From the Department of Medical Biochemistry & Biophysics
Division of Biochemistry
Karolinska Institutet, Stockholm, Sweden

STUDIES OF THE THIOREDOXIN SYSTEM IN REDOX SIGNALING AND OXIDATIVE STRESS

Belén Espinosa Fernández

Karolinska Institutet

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Studies of the thioredoxin system in redox signaling and oxidative stress

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By

Belén Espinosa Fernández

Principal Supervisor:
Professor Elias S.J. Arnér
Karolinska Institutet
Department of Medical Biochemistry & Biophysics
Division of Biochemistry

Co-supervisors:
Professor Stig Linder
Linköping University
Department of Biomedical and Clinical Sciences
Division of Division of Drug Research

Opponent:
Professor C. Alicia Padilla Peña
Universidad de Córdoba
Department of Biochemistry & Molecular Biology

Examination Board:
Professor Anna-Liisa Levonen
University of Eastern Finland
A.I. Virtanen Institute for Molecular Sciences

Professor Martin Bergö
Karolinska Institutet
Department of Department of Biosciences and Nutrition

Professor Gunter Schneider
Karolinska Institutet
Department of Department Medical Biochemistry and Biophysics
Division of Molecular Structural Biology

To my family, friends and every single person
that helped me out until the last full stop

La investigación puede ser muy frustrante. La investigación no entiende de horarios, no le importa si es domingo, festivo, o si se casa tu prima la del pueblo. Te encuentras continuamente con experimentos fallidos una y otra vez, una y otra vez… Sin embargo, al final del día querido amigo, generas conocimiento y esa sensación es impagable y muy poca gente
en este mundo puede llegar a sentirlo.

ABSTRACT

Reactive oxygen species exert reversible posttranslational modifications in proteins containing redox sensitive thiols, thereby affecting several cellular processes and protein functions. Reductive enzyme systems, such as the thioredoxin system, can reverse most of these cysteine modifications. Therefore, a tight control between oxidizing and reducing events is the central principle of redox signaling. The studies within this thesis have covered several aspects of the Trx system in the modulation of cellular signaling pathways.

In **Paper I**, we evaluated how b-AP15, a small inhibitor of proteasome-associated DUB, exerts redox perturbations in tumor cells. It is known that b-AP15 triggers an increase level of reactive oxygen species (ROS) and proteotoxic stress in cancer cells. However, its efficacy inducing apoptosis diminished by antioxidants. To identify the precise mechanism by which b-AP15 induces redox perturbations, we generated cells deprived of mitochondrial DNA. We found that in cells lacking mitochondria, the oxidative stress generated by b-AP15 was completely abrogated. Furthermore, to exclude that the observed increase in the levels of oxidative stress were due to an inhibition of TrxR1, we evaluated a number of proteasome associated DUBs inhibitors that did not inhibit TrxR1. Similarly, to b-AP15 all the inhibitors tested induced oxidative stress and the expression of HO-1. In parallel, we observed mitochondrial dysfunction, measuring the levels of COX5b and TOMM34, in both cases their respective levels decreased in those cells treated with b-AP15. Based in all the results we could conclude that the source of ROS in cells treated with b-AP15 was of mitochondrial origin.

In **Paper II**, we performed a drug-screen of compounds sharing a common enone motif with b-AP15 and many natural products with antineoplastic effect. Through biochemical and structural analyses, we could demonstrate the binding of the enone containing compounds to the proteasome-associated cysteine deubiquitinase, USP14, inhibiting its activity. Additionally, we further analyzed a subset of those compounds in a zebrafish embryo model where they showed antineoplastic activity. These findings suggest that DUB inhibition is a relatively common mode of action by cytotoxic compounds containing motifs and it helps to explain the antineoplastic effects of natural products containing such functional group.

In **Paper III**, we identified HRI as a redox-regulated protein, which becomes oxidized when activated upon As(III) exposure. TrxR1 associates with HRI in cells and together with TRP14 and Trx1 reduces HRI in vitro. Moreover, several specific inhibitors of TrxR1 lead to HRI-dependent eIF2α phosphorylation, translation suppression and stress granule formation. Based on our finding that HRI-mediated translation suppression is essential for cellular survival under conditions of high As(III), we revealed the Trx system as a regulator of the HRI dependent translational stress response.

In **Paper IV**, we evaluated the role of TRP14 in the regulation of different redox-regulated transcriptional factors using our unique tool pTRAF (plasmid for transcription factor reporter activation based upon fluorescence). We discovered that using TRP14 knockdown HEK293 cells, NRF2 activation increased upon treatment with auranofin, we also uncovered that TRP14 is crucial for HIF activation upon TNF-α stimulation in hypoxic conditions. Furthermore, endogenous TRP14 levels increased under hypoxia or TNF-α treatment, suggesting that TRP14 could itself be regulated by NFκB and HIF, which is compatible with the presence of the corresponding response elements in the proximal TXNDC17 promoter region. Surprisingly, using TRP14 knockout HEK293 cells we found that global protein translation was reduced, which could be reverted with methionine or N-acetylcysteine supplementation. TRP14 knockout cells were also, in contrast to controls, highly sensitive to PPG. We conclude that TRP14 has several roles in control of redox signaling pathways, and that TRP14 is the main intracellular reductase for liberation of cysteine from cystine.

In **Paper V**, we de novo synthesized eight vinyl sulfone compounds and evaluated their capacity to activate NRF2, NFκB and HIF1 in comparison with DMF using our previously mention tool pTRAF. We selected a set of compounds that activate NRF2 more selectively than DMF and characterize their downstream effects using in vitro and in vivo models. Our selected compounds display a more selective oligodendrocyte associated effect which could be explore in the future as a regenerative drug in demyelinating disorders.

LIST OF SCIENTIFIC PAPERS

I. Xiaonan Zhang, **Belén Espinosa**, Amir Ata Saei, Padraig D'Arcy, Roman A Zubarev, and Stig Linder. Oxidative Stress Induced by the Deubiquitinase Inhibitor b-AP15 Is Associated with Mitochondrial Impairment. *Oxid Med Cell Longev. 2019 Jun 10;2019:1659468. doi: 10.1155/2019/1659468. eCollection 2019.PMID:31281566*

II. Karthik Selvaraju, Arjan Mofers, Paola Pellegrini, Johannes Salomonsson, Alexandra Ahlner, Vivian Morad, Ellin-Kristina Hillert, **Belén Espinosa**, Elias S. J. Arnér, Lasse Jensen, Jonas Malmström, Maria V. Turkina, Padraig D'Arcy, Michael A. Walters, Maria Sunnerhagen & Stig Linder. Cytotoxic unsaturated electrophilic compounds commonly target the ubiquitin proteasome system. *Sci Rep. 2019 Jul 8;9(1):9841. doi: 10.1038/s41598-019-46168-x. PMID:31285509*

III. Bogdan Jovanovic, **Belén Espinosa**, Shawn M. Lyons, Nga Ly-Hartig, Tobias Dick, Elias Arnér and Georg Stoecklin. Cellular protection from arsenic compounds through control of global protein synthesis by TrxR1-mediated HRI kinase activation. *Submitted manuscript*

IV. **Belén Espinosa**, Irina Pader, Marcus Cebula, Katarina Johansson, Elias S. J. Arnér. Thioredoxin related protein of 14 kDa (TRP14, TXNDC17) represses NRF2 and NFκB activities and augments HIF activation. *Manuscript*

V. Karl E Carlström, Praveen K Chinthakindi, **Belén Espinosa**, Faiez Al Nimer, Katarina Johansson, Elias S J Arnér , Per I Arvidsson , Fredrik Piehl. Novel vinyl sulfone compounds are more specific NRF2 activators in the in vitro and in the central nervous system than dimethyl fumarate. *Accepted in Neurotherapeutics*

ARTICLES NOT INCLUDED IN THIS THESIS

VI. Éva Dóka, Yumi Abiko, Markus Dagnell, Tomoaki Ida, Nho Luong Cong, Noémi Balog, **Belén Espinosa**, Akira Nishimura, Yosuke Funato, Hiroaki Miki, Jon Fukuto, Justin R. Prigge, Edward E. Schmidt, Elias S. J. Arnér, Yoshito Kumagai, Takaaki Akaike, Péter Nagy. Control of protein function through oxidation and reduction of persulfidated states. *Sci Adv. 2020 Jan 1;6(1):eaax8358. doi:10.1126/sciadv.aax8358. eCollection 2020 Jan. PMID:31911946*

VII. S. Busker, W. Qian, M. Haraldsson, **B. Espinosa**, L. Johansson, S. Attarha, *I.* Kolosenko, J. Liu, M. Dagnell, D. Grandér, E. S. J. Arnér, K. Pokrovskaja Tamm, B. D. G. Page. Irreversible TrxR1 inhibitors block STAT3 activity and induce cancer cell death. *Sci Adv. 2020 Mar; 6(12): eaax7945. Published online 2020 Mar 20. doi: 10.1126/sciadv.aax7945 PMCID: PMC7083616*

VIII. **Belén Espinosa**, Elias S. J. Arnér. Thioredoxin-related protein of 14 kDa as a modulator of redox signalling pathways. *Br J Pharmacol. 2019 Feb;176(4):544-553. doi: 10.1111/bph.14479. Epub 2018 Oct 6. Review. PMID:30129655*

CONTENTS

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
ARE	Antioxidant responsive element
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine-5'-triphosphate
BCL-3	B-cell lymphoma 3
bZIP	Basic Leucine Zipper
CBS	Cystathionine-β-synthase
CBP	CREB-binding protein
CSE	Cystathionine-γ-lyase
COX-2	Cyclooxygenase-2
Crm1	Chromosome region maintenance 1
CT-B	Cholera toxin subunit B
Cys	Cysteