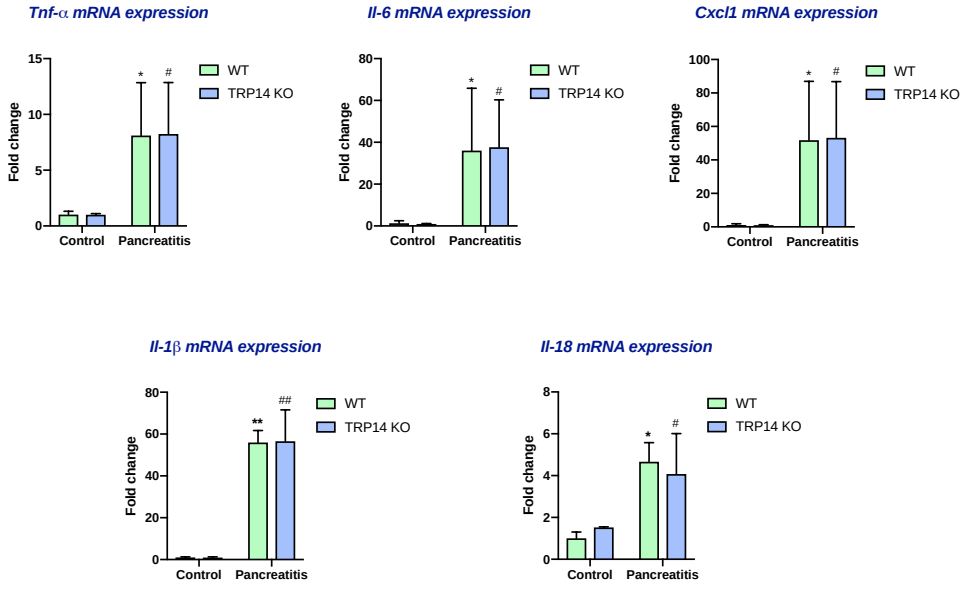

Figure 49. mRNA levels of proinflammatory cytokines in pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. *Tnf-α: tumor necrosis factor alpha; Il-6: interleukin 6; Cxcl-1: C-X-C motif ligand 1; Il-1β: interleukin 1-beta; Il-18: interleukin 18. * P < 0.05 vs. WT control; ** P < 0.005 vs. WT control; # P < 0.05 vs. KO control; ## P < 0.005 vs. KO control* (n=6).

4.2.10. TRP14 deficiency decreased tissue damage in pancreas upon pancreatitis

Sections of pancreatic tissue from wild type and TRP14 knockout mice were analyzed by conventional brightfield microscopy, both under basal conditions and after acute pancreatitis induction, to assess the presence of tissue edema and inflammatory infiltrate as the main indicators of tissue damage. Hematoxylin-eosin staining clearly revealed tissue edema and inflammatory infiltrate in the pancreas from wild type mice with pancreatitis (**Figure 50**). However, in TRP14 knockout mice with pancreatitis, only slight edema was observed with almost no infiltrate present in the pancreas

(**Figure 50**). No changes in the histological analysis were observed at the basal level between wild type and TRP14 knockout mice (**Figure 50**).

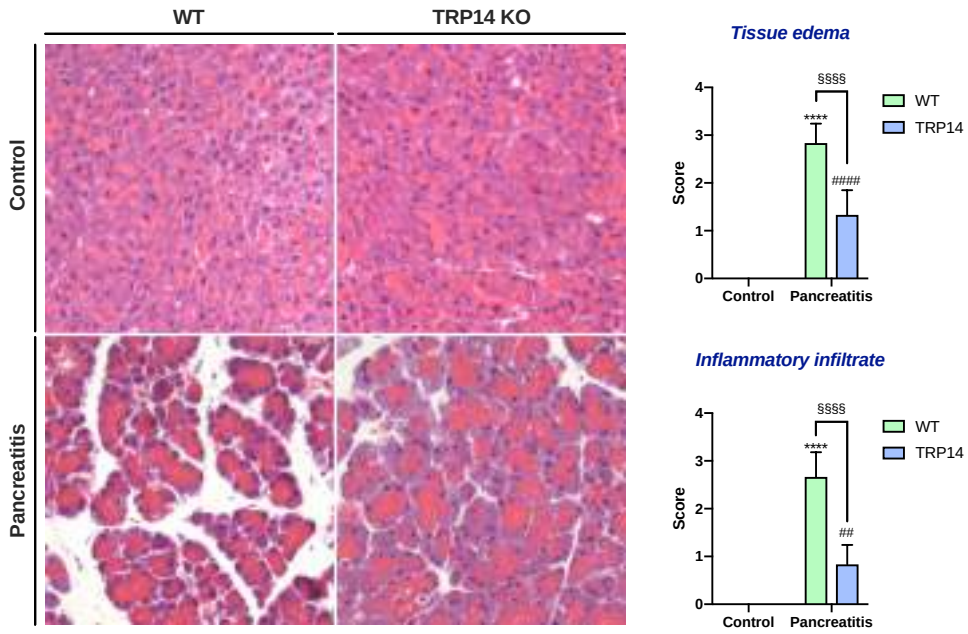

Figure 50. Hematoxylin-eosin staining of pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. A representative image from each group is shown in each panel. On the right side of the figure, the histological scores of tissue edema and inflammatory infiltration corresponding to the analysis of the tissue sections are shown (10 fields per sample). **** *P < 0.00005 vs. WT control;* ## *P < 0.005 vs. KO control;* #### *P < 0.00005 vs. KO control;* §§§§ *P < 0.00005 vs. WT pancreatitis* (n=6).

In addition, biochemical parameters were measured in plasma and pancreas, and they correlated with what was observed in the histological analysis. Both plasma amylase and lipase levels increased significantly after acute pancreatitis induction. However, the activities of both enzymes in TRP14 knockout mice with pancreatitis were significantly lower compared to wild type mice with pancreatitis (**Figure 51**). Myeloperoxidase (MPO) activity was measured in pancreatic tissue to quantify the degree of inflammatory infiltration (Van Laethem *et al.,* 1998; Perez *et al.,* 2019). MPO activity in

pancreas markedly increased in pancreatitis (**Figure 52**). However, again, TRP14 knockout mice with pancreatitis showed much lower pancreatic myeloperoxidase activity than wild type mice with pancreatitis (**Figure 52**). No significant differences were found under basal conditions concerning any of these parameters.

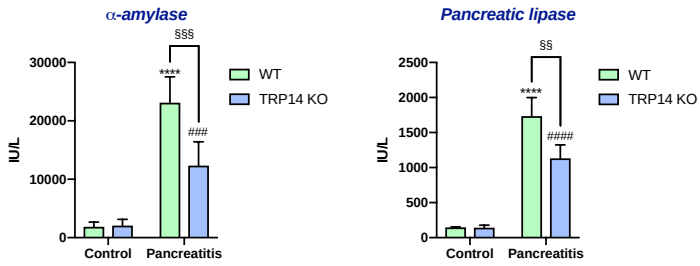

Figure 51. α-amylase and pancreatic lipase levels in serum from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. **** *P < 0.00005 vs. WT control;* ### *P < 0.0005 vs. WT control;* #### *P < 0.00005 vs. KO control;* §§ *P < 0.005 vs. WT pancreatitis;* §§§ *P < 0.0005 vs. WT pancreatitis* (n=6).

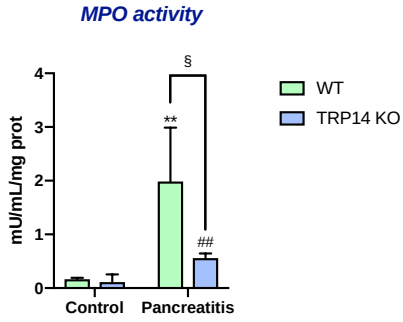

Figure 52. MPO activity in pancreas from wild type and TRP14 knockout mice under basal conditions and in cerulein-induced acute pancreatitis. *MPO: myeloperoxidase.* ** *P < 0.005 vs. WT control;* ## *P < 0.005 vs. WT control;* § *P < 0.05 vs. WT pancreatitis* (n=6).

V. DISCUSSION

5. DISCUSSION

5.1. TRP14 and thioredoxin 1 regulate protein cysteinylation

We have previously highlighted the importance of *S*-thiolation as a regulatory and protective mechanism against oxidative stress, the most common and abundant modification being the formation of mixed disulfides with glutathione (Mailloux, 2020). However, some organisms lack glutathione, as it is the case with many Gram-positive bacteria (Hochgräfe *et al.,* 2007; Loi, Rossius and Antelmann, 2015). For instance, in the case of *Bacillus subtilis*, cysteine is the most abundant low-molecular-weight thiol, and when oxidized to cystine, it leads to protein cysteinylation (Hochgräfe *et al.,* 2007). The same process was also observed in mammals under certain oxidative stress conditions, including necrotizing acute pancreatitis, which favored the oxidation of cysteine to cystine allowing the disulfide to react with free thiols within proteins, giving rise to mixed disulfides, specially protein cysteinylation (Moreno *et al.,* 2014).

In 2014, our laboratory proposed a model of disulfide stress defined as a specific type of oxidative stress associated with an acute inflammatory process, such as necrotizing acute pancreatitis (Moreno *et al.,* 2014). Based on these previous results showing that cysteine oxidation to cystine and protein cysteinylation are involved in the course of necrotizing acute pancreatitis, we decided to explore the role of TRP14 in regulating protein cysteinylation *in vitro* and *in vivo*, since it was reported that TRP14 shows cystine reductase activity (Pader *et al.,* 2014). For this purpose, we performed first *in vitro* experiments using HEK-293 control cells and stably TRP14 knockdown cells incubated with Cys-BIO, which allowed us to assess

the incorporation of labeled cysteine to proteins. As expected, TRP14 knockdown cells presented higher protein cysteinylation levels than their controls incubated with Cys-BIO. The obtained Western Blot images showed apparently a non-specific generalized cysteinylation pattern, with labeling observed at virtually all molecular weights.

The next step was to investigate whether these cysteinylated lysates obtained from TRP14 knockdown cells could be used as substrate for the TRP14 system. For this reason, these lysates were incubated with the components of the TRP14 system separately and in different combinations, being the greatest de-cysteinylanting activity observed when samples were incubated with all the components of the system together. Hence, these results suggest, for the first time, that TRP14 regulates protein cysteinylation *in vitro*.

Subsequently, we studied whether TRP14 exhibits any specificity towards particular proteins when promoting their de-cysteinylation. For this purpose, we performed a proteomic study to identify cysteinylation targets. The differential expression study showed 42 proteins whose cysteinylation was significantly increased in TRP14 knockdown cells incubated with Cys-BIO. Among them, we found proteins involved in the translation process, such as elongation factor 2, or in the detoxification of H_2O_2, such as Prx2. Therefore, these findings point to a role for TRP14 in the regulation of such important processes as translation and protein synthesis or the control of the cell's antioxidant defense.

We then determined the de-cysteinylating activity of TRP14 using peroxiredoxin 2 (Prx2) as substrate as well as the effect of cysteinylation on peroxiredoxn 2 function. We chose Prx2 as the model protein to perform our studies since it was one of the cysteinylation targets found in TRP14

knockdown cells. Human recombinant Prx2 cysteinylated in cysteine 172 lost its ability to clear H_2O_2 from the medium compared to its non-cysteinylated control. The activity of cysteinylated Prx2 was fully recovered after incubation with TRP14 and the rest of the components of the system. Furthermore, as with the cysteinylated lysates obtained from TRP14 knockdown cells, the greatest de-cysteinylating effect was observed when cysteinylated Prx2 was incubated with all components of the TRP14 system. Hence, this finding indicates on the one hand that TRP14 is capable of decysteinylating Prx2, and on the other that cysteinylation is a reversible post-translational protein modification that affects the enzymatic activity of Prx2. Our *in vitro* cysteinylation experiments with recombinant Prx2 show a new activity of TRP14 in addition to the regulation of protein persulfidation recently described by Dóka *et al.* (Dóka *et al.,* 2020). TRP14 can reduce inorganic polysulfides as well as persulfidated proteins, such as PTP1B or Prx2 (Dóka *et al.,* 2016; Dóka *et al.,* 2020). Both PTP1B and Prx2 were inactive when persulfidated (Dóka *et al.,* 2020). In both cases, subsequent incubation with TRP14 in presence of all components of the system reduced the persulfidated forms, thus recovering the normal activity of both enzymes (Dóka *et al.,* 2020). Moreover, in the case of Prx2, persulfidation of Cys172 conferred protection against irreversible oxidation of this cysteine residue (Dóka *et al.,* 2020).

As Trx1 has been described to reduce L-cystine, we also checked whether cysteinylated Prx2 could be a substrate for Trx1 as well. Our results show that the Trx1 system was able to reduce cysteinylated Prx2 even more efficiently than the TRP14 system, since only a five-minute incubation was needed to show a marked reduction in Prx2 cysteinylation compared to the 30 minutes required in the case of the TRP14 system. This result is surprising since Trx1 is five times less efficient than TRP14 in reducing

cystine (Pader *et al.,* 2014). However, based on our results, the Trx1 system may reduce cysteinylated Prx2 more efficiently than the TRP14 system. Whether the observed effect is specific to cysteinylated Prx2 or generalized to any cysteinylated substrate should be further studied.

Therefore, the results discussed above show that protein cysteinylation, which is regulated by the TRP14 and thioredoxin 1 systems, is a mechanism capable of controlling the activity of the enzymes it affects. We wondered what impact this post-translational modification might have on the inflammatory response *in vivo*.

5.2. TRP14 regulates disulfide stress in acute pancreatitis

Disulfide stress is characterized by oxidation of the redox pairs cysteine/cystine, γ-glutamylcysteine/γ-glutamylcystine, and homocysteine/homocystine, as well as by an increase in protein cysteinylation but without glutathione oxidation and therefore without changes in protein glutathionylation (Moreno *et al.,* 2014). The term disulfide stress had previously been used in bacteria to describe the generalized thiol oxidation induced by diamide, which also caused glutathione oxidation (Hochgräfe *et al.,* 2007; Loi, Rossius and Antelmann, 2015). However, in the model of disulfide stress associated with an acute inflammatory process in mammals proposed by Moreno *et al.*, no such effects are observed (Moreno *et al.,* 2014). Besides, the results obtained in our laboratory support the hypothesis raised in Dr. Toledano's laboratory, who suggested that control of the redox state of protein thiols does not depend critically on glutathione levels (Kumar *et al.,* 2011).

The taurocholate-induced necrotizing acute pancreatitis model in rats used in the study by Moreno *et al.* showed the characteristic depletion of GSH levels during the early course of the disease (Moreno *et al.,* 2014). In addition, they observed an increase in the levels of cysteine and cystine in pancreas upon acute pancreatitis (Moreno *et al.,* 2014). It is precisely the high levels of the latter, the disulfide, that seem to be responsible for the increase in protein cysteinylation observed in the course of acute pancreatitis (Moreno *et al.,* 2014) since free protein thiols can react with disulfides such as cystine to form mixed disulfides (Hochgräfe *et al.,* 2007).

In addition to protein cysteinylation, a widespread increase in the oxidation of protein thiols was observed upon pancreatitis (Moreno *et al.,* 2014). Two principal targets of disulfide stress were identified: redox buffers and proteins involved in redox signaling. Among the former, the ribonuclease inhibitor and albumin were identified, while among the latter, thioredoxin 1, APE1/Ref1, PTPs, and PP2A were identified (Moreno *et al.,* 2014). It is interesting to note that, as a consequence of the oxidation of cysteine residues in the active center, a decrease in the activity of protein phosphatases was observed after the induction of acute pancreatitis (Moreno *et al.,* 2014). Furthermore, in the case of PP2A, the loss of its activity was prevented by the administration of *N*-acetylcysteine, indicating that its inactivation was due to a reversible oxidative process (Moreno *et al.,* 2014). Moreover, activation of the NRF2 pathway, as confirmed by PCR of its targets (*Ho-1* and *Nqo1*), was observed upon Keap1 oxidation in taurocholate-induced necrotizing acute pancreatitis in rats (Moreno *et al.,* 2014). The role of NRF2 activation in cerulein-induced pancreatitis and its relationship with TRP14 will be described in detail later on.

The cerulein-induced acute pancreatitis experimental model in mice used in this Thesis differs from the taurocholate model of acute pancreatitis in rats

used by Moreno *et al.* to describe disulfide stress for the first time (Moreno *et al.,* 2014; Pérez *et al.,* 2019). Both models show the characteristic depletion of GSH levels after the induction of pancreatitis; however, the increase in cystine levels and protein cysteinylation was only found in taurocholate-induced pancreatitis and it does not occur in cerulein-induced pancreatitis in mice. Different mechanisms of GSH hydrolysis or depletion in pancreas during pancreatitis might be involved in order to explain the marked differences in the profiles of cystine and mixed disulfides in these experimental models in wild type animals.

Although Moreno *et al.* did not detect cysteinylated proteins in pancreas under basal conditions, in our model developed in this Thesis, we did detect cysteinylated proteins in pancreas from wild type sham mice. Surprisingly, in our model, TRP14 knockout mice showed significantly lower levels of cysteinylated proteins in pancreas than wild type sham mice under basal conditions, which may be paradoxical since TRP14 shows cystine reductase activity (Pader *et al.,* 2014) and de-cysteinylating activity as we have described here. Therefore, cystine accumulation would be expected in the TRP14 knockout mouse leading to increased protein cysteinylation. However, cystine levels did not show significant differences between wild type and knockout TRP14 sham mice under basal conditions, so the drop observed in cysteinylation levels might be due to some other factor, such as a potential induction of thioredoxin 1 which, as we have demonstrated in this Thesis, is also able to reduce the disulfide bonds of cysteinylated proteins. In acute pancreatitis there was a marked increase in protein cysteinylation levels in pancreas from TRP14 knockout mice, while there was a decrease in cysteinylation levels in pancreas from wild type mice, both compared to their respective sham groups. This time, as expected, the changes in protein cysteinylation profile flawlessly matched cystine levels in the pancreas.

Therefore, although in pancreas from sham animals we did not observe the expected increase in protein cysteinylation, after the induction of acute pancreatitis in TRP14 knockout mice there was a significant increase in cysteinylation levels, one of the main characteristics of disulfide stress (Moreno *et al.,* 2014).

Other than in experimental acute pancreatitis, the detection of cysteinylated proteins has been considered as a marker of oxidative stress in some diseases (Nagumo *et al.,* 2014; Tanaka, R. *et al.,* 2020). For example, the detection of cysteinylated albumin in plasma from patients with end-stage renal failure was associated with increased oxidative stress (Regazzoni *et al.,* 2013). Interestingly, after hemodialysis cysteinylated albumin levels were significantly decreased in these patients (Regazzoni *et al.,* 2013). The same effects were observed after kidney transplant in this type of patients (Tanaka *et al.,* 2020). Increased plasma levels of cysteinylated albumin were also observed in chronic liver diseases, such as cirrhosis, associated with a poor prognosis in hospitalized patients (Domenicali *et al.,* 2014). It is also interesting to mention that gestational diabetes resulted in increased cysteinylated albumin levels in the amniotic fluid after 15 weeks of pregnancy (Boisvert, Koski and Skinner, 2010).

Moreno *et al*. also described for the first time protein γ-glutamylcysteinylation, which increased in pancreas during acute pancreatitis (Moreno *et al.,* 2014). In our model, protein γ-glutamylcysteinylation was detected in sham mice, with no changes observed between wild type and TRP14 knockout mice. However, in acute pancreatitis protein γ-glutamylcysteinylation levels in TRP14 knockout mice significantly increased, in agreement again with another main characteristic of disulfide stress (Moreno *et al.,* 2014). In this case, the profile of protein γ-

glutamylcysteinylation levels matched the levels of γ-glutamylcystine, the oxidized form –the disulfide– being once more apparently in charge of regulating the process of mixed disulfide formation.

Regarding protein glutathionylation in pancreas, no changes were observed in the levels of any of the groups studied in this Thesis, either in controls or after pancreatitis induction, precisely in accordance to Moreno *et al.* (Moreno *et al.,* 2014). It is remarkable that no changes in protein glutathionylation were observed in spite of the significant increase in GSSG levels detected in pancreas from TRP14 knockout mice with pancreatitis. However, as it will be discussed later, GSH levels also increased in that group, so the GSSG/GSH ratio did not vary in TRP14 knockout mice with pancreatitis.

Once it had been proven that cerulein-induced acute pancreatitis developed with the main characteristics of disulfide stress in TRP14-deficient mice, we analyzed the state of the main redox pairs as well as the key metabolites of the transsulfuration pathway.

5.3. TRP14 deficiency avoids GSH depletion and up-regulates the transsulfuration pathway in pancreas in acute pancreatitis

The redox pair that we consider to be fundamental in this Thesis is that formed by cysteine and cystine, since TRP14 has cystine reductase activity and, therefore, is key for the reduction of cystine to cysteine (Pader *et al.,* 2014). Under basal conditions no significant differences in cysteine levels between wild type and TRP14 knockout mice were observed. However, after acute pancreatitis induction, a significant drop in cysteine levels occurred in wild type mice while levels remained stable in TRP14 knockout mice,

compared to their respective sham groups. A rather similar profile was observed for the oxidized form: in sham animals, no change in cystine levels was observed between wild type and TRP14 knockout mice, while pancreatitis caused a decrease in cystine levels of wild type mice. However, cystine levels were significantly increased in TRP14 knockout mice with pancreatitis. As for the cystine/cysteine ratio, a significant increase was observed in TRP14 knockout mice with pancreatitis, indicating a prevalence of the oxidized form. Therefore, TRP14 plays a role in the regulation of disulfide stress associated with acute inflammation by regulating cystine levels.

We have previously discussed the marked decrease in GSH levels that occurred after the induction of acute pancreatitis in wild type mice (Moreno *et al.,* 2014; Pérez *et al.,* 2015; Pérez *et al.,* 2019). However, it is very interesting to note that, in TRP14 knockout mice with pancreatitis, a significant increase in pancreatic GSH levels was detected. In turn, pancreatic GSSG levels –the oxidized form– also increased markedly in TRP14 knockout mice with pancreatitis, with no significant changes observed among the other groups. When calculating the GSSG/GSH ratio, which indicates how oxidized the redox pair is, no significant changes were observed between groups. This is because these increases in the reduced form and in the oxidized form in pancreas from TRP14 knockout mice were similar. Therefore, when interpreting the results, we should not overlook the increase in GSH levels in TRP14-deficient mice with pancreatitis, as these levels are a clear sign that GSH synthesis was induced.

Both γ-glutamylcysteine and γ-glutamylcystine levels were increased in pancreas from TRP14 knockout mice with pancreatitis. However, there was a marked drop in pancreatic γ-glutamylcysteine levels in wild type mice after acute pancreatitis induction –as we recently described (Rius-Perez *et al.,*

2020)–, with no change in γ-glutamylcystine levels. Therefore, the γ-glutamylcystine/γ-glutamylcysteine ratio remained unchanged between groups except for a significant increase in the case of wild type mice with pancreatitis due to the sharp drop in the levels of the reduced form rather than the accumulation of the oxidized form.

The last of the redox pairs studied in this Thesis was the one formed by homocysteine and homocystine. In this case, differences under basal conditions between wild type and TRP14 knockout mice were observed. Deficiency in TRP14 resulted in the accumulation of homocysteine in the pancreas compared to wild type mice under basal conditions and also in acute pancreatitis. Induction of acute pancreatitis did not alter significantly homocysteine levels, maintaining similar differences between wild type and TRP14 knockout mice. As for homocystine levels, no differences were observed between groups, either in control mice or after pancreatitis induction.

Taking into account our results that suggested upregulation of the transsulfuration pathway in TRP14 knockout mice with pancreatitis –increased cysteine, γ-glutamylcysteine, and GSH levels–, we studied methionine levels. Interestingly, already under basal conditions a decrease in methionine levels was observed in TRP14 knockout mice compared to wild type mice. Furthermore, after acute pancreatitis induction, methionine levels exhibited a dramatic drop in wild type mice –as we recently described (Rius-Perez *et al.,* 2020)–, being this drop even more pronounced in TRP14 knockout mice.

These observations are consistent with an increase in the flow of the transsulfuration pathway in TRP14 knockout mice with pancreatitis, just as it happens, for example, in experimental models of sepsis (Malmezat *et al.,*

2000; Semmler *et al.,* 2008). That increase would lead to depletion of methionine, the precursor of the remaining metabolites of the pathway, and accumulation of the final product, cysteine. Cysteine would then be used in the synthesis of GSH whose levels also increase in pancreas upon TRP14 deficiency and pancreatitis. Besides, we observed an increase in cystathionine levels in pancreas from TRP14-deficient mice with pancreatitis likely as a result of CBS induction, as revealed by western blot, confirming in this way the induction of the pathway.

As for the role of CBS in gastrointestinal pathology, it seems that its deficiency is harmful as severe manifestations of Chron's disease have been associated with CBS deficiency (Alsahli *et al.,* 2018), and patients with ulcerative colitis showed in their biopsies a lower expression of CBS that could contribute to the aggravation of inflammatory lesions caused to the intestinal barrier (Chen *et al.,* 2019). Patients with homocystinuria associated with CBS deficiency had elevated levels of pro-inflammatory cytokines, such as IL-1α, IL-6, TNF-α, Il-17, IL-12, IL-9, and TGF-α (Keating *et al.,* 2011). Furthermore, we have recently shown that blockade of CBS by nitration occurs in experimental cerulein-induced acute pancreatitis in association with the inflammatory response (Rius-Perez *et al.,* 2020). The increased CBS levels in TRP14-deficient mice after pancreatitis induction may have a beneficial effect. This effect could be mediated by an increase in the levels of hydrogen sulfide, whose anti-inflammatory effect on the gastrointestinal tract has been widely described (Gemici and Wallace, 2015). Anyhow, whether the observed beneficial effects are due to an increase in hydrogen sulfide following the induction of CBS or caused by any other factor should be studied further.

5.4. TRP14 deficiency triggers NRF2 activation in acute pancreatitis reducing tissue damage

We explored the mechanistic explanation for the differences between wild type and TRP14 knockout mice after acute pancreatitis induction. The expression of the messenger RNA (mRNA) of the enzymes Trx1, TrxR1, and glutathione reductase was up-regulated in pancreas after the induction of acute pancreatitis with cerulein in wild type mice, this increase being markedly higher in the case of TRP14 knockout mice with pancreatitis. This increase in mRNA expression observed in TRP14 knockout mice is particularly interesting since all these three enzymes are NRF2 downstream targets. Hence, the induction of these targets in TRP14-deficient mice with pancreatitis would indicate activation of the NRF2 transcription factor in the absence of TRP14, as further discussed below.

As we had evidence of possible NRF2 activation in pancreas from TRP14 knockout mice with pancreatitis, we measured the expression of other classical NRF2 targets. Given the scope of this Thesis, it is particularly interesting to mention that NRF2 regulates the expression of proteins from the thioredoxin family, namely thioredoxin 1 and thioredoxin reductase, as well as peroxiredoxins and sulfiredoxin (Cuadrado *et al.,* 2019). Among NRF2 targets, we also find enzymes that regulate the participation of quinones in redox cycling reactions –NQO1 being a clear example– as well as in glutathione depletion –such as GCLc–, as well as enzymes in charge of bilirubin and biliverdin synthesis, such as heme oxygenase-1 and biliverdin reductase respectively (Sedlak *et al.,* 2009; Cuadrado *et al.,* 2019). Concerning *Nqo-1*, *Gclc*, and *Ho-1* mRNA levels, no changes were observed under basal conditions in sham TRP14 knockout mice, but a significant increase in the expression of all three targets was detected upon acute

pancreatitis induction, with further increases in expression in TRP14 knockout mice with pancreatitis. Such differences in the expression of NRF2 targets might be sufficient to state that NRF2 was much more active upon the absence of TRP14, nevertheless, we also decided to study the nuclear translocation of NRF2. In sham wild type mice, nuclear NRF2 levels in the pancreas were much higher than in TRP14 knockout mice, where they were almost non-detected. However, after induction of acute pancreatitis with cerulein, nuclear NRF2 levels unexpectedly decreased in pancreas from wild type mice compared to basal conditions, while they increased markedly in TRP14 knockout mice. It is known that when NRF2 translocates to the nucleus, it binds to ARE regions of target gene promoters in presence of p300, which decreases the availability of p300 to bind to p65, thus decreasing the expression of NF-κB controlled genes so that the activation of NRF2 can be considered to have an anti-inflammatory effect (Kim, S. *et al.,* 2013; Cuadrado *et al.,* 2019). Probably, in wild type mice with pancreatitis, the presence of some other transcription factor in the nucleus, such as NF-κB, could prevent NRF2 transactivation of target genes. Anyway, such translocation in pancreas from TRP14-deficient mice with pancreatitis would explain the marked induction of NRF2 targets in this group while being consistent with the observations of Moreno *et al.*, who suggested that the oxidation of Keap-1 under disulfide stress conditions would lead to nuclear translocation of NRF2 (Moreno *et al.,* 2014).

Regarding the main enzymes in charge of the antioxidant defense, it is interesting that under basal conditions the mRNA levels of *Sod1* and *Sod2* were lower in pancreas from TRP14 knockout mice than in wild type mice. However, in the case of catalase, no significant changes were observed. Interestingly, despite these lower expression levels in TRP14-deficient mice under basal conditions, after induction of acute pancreatitis, the expression

of all three enzymes markedly increased in pancreas from TRP14 knockout mice compared to wild type mice with pancreatitis that already showed a significant increase compared to their controls.

Regarding the most important pro-inflammatory cytokines involved in the inflammatory response of acute pancreatitis, it should be noted that no differences in their mRNA expression were found between wild type and TRP14 knockout mice. A similar increase was observed in both wild type and TRP14-deficient mice upon acute pancreatitis induction. Whether this equal expression of cytokines in both groups is due to NRF2 acting through AREs in the promoter regions of the genes of these cytokines (Cuadrado *et al.,* 2019) or due to some other factors, should be studied more in detail.

At the histological level, the study of pancreas sections stained with hematoxylin/eosin clearly revealed lower tissue edema and inflammatory infiltrate in pancreas from TRP14 knockout mice with pancreatitis. This finding is relevant because it would indicate less pancreatic damage in acute experimental pancreatitis in knockout mice. Besides, the activities of the pancreatic enzymes amylase and lipase in serum were lower in TRP14 knockout mice with pancreatitis. The same pattern was observed for MPO activity in pancreas (Pérez *et al.,* 2019), indicating inflammatory infiltrate in TRP14 knockout mice. This reduced severity of pancreatitis observed in TRP14-deficient mice could be due to activation of NRF2 and its anti-inflammatory potential. In agreement with our hypothesis, a recent study showed that overexpression of NRF2 protected against experimental acute pancreatitis induced by LPS or cerulein (Fu *et al.,* 2020). In rats in which *Nrf2* was overexpressed when treated with cerulein or LPS to induce acute pancreatitis, in both cases a decrease in pancreatic damage was observed as well as a decrease in serum amylase activity and c-reactive protein levels, demonstrating the beneficial effect of NRF2 activation in acute pancreatitis

(Fu *et al.,* 2020). AR42J rat pancreas acinar cells were also stimulated with 1 mg/L LPS, 10 nmol/L cerulein, or a combination of both and by overexpressing *Nrf2* in these cells, amylase activity as well as malondialdehyde and c-reactive protein levels decreased in stimulated cells, while SOD activity increased (Fu *et al.,* 2020).

Therefore, NRF2 may be responsible for the protection we observe in TRP14 knockout mice after acute pancreatitis induction, presenting a much milder form of acute pancreatitis than wild type mice, as it has been thoroughly described before. To date, three different mechanisms have been described by which NRF2 can develop its remarkable anti-inflammatory activity: its ability to regulate redox metabolism, to interact with NF-κB, and to directly down-regulate the production of pro-inflammatory cytokines (Cuadrado *et al.,* 2019). In our model, TRP14 deficiency favored the activation and nuclear translocation of NRF2 after the induction of acute pancreatitis with cerulein, which was not observed in wild type mice and, therefore, due to its strong protective and anti-inflammatory capacity, a milder form of the disease developed.

VI. CONCLUSIONS

6. CONCLUSIONS

In our study of the role of TRP14 in the regulation of protein cysteinylation *in vitro* and *in vivo* during cerulein-induced acute pancreatitis as a representative model of acute inflammation associated with disulfide stress, the following conclusions were obtained:

1. TRP14 regulates protein cysteinylation, both *in vitro* and *in vivo*:

   a. TRP14 and thioredoxin 1 both exhibit decysteinylating activity towards cysteinylated peroxiredoxin 2 in presence of thioredoxin reductase and NADPH, being the rate of decysteinylation higher with thioredoxin 1 than with TRP14.

   b. Peroxiredoxin 2 cysteinylated in cysteine 172 is inactive and the TRP14 system restores the activity of cysteinylated Prx2 by reduction of the disulfide bond of the mixed disulfide.

   c. Among the most affected targets of protein cysteinylation, we found proteins involved in RNA translation and protein synthesis, as well as in the elimination of oxidizing agents like hydrogen peroxide.

2. TRP14 deficiency protects against cerulein-induced acute pancreatitis in mice by triggering up-regulation of GSH synthesis and NRF2 activation.

   a. TRP14 knockout mice with pancreatitis exhibit less tissue edema, and inflammatory infiltrate and MPO activity in the pancreas than wild type mice with pancreatitis.

b. TRP14 deficiency leads to up-regulation of the transsulfuration pathway and glutathione synthesis in acute pancreatitis, resulting in increased GSH levels in the pancreas of TRP14 knockout mice with pancreatitis.

c. The nuclear levels of NRF2 in pancreas of TRP14 knockout mice with pancreatitis increased compared to wild type mice, leading to a marked up-regulation of target genes involved in the antioxidant defense, without changes in the expression of the main pro-inflammatory cytokines.

VII. BIBLIOGRAPHY

7. BIBLIOGRAPHY

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ANNEX

RESUMEN

Regulación de la cisteinilación de proteínas y de la cascada inflamatoria por la proteína de 14 kDa relacionada con la tiorredoxina (TRP14) en el páncreas

1. INTRODUCCIÓN

Estrés oxidativo, oxidación de proteínas y defensa antioxidante

Como consecuencia de la vida aeróbica se forman especies reactivas del oxígeno (ERO) que juegan un papel clave en la señalización celular normal y en procesos fisiológicos, como la activación de ciertos factores de transcripción o la dinámica redox de la célula (Stamati, Mudera and Cheema, 2011; Semenza, 2009). No obstante, se han relacionado las ERO con condiciones pro-oxidantes que conllevan deterioro de la función mitocondrial, daño celular a membranas y estructuras o mal funcionamiento de las enzimas (Halliwell, 1996).

Las ERO desempeñan un papel importante en numerosos procesos fisiológicos, como el control de la función celular (Dröge, 2002), su participación en el ejercicio físico (Sastre *et al.,* 1992; Magherini *et al.,* 2019) o en el envejecimiento (Liochev, 2013; Santos, Sinha and Lindner, 2018). Sin embargo, en condiciones de estrés oxidativo, cuando los niveles de ERO aumentan considerablemente son responsables del daño oxidativo a macromoléculas como ácidos nucleicos, lípidos, glúcidos y proteínas (Lambeth, 2007). En este sentido, se ha relacionado una amplia gama de

enfermedades con el aumento de los niveles de ERO, aunque actualmente aún no se ha establecido si las ERO elevadas son causa o consecuencia del proceso patológico (Lugrin *et al.,* 2014; Kudryavtseva *et al.,* 2016; Krata *et al.,* 2018).

En el interior de la célula se producen constantemente ERO como consecuencia de la respiración celular y de los procesos metabólicos. Como consecuencia, las células desarrollaron sistemas de defensa antioxidante para protegerse frente a las especies oxidantes. En 1995, Halliwell y Gutteridge definieron el término antioxidante como “cualquier sustancia que, cuando está presente a un nivel bajo en comparación con un sustrato susceptible de ser oxidado, previene o retrasa significativamente la oxidación de dicho sustrato” (Halliwell and Gutteridge, 1995). Atendiendo a sus propiedades químicas, los antioxidantes pueden ser enzimáticos o no enzimáticos.

Entre los antioxidantes enzimáticos encontramos la catalasa, que participa en la eliminación del peróxido de hidrógeno dando lugar a agua y oxígeno molecular (Chance, Sies and Boveris, 1979); las glutatión peroxidasas, que convierten el peróxido de hidrógeno y los lipoperóxidos en moléculas no reactivas (Chance, Sies and Boveris, 1979); y las superóxido dismutasas, que transforman el radical superóxido en peróxido de hidrógeno (McCord, Keele and Fridovich, 1971; Fridovich, 1978). Además de los anteriores, el sistema tiorredoxina/peroxirredoxina desempeña un papel fundamental en la detoxificación de ERO y en la reducción de modificaciones oxidativas de proteínas, como se detallará más adelante debido a su importancia en el marco de esta Tesis.

Los antioxidantes no enzimáticos son moléculas que no requieren apoyo enzimático para reaccionar directamente con las especies oxidantes,

aunque algunos de ellos pueden participar también en procesos antioxidantes enzimáticos. El glutatión reducido, la vitamina C, la vitamina E, los betacarotenos y el ácido úrico son típicos ejemplos de antioxidantes no enzimáticos. Aunque todos ellos desempeñan funciones importantes, el glutatión reducido (GSH) es el antioxidante tiólico no enzimático más abundante en la célula, donde se encarga del mantenimiento de la homeostasis redox (Meister and Anderson, 1983; Deponte, 2013; Couto, Wood and Barber, 2016).

El GSH se sintetiza en el citosol de las células de mamífero a partir de sus aminoácidos precursores: ácido glutámico, cisteína y glicina. La síntesis de glutatión es un proceso catalizado por dos enzimas. La primera es la glutamato-cisteína ligasa (GCL), que cataliza la síntesis de γ-glutamilcisteína a partir de ácido L-glutámico y L-cisteína. El segundo paso en la síntesis de GSH está catalizado por la glutatión sintetasa (GSS), que une la glicina con la γ-glutamilcisteína para formar el tripéptido γ-glutamilcisteinil glicina. La síntesis de glutatión está controlada por un mecanismo de retroalimentación negativo, ya que los niveles elevados de GSH inhiben la GCL y, por tanto, su propia síntesis (Viña, Hems and Krebs, 1978; Lu, S. C., 2013).

En condiciones fisiológicas, el factor limitante en la síntesis de glutatión es la disponibilidad de cisteína (Lu, 2013). La cisteína es un aminoácido que puede obtenerse de diferentes fuentes como la dieta, la proteólisis o por síntesis a partir de la metionina a través de la vía de la transulfuración (Sbodio, Snyder and Paul, 2019). La vía de la transulfuración tiene lugar en numerosos tejidos, aunque principalmente en el hígado, y consta de cinco reacciones: 1) activación de la metionina a *S*-adenosil metionina, 2) desmetilación de la *S*-adenosil metionina para formar *S*-adenosil homocisteína, 3) eliminación de la fracción *S*-adenosil para formar

homocisteína, 4) formación de cistationina a partir de homocisteína y serina y 5) escisión de la cistationina para dar cisteína y α-cetobutirato. Las reacciones más importantes en la regulación de la vía de la transulfuración son la primera, catalizada por la metionina *S*-adenosil transferasa, y la última, catalizada por la γ-cistationasa, y que es irreversible (Sbodio, Snyder and Paul, 2019).

Las proteínas son una de las principales dianas celulares de las ERO debido a la gran cantidad de residuos susceptibles de ser oxidados presentes en las cadenas laterales de los aminoácidos (Winterbourn and Kettle, 2013). Aunque ciertas modificaciones oxidativas pueden ser reversibles y desempeñan un papel importante en los procesos de señalización celular, las proteínas pueden sufrir modificaciones irreversibles en sus cadenas laterales, como la carbonilación, la hiperoxidación de los grupos tiólicos o la formación de ditirosina, lo que puede dar lugar a un plegamiento erróneo o a la pérdida de su función (Winterbourn and Kettle, 2013).

El grupo tiol de los residuos de cisteína se oxida primero a ácido sulfénico (–SOH), altamente reactivo, que a su vez puede oxidarse aún más a ácido sulfínico ($–SO_2H$) y sulfónico ($–SO_3H$) (Lo Conte and Carroll, 2013). Las formas sulfénica y sulfínica del grupo tiol de la cisteína son formas oxidadas reversibles, mientras que el ácido sulfónico se trata de un estado irreversible de oxidación del grupo tiol (Lo Conte and Carroll, 2013). Además, en condiciones de estrés oxidativo se pueden formar enlaces disulfuro entre dos proteínas (enlaces disulfuro intermoleculares) o entre residuos de cisteína de la misma proteína (enlaces disulfuro intramoleculares), dando lugar a cambios estructurales (principalmente cambios de conformación, mal plegamiento y agregación de proteínas) o a cambios en la actividad de las proteínas (Lo Conte and Carroll, 2013). Los tioles proteicos también

pueden reaccionar con tioles de bajo peso molecular como la cisteína libre o el GSH, así como con homocisteína, γ-glutamilcisteína o cisteinilglicina, dando lugar a disulfuros mixtos (Brigelius-Flohé, 2016). Estas modificaciones pueden revertirse mediante la reducción de los enlaces disulfuro formados entre las proteínas y los tioles de bajo peso molecular por la acción de las disulfuro reductasas como los sistemas tiorredoxina y glutarredoxina (Brigelius-Flohé, 2016). Dado que el GSH y la cisteína libre son los tioles de bajo peso molecular más abundantes, los disulfuros mixtos más comunes son las proteínas *S*-glutationiladas y *S*-cisteiniladas (Ghezzi, 2013).

Si el glutatión es el tiol de bajo peso molecular más abundante en las células de los mamíferos, la cisteína es el tiol de bajo peso molecular más abundante en los fluidos extracelulares y en el plasma, donde está presente principalmente en su forma de disulfuro: cistina (Giustarini *et al.,* 2009; Held, 2019). Debido a esta diferencia en la abundancia relativa de ambas especies, la glutationilación es la modificación proteica intracelular predominante en respuesta al estrés oxidativo, mientras que la cisteinilación se observa comúnmente en las proteínas plasmáticas circulantes (Giustarini *et al.,* 2009; Held, 2019). En este sentido, la albúmina sérica o la hemoglobina son susceptibles de ser cisteiniladas en condiciones de estrés oxidativo y se pueden detectar en plasma (Matsui *et al.,* 2019). Se han encontrado niveles elevados de cisteinilación de proteínas en diferentes patologías como Alzheimer (Poulsen *et al.,* 2014; Costa *et al.,* 2019), enfermedad renal crónica (Regazzoni *et al.,* 2013), cirrosis hepática (Domenicali *et al.,* 2014), enfermedades cardiovasculares (Belcastro *et al.,* 2017; Wakabayashi *et al.,* 2004), artritis reumatoide (Seward *et al.,* 2011) y pancreatitis aguda (Moreno *et al.,* 2014). A diferencia de la glutationilación de proteínas, la regulación de la cisteinilación de proteínas todavía no se ha

estudiado, y aún se desconoce. Sin embargo, este tipo de modificación oxidativa puede desempeñar un papel fundamental en la regulación redox de las proteínas que contienen grupos tiólicos en su centro activo y, al mismo tiempo, puede ser un mecanismo de protección contra la oxidación irreversible de las proteínas en condiciones de estrés oxidativo además de la glutationilación o cuando se depleciona el GSH (Hochgräfe *et al.,* 2007).

El sistema tiorredoxina y el papel de TRP14

El sistema de tiorredoxina es, junto con el glutatión, uno de los sistemas antioxidantes tiólicos más importantes en las células de los mamíferos. Este sistema disulfuro reductasa está compuesto por tiorredoxina (Trx), tiorredoxina reductasa (TrxR) y NADPH (Lu, J. and Holmgren, 2014). En mamíferos, existen dos sistemas de tiorredoxina diferentes: el sistema citosólico (Trx1) y el mitocondrial (Trx2) (Lu and Holmgren, 2014). Se han descrito tres isoformas diferentes de TrxR en mamíferos: TrxR1 (citosólica), TrxR2 (mitocondrial), y una isoforma especial en los testículos denominada tiorredoxina glutatión reductasa (TGR), que presenta un dominio glutarredoxina adicional en su estructura (Lu, J. and Holmgren, 2009; Cheng, Qing *et al.,* 2009).

A fin de cumplir su actividad antioxidante, el sistema tiorredoxina transfiere electrones principalmente a peroxirredoxinas, metionina sulfóxido reductasas y factores de transcripción sensibles al estado redox (Lu and Holmgren, 2014).

Las peroxirredoxinas (Prxs) son peroxidasas dependientes de Trx. En los seres humanos se han descrito seis isoformas diferentes presentes en distintos compartimentos celulares (Rhee, Woo and Kang, 2018). Según el

número de residuos de cisteína presentes en sus centros activos, las peroxirredoxinas pueden clasificarse como peroxirredoxinas de 2-Cys (Prx1, Prx2, Prx3 y Prx4), una peroxirredoxina atípica de 2-Cys (Prx5) y una peroxirredoxina de 1-Cys (Prx6) (Rhee, 2016). Las peroxirredoxinas de 2-Cys son las únicas isoformas capaces de aceptar electrones del sistema tiorredoxina para apoyar su actividad enzimática: la eliminación de peróxido de hidrógeno, hidroperóxidos y peroxinitrito (Rhee, 2016). Contienen dos residuos de cisteína en su centro activo: la cisteína N-terminal se conoce como cisteína peroxidática (Cys_P), que se desprotona a pH neutro debido a su bajo valor de pKa (alrededor de 5-6) formando un tiolato, mientras que la cisteína C-terminal recibe el nombre de cisteína resolutiva (Cys_R) (Rhee and Woo, 2020). Las peroxirredoxinas se reciclan mediante el sistema tiorredoxina, que se encarga de reducir el enlace disulfuro formado como consecuencia de su mecanismo catalítico (Rhee, 2016).

La TRP14 es una proteína citosólica de 14 kDa relacionada con la tiorredoxina, compuesta por 123 aminoácidos y que se expresa en una amplia variedad de células y tejidos. La TRP14 humana presenta un motivo WCPDC bien conservado en el centro activo, típico de las Trx; sin embargo, la homología de la secuencia con Trx1 es de tan sólo el 20% (Jeong, Yoon *et al.,* 2004). La estructura de TRP14 contiene cinco residuos de cisteína, aunque únicamente dos de ellas, las presentes en el centro activo WCPDC, se encuentran expuestas y son sensibles al estado redox (Jeong, Yoon *et al.,* 2004). Cuando TRP14 se oxida, inmediatamente se reduce por acción de la TrxR1 citosólica, pero no por la TrxR2 mitocondrial, como sí que sucede en el caso de Trx1 y Trx2 que se reducen de forma efectiva por ambas isoformas (Jeong, Yoon *et al.,* 2004).

Desde el punto de vista bioquímico, TRP14 es una disulfuro reductasa, al igual que Trx1. Sin embargo, TRP14 no puede reducir los sustratos típicos

de Trx1 como la insulina, la ribonucleótido reductasa, peroxirredoxinas o la metionina sulfóxido reductasa, lo que sugiere que TRP14 y Trx1 pueden tener diferentes funciones y, por tanto, actúan específicamente sobre sustratos diferentes (Jeong *et al.,* 2004). Las diferencias observadas a nivel de sustrato pueden deberse a la existencia de una hélice adicional en la estructura de TRP14, así como a una distribución de carga diferente de los residuos en comparación con Trx1 (Woo *et al.,* 2004).

El sistema TRP14 (TRP14, TrxR1 y NADPH) es capaz de reducir la cistina al romper su enlace disulfuro, dando lugar a dos moléculas de cisteína. La TrxR1 participa en el mecanismo al reducir el disulfuro que se forma en el centro activo de TRP14 como consecuencia de su actividad enzimática. Además, Pader y cols. demostraron que la TRP14 es más activa como cisteína reductasa que la Trx1, ya que es cinco veces más eficiente reduciendo L-cistina (Pader *et al.,* 2014).

Además, la TRP14 está involucrada en el control de numerosos procesos de señalización redox. En 2004 se descubrió que podía jugar un papel como inhibidor de NF-κB (Jeong, Chang *et al.,* 2004). De hecho, aunque Trx1 modula numerosos factores de transcripción, incluido NF-κB, TRP14 es un inhibidor más potente de la señalización de NF-κB que Trx1 a pesar de sus niveles de expresión más bajos en comparación con Trx1 (Jeong, Chang *et al.,* 2004). También se ha descrito su capacidad para reducir proteínas nitrosiladas al igual que Trx1, jugando un papel clave en los procesos de autofagia (Zhang *et al.,* 2015). De un modo similar, TRP14 es capaz de reducir tanto cadenas de polisulfuros inorgánicos como proteínas persulfuradas, jugando un papel esencial en la regulación de la señalización por sulfuro de hidrógeno (Dóka *et al.,* 2020). Otro caso algo más específico es la capacidad de TRP14 para reducir a PTP1B, que se oxida fácilmente

por la acción de las NOXs, involucrándose de este modo en el control de las cascadas de fosforilación de tirosinas (Dagnell *et al.,* 2013).

Pancreatitis aguda, mediadores inflamatorios y señalización redox

La pancreatitis aguda es un proceso inflamatorio de la glándula pancreática que cursa con un amplio espectro de manifestaciones clínicas derivadas de la afectación local y sistémica, y en la mayoría de los casos es leve. Sin embargo, la pancreatitis aguda puede evolucionar hacia una forma grave que no responde a ninguna estrategia terapéutica, lo que tiene consecuencias fatales en alrededor del 20 al 30% de los casos (Hines and Pandol, 2019).

Según la Clasificación de Atlanta revisada, la pancreatitis aguda se define como un "proceso inflamatorio agudo del páncreas, que puede afectar de forma variable a otros tejidos circundantes o incluso a otros órganos y sistemas, cuyas manifestaciones se caracterizan por la aparición repentina de dolor abdominal, frecuentemente acompañado de náuseas y vómitos, fiebre, taquicardia, leucocitosis, así como un aumento de las enzimas pancreáticas en la sangre y/o la orina" (Banks *et al.,* 2013).

En alrededor del 5-10% de los casos se desarrolla una necrosis del parénquima pancreático, del tejido peripancreático o de ambos (Banks *et al.,* 2013). Los pacientes que desarrollan pancreatitis necrotizante muestran un aumento de la mortalidad y de las tasas de intervención en comparación con los pacientes que desarrollan pancreatitis edematosa. La tasa de mortalidad de la pancreatitis necrotizante aguda es de alrededor del 17%, en comparación con sólo el 2% de mortalidad en los casos de pancreatitis edematosa intersticial aguda (Forsmark, Vege and Wilcox, 2016; van Dijk *et*

al., 2017). La tasa de mortalidad de la pancreatitis necrotizante es mucho mayor entre los pacientes que desarrollan una necrosis con infección del tejido afectado (aproximadamente el 30%) en comparación con los que desarrollan una necrosis estéril (alrededor del 12%) (Hines and Pandol, 2019).

La pancreatitis aguda es actualmente una de las principales causas de ingreso hospitalario en relación con los trastornos gastrointestinales, y su incidencia ha aumentado considerablemente durante la última década (Hines and Pandol, 2019). Aunque la etiología de la pancreatitis aguda es bastante diversa, los cálculos biliares y el abuso del alcohol son factores clave que desencadenan los mecanismos fisiopatológicos que dan lugar al desarrollo de la pancreatitis aguda (Lankisch, Paul Georg, Apte and Banks, 2015).

Los mecanismos que intervienen en la aparición y el desarrollo de la enfermedad comprenden una compleja cascada de eventos que se inicia en las células pancreáticas acinares, generalmente como resultado de una lesión de las células acinares y la consiguiente liberación de sus proteasas activas al tejido pancreático. La conversión del tripsinógeno en tripsina y su consiguiente acumulación en el parénquima pancreático inicia un proceso autodigestivo que estimula una respuesta inflamatoria. Las citoquinas proinflamatorias, las quimioquinas, las ERO y el calcio (Ca^{2+}) se consideran los principales mediadores de las complicaciones locales y sistémicas, dando lugar al fallo orgánico múltiple y muerte. Por lo tanto, la activación de las enzimas proteolíticas y la respuesta inflamatoria desencadenada en las células acinares podrían considerarse los principales procesos moleculares que conducen al desarrollo de la pancreatitis aguda (Bhatia *et al.,* 2005; Lankisch, P. G., Apte and Banks, 2015; Pérez *et al.,* 2015).

En cuanto al papel del estrés oxidativo en la pancreatitis aguda, en las últimas décadas, numerosos estudios han relacionado el daño pancreático observado en la pancreatitis aguda con el estrés oxidativo (Pérez *et al.,* 2015). Una característica común observada en todos los modelos experimentales de pancreatitis aguda es la marcada disminución de los niveles de GSH en páncreas. Dicha depleción de GSH puede evitarse tratando previamente a los animales con GSH monoetil éster, *N*-acetilcisteína o pentoxifilina, disminuyendo así el daño pancreático (Sandoval *et al.,* 2009).

Aparte de los niveles de GSH, otros parámetros que reflejan el estrés oxidativo se alteran durante el curso de la pancreatitis aguda, como los lípidos oxidados o las proteínas carboniladas, lo que indica que la pancreatitis aguda y el estrés oxidativo están estrechamente relacionados (Park *et al.,* 2003; Winterbourn *et al.,* 2003; Hernández *et al.,* 2011; Pérez *et al.,* 2015). De hecho, la gravedad de la enfermedad se ha correlacionado con la concentración de algunos de estos parámetros, como la carbonilación de proteínas, la peroxidación lipídica y los niveles de ácido ascórbico (Abu-Zidan, Bonham and Windsor, 2000).

El estrés oxidativo se considera un modulador clave de la señalización celular y desempeña un papel crucial en el desarrollo de la cascada inflamatoria (Pérez *et al.,* 2015). La presente Tesis se enmarca entre los estudios encaminados a aclarar la contribución precisa del estrés oxidativo en el proceso inflamatorio en respuesta al daño tisular producido en la pancreatitis aguda para poder diseñar estrategias terapéuticas efectivas basadas en el tratamiento con antioxidantes o moduladores del tono redox.

2. OBJETIVOS

Basándonos en el contexto descrito anteriormente sobre la regulación de los disulfuros mixtos, nuestros objetivos en esta Tesis fueron los siguientes:

1. Determinar si la proteína de 14 kDa relacionada con la tiorredoxina (TRP14) regula la cisteinilación de proteínas *in vitro* e *in vivo* e identificar sus sustratos.

2. Estudiar el papel de la TRP14 y la cisteinilación de proteínas en la regulación de la respuesta inflamatoria en la pancreatitis aguda experimental como modelo *in vivo* de inflamación aguda asociada con disulfuros mixtos.

3. MATERIAL Y MÉTODOS

Para el desarrollo de esta Tesis se utilizaron células HEK-293 y células *knockdown* de TRP14. Las células *knockdown* de TRP14 fueron desarrolladas y proporcionadas por el Profesor Elias Arnér del Departamento de Bioquímica Médica y Biofísica del Instituto Karolinska, Estocolmo (Suecia).

Los ratones C57BL/6 *wild type* de *Txndc17* que codifican TRP14, así como los ratones *knockout* de *Txndc17* empleados en esta Tesis fueron proporcionados por el Profesor Ed Schmidt del Departamento de Microbiología e Inmunología de la Universidad Estatal de Montana, Bozeman (Montana, EE.UU.). Los animales se estabularon en la Sección de Producción animal del Servicio Central de Apoyo a la Investigación Experimental (SCSIE) de la Universitat de València.

Todos los procedimientos y experimentos descritos en esta Tesis se realizaron en cumplimiento de la normativa del Parlamento Europeo y del Consejo de 22 de septiembre de 2010 sobre la protección de los animales utilizados con fines científicos (Directiva 2010/63/UE), incorporada a la legislación española por el Real Decreto 53/2013 del 1 de febrero de 2013. Los protocolos experimentales se aprobaron por el Comité de Ética y Bienestar Animal de la Universitat de València.

La expresión y purificación de las proteínas recombinantes empleadas en esta Tesis fue llevada a cabo por el Dr. Qing Cheng en el laboratorio del Profesor Elias Arnér en el Instituto Karolinska.

La identificación y cuantificación de las dianas de cisteinilación de proteínas se realizó *in vitro* en células *knockdown* de TRP14 incubadas con cisteína biotinilada, seguida de la purificación por cromatografía de afinidad con estreptavidina y la identificación y cuantificación por espectrometría de masas MS/MS de los lisados celulares. El análisis proteómico se llevó a cabo en la Sección de Proteómica del SCSIE de la Universitat de València.

Desarrollamos también un método *in vitro* en el laboratorio del profesor Elias Arnér en el Instituto Karolinska para cisteinilar Prx2 humana recombinante. La cisteinilación de la proteína se logró *in vitro* tras la incubación de peroxirredoxina 2 humana (hPrx2) recombinante con BODIPY® FL L-Cistine (Thermo Fisher, Massachusetts, EE.UU.). La actividad de Prx2 se evaluó monitorizando la eliminación de H_2O_2 del medio en presencia del sistema tiorredoxina empleando el ensayo de oxidación del hierro-anaranjado de xilenol (FOX).

Para la inducción de la pancreatitis aguda, se emplearon ratones de 12 semanas de edad a los que se les administraron siete inyecciones

intraperitoneales de ceruleína (Merck, Nueva Jersey, EE.UU.) (50 µg/kg de peso corporal) a intervalos de 1 h. Tras 1 h de la última inyección, los animales fueron sacrificados mediante exanguinación bajo anestesia con isoflurano al 3%, y el páncreas fue inmediatamente extraído y procesado según la técnica a emplear en las diferentes determinaciones. La muerte fue confirmada por dislocación cervical. El grupo control recibió siete inyecciones intraperitoneales de solución salina al 0,9% (B. Braun, Melsungen, Alemania) a intervalos de una hora.

Además, se emplearon las siguientes técnicas:

- Espectrometría de masas UHPLC MS/MS para la detección de metabolitos de la vía de la transulfuración, pares redox y disulfuros mixtos.
- *Western Blot*.
- Análisis de la expresión génica mediante RT-PCR.
- Medida de la actividad mieloperoxidasa mediante colorimetría.
- Medida de las actividades amilasa y lipasa en suero mediante colorimetría.
- Tinción de hematoxilina/eosina para análisis histológico del páncreas mediante microscopía óptica.

Los resultados se expresaron como media ± desviación estándar. Para el análisis estadístico de los resultados, se realizó primero un ANOVA de dos vías para comparar la media de los diferentes grupos. Cuando se observaron diferencias significativas, se determinó la diferencia entre los

grupos individuales mediante la prueba de Scheffé. Se consideraron significativos aquellos análisis en los que se obtuvo un $P < 0,05$.

4. RESULTADOS Y DISCUSIÓN

Partiendo de resultados previos obtenidos en nuestro laboratorio que describieron por vez primera el estrés por disulfuro mostrando la oxidación del par cisteína/cistina y la cisteinilación de proteínas durante la pancreatitis aguda necrotizante (Moreno *et al.*, 2014), decidimos explorar el papel de TRP14 en la regulación de la cisteinilación *in vitro*, debido a su actividad cistina reductasa (Pader *et al.,* 2014). Para ello, realizamos experimentos *in vitro* utilizando células HEK-293 y células *knockdown* de TRP14 incubadas con cisteína biotinilada (Cys-BIO), lo que nos permitió evaluar la incorporación de cisteína marcada a las proteínas. Las células deficientes en TRP14 presentaron niveles de cisteinilación de proteínas más altos que sus controles incubados con Cys-BIO. Las imágenes de *Western Blot* obtenidas mostraron un patrón de cisteinilación generalizado, observándose marcaje en prácticamente todos los pesos moleculares.

En un siguiente paso investigamos si estos lisados cisteinilados correspondientes a las células *knockdown* de TRP14 podrían emplearse como sustrato para el sistema TRP14, formado por TRP14, tiorredoxina reductasa 1 y NADPH. Por esta razón, se incubaron los lisados con los diversos componentes del sistema TRP14 por separado y en diferentes combinaciones, observándose el mayor efecto descisteinilante cuando todos los componentes del sistema estaban presentes. Así pues, estos resultados sugieren, por primera vez, que TRP14 regula el proceso de cisteinilación de proteínas *in vitro*.

A continuación, nos planteamos si TRP14 muestra alguna especificidad hacia proteínas particulares a la hora de regular su estado de cisteinilación *in vitro*. Para este propósito, realizamos un análisis proteómico para identificar posibles dianas de cisteinilación. El estudio de expresión diferencial mostró 42 proteínas cuya cisteinilación fue significativamente mayor en las células *knockdown* de TRP14 incubadas con Cys-BIO. Entre las proteínas identificadas encontramos proteínas involucradas en el proceso de traducción, como el factor de elongación 2, o en la eliminación del H_2O_2, como Prx2. Estos resultados apuntan a la participación de TRP14 en la regulación de procesos tan importantes como la traducción y la síntesis de proteínas, o el control de la defensa antioxidante.

Decidimos por tanto determinar la actividad decisteinilante de TRP14 sobre un sustrato específico y estudiar el efecto de la cisteinilación en la función de las proteínas. Para ello, elegimos la Prx2 como proteína modelo para realizar nuestros estudios, ya que fue una de las dianas de cisteinilación identificadas en las células HEK-293 *knockdown* de TRP14. Como en las incubaciones de lisados cisteinilados, el mayor efecto descisteinilante se observó cuando se incubó la Prx2 cisteinilada con todos los componentes del sistema TRP14. La Prx2 humana recombinante, cisteinilada en la cisteína 172, perdió su capacidad de eliminar el H_2O_2 del medio comparado con su control no cisteinilado. La actividad de la Prx2 cisteinilada se recuperó después de la incubación con TRP14 y el resto de los componentes del sistema enzimático.

Dado que Trx1 también reduce la L-cistina (Pader *et al.,* 2014), también estudiamos si la Prx2 cisteinilada podría ser un sustrato de Trx1. Nuestros resultados mostraron que el sistema de Trx1 es capaz de reducir la Prx2 cisteinilada, incluso con mayor actividad que el sistema de TRP14.

En nuestro modelo de pancreatitis aguda inducida por ceruleína, los ratones *knockout* de TRP14 mostraron sorprendentemente niveles más bajos de proteínas cisteiniladas que los ratones *wild type* en condiciones basales. Sin embargo, los niveles de cistina no mostraron diferencias entre los ratones *wild type* y los *knockouts* de TRP14 en condiciones basales, por lo que la disminución observada en los niveles de cisteinilación podría deberse a algún otro factor, como una posible inducción de la tiorredoxina 1 que, como hemos demostrado anteriormente, también es capaz de reducir los enlaces disulfuro de las proteínas cisteiniladas. Tras la inducción de la pancreatitis aguda, se produjo un marcado aumento de los niveles de cisteinilación de proteínas y de cistina en el páncreas de los ratones *knockout* de TRP14, mientras que se produjo una disminución de los niveles de cisteinilación de proteínas y de cistina en el páncreas de los ratones *wild type*. Es decir, tras la inducción de pancreatitis aguda en los ratones deficientes en TRP14, se produjo un marcado aumento en los niveles de cisteinilación, una de las principales características del estrés por disulfuro (Moreno *et al.*, 2014).

No se observaron cambios en los niveles de γ-glutamil cisteinilación de proteínas en páncreas en condiciones basales entre ratones *wild type* y *knockout*. Sin embargo, tras la inducción de pancreatitis aguda, los niveles de γ-glutamil cisteinilación de proteínas en el páncreas de los ratones *knockout* de TRP14 aumentaron de forma considerable, concordando de nuevo con otra característica principal del estrés por disulfuro. En este caso, el perfil de los niveles de γ-glutamil cisteinilación de proteínas coincidió con los niveles de γ-glutamil cistina, aparentemente responsable de la formación de estos disulfuros mixtos. En cuanto a la glutationilación de proteínas en páncreas, no se observaron cambios en sus niveles en ninguno de los

grupos estudiados, ni en condiciones basales ni tras la inducción de pancreatitis.

El estudio del par redox que consideramos fundamental en esta Tesis es el formado por la cisteína y la cistina. En condiciones basales no se observaron diferencias significativas en los niveles pancreáticos de cisteína entre ratones *wild type* y *knockout*. Sin embargo, tras la inducción de pancreatitis aguda, se produjo una disminución de los niveles de cisteína en el páncreas de los ratones *wild type*, mientras que sus niveles se mantuvieron estables en los ratones *knockout* de TRP14. Respecto a la forma oxidada, se observó un perfil bastante similar: en condiciones basales, no hubo cambios en los niveles pancreáticos de cistina, mientras que la pancreatitis causó una disminución de los niveles de cistina en el páncreas de los ratones *wild type*. Sin embargo, los niveles de cistina aumentaron marcadamente en el páncreas de los ratones deficientes en TRP14 con pancreatitis. En cuanto al cociente cistina/cisteína, se observó un claro aumento en el páncreas de los ratones *knockout* de TRP14 con pancreatitis, indicando la prevalencia de la forma oxidada. Por lo tanto, TRP14 regula el estrés por disulfuro asociado a la inflamación aguda mediante la regulación de los niveles de cistina y de la cisteinilación de proteínas en el páncreas.

Resulta interesante que en el páncreas de los ratones *knockout* de TRP14 con pancreatitis se detectó un aumento de los niveles de GSH. A su vez, los niveles pancreáticos de GSSG también aumentaron notablemente en los ratones *knockout* con pancreatitis, sin que se observaran cambios significativos entre los demás grupos. Al calcular el cociente GSSG/GSH, indicativo del grado de oxidación del glutatión, no se observaron cambios entre los grupos.

Tanto los niveles de γ-glutamil cisteína como de γ-glutamil cistina se incrementaron en el páncreas de los ratones *knockout* de TRP14 con pancreatitis. Sin embargo, hubo un marcado descenso en los niveles de γ-glutamil cisteína en el páncreas de los ratones *wild type* tras la inducción de pancreatitis aguda, sin cambios en los niveles de γ-glutamil cistina. El cociente γ-glutamil cistina/γ-glutamil cisteína se mantuvo sin cambios entre los grupos, excepto por un aumento significativo en el caso de los ratones *wild type* con pancreatitis debido a la fuerte caída de los niveles de la forma reducida.

El último de los pares redox estudiados fue el formado por homocisteína y homocistina. La deficiencia de TRP14 dio lugar a la acumulación de homocisteína en el páncreas en comparación con los ratones *wild type*. La inducción de la pancreatitis aguda no alteró los niveles de homocisteína, y se mantuvieron las mismas diferencias entre ratones *wild type* y *knockout*. En cuanto a los niveles pancreáticos de homocistina, no se observaron diferencias entre los grupos.

Como nuestros resultados apuntaban a una inducción de la vía de la transsulfuración con la deficiencia de TRP14, decidimos medir los niveles de metionina. En condiciones basales, se observó una disminución en los niveles de metionina en el páncreas de los ratones *knockout* de TRP14. Tras la inducción de pancreatitis aguda, los niveles de metionina experimentaron una dramática caída en el páncreas de los ratones *wild type*, siendo esta caída aún más acusada en los ratones *knockout* de TRP14. Estas observaciones son consistentes con un aumento de la actividad de la vía de transsulfuración que llevaría al agotamiento de la metionina y a la acumulación del producto final, la cisteína, que se emplearía para la síntesis de GSH. Además, observamos un aumento en los niveles de cistationina en páncreas de los ratones *knockout* de TRP14 con pancreatitis respecto a los

wild type, como resultado de la inducción de la cistationina β-sintasa, confirmando de esta manera la inducción de la vía.

La expresión del ARN mensajero de *Trx1*, *Trxr1* y *Gr* aumentó en el páncreas de los ratones *wild type* tras la inducción de pancreatitis aguda, siendo este incremento marcadamente mayor en el caso de los ratones *knockout* de TRP14 con pancreatitis. Estas tres enzimas son dianas de NRF2, lo que indicaría una mayor activación de dicho factor de transcripción durante la pancreatitis en ausencia de TRP14. En lo que respecta a los niveles de ARNm de *Nqo-1*, *Gclc* y *Ho1*, todas ellas dianas típicas de NRF2, no se observaron cambios en condiciones basales, pero se detectó un aumento de su expresión tras la inducción de pancreatitis aguda, con incrementos más marcados en ratones *knockout* de TRP14 con pancreatitis. Los niveles nucleares de NRF2 en el páncreas, que se midieron para confirmar su activación, fueron mucho más altos en el páncreas de los ratones *wild type* en condiciones basales que en los ratones *knockout*. Sin embargo, tras la inducción de pancreatitis aguda, los niveles nucleares de NRF2 disminuyeron en los ratones *wild type*, mientras que aumentaron marcadamente en los ratones *knockout* de TRP14.

En cuanto a las principales enzimas encargadas de la defensa antioxidante, en condiciones basales los niveles de ARNm de *Sod1* y *Sod2* fueron menores en el páncreas de los ratones *knockout* de TRP14. Sin embargo, en el caso de la catalasa, no se observó ningún cambio. Curiosamente, a pesar de estos niveles de expresión más bajos en ratones deficientes de TRP14, tras la inducción de pancreatitis aguda, la expresión de las tres enzimas aumentó marcadamente en el páncreas de los ratones *knockout* de TRP14 en comparación con los ratones *wild type*.

Por lo que respecta a las principales citoquinas proinflamatorias, no se observaron cambios en la expresión de sus ARN mensajeros entre ratones *wild type* y *knockout* de TRP14 en condiciones basales, y se observó un aumento similar en ambos grupos de en la expresión de *Tnf-α*, *Il-6*, *Il-1β*, *Cxcl-1* e *Il-18* tras la inducción de pancreatitis aguda.

A nivel histológico, el estudio de las secciones de páncreas teñidas con hematoxilina/eosina reveló menor edema tisular e infiltrado inflamatorio en ratones *knockout* de TRP14 con pancreatitis. Este hallazgo es relevante porque el daño tisular en la pancreatitis aguda experimental en ratones se caracteriza por una marcada presencia de edema tisular e infiltrado inflamatorio. Se observó el mismo patrón para la actividad mieloperoxidasa, lo que confirmó el menor infiltrado inflamatorio. Esta posible disminución de la gravedad de la pancreatitis que se observó en el páncreas de los ratones deficientes en TRP14 podría deberse a la activación del NRF2 y su potencial antiinflamatorio (Cuadrado *et al.,* 2019).

5. CONCLUSIONES

En nuestro estudio de la función de TRP14 en la regulación de la cisteinilación de proteínas *in vitro* e *in vivo* durante la pancreatitis aguda como modelo de inflamación aguda asociada con estrés por disulfuro, se obtuvieron las siguientes conclusiones:

1. TRP14 regula la cisteinilación de las proteínas, tanto *in vitro* como *in vivo*:

    a. Tanto TRP14 como tiorredoxina 1 presentan actividad descisteinilante hacia la peroxirredoxina 2 cisteinilada en

presencia de tiorredoxina reductasa 1 y NADPH, siendo la tasa de descisteinilación mayor para la tiorredoxina 1 que para la TRP14.

b. La peroxirredoxina 2 cisteinilada en la cisteína 172 es inactiva y el sistema de TRP14 restaura la actividad de la peroxirredoxina 2 cisteinilada mediante la reducción del enlace disulfuro presente en el disulfuro mixto.

c. Entre las dianas de la cisteinilación de proteínas, encontramos proteínas implicadas en la traducción del ARN y en la síntesis de proteínas, así como en la eliminación de agentes oxidantes como el peróxido de hidrógeno.

2. La deficiencia de TRP14 protege contra la pancreatitis aguda inducida por ceruleína en ratones al inducir la síntesis de GSH y la activación de NRF2:

a. Los ratones *knockout* de TRP14 con pancreatitis presentan menos edema tisular e infiltrado inflamatorio en el páncreas, así como menor actividad MPO en el páncreas que los ratones *wild type* con pancreatitis.

b. La deficiencia de TRP14 conduce a una inducción de la vía de transsulfuración y de la síntesis de glutatión en la pancreatitis aguda, lo que da lugar a un aumento de los niveles de GSH en el páncreas de ratones *knockout* de TRP14 con pancreatitis.

c. Los niveles nucleares de NRF2 en el páncreas de los ratones *knockout* de TRP14 con pancreatitis aumentaron en

comparación con los ratones *wild type*, dando lugar a un marcado aumento de la expresión de sus genes diana implicados en la defensa antioxidante, sin cambios en la expresión de las principales citoquinas proinflamatorias.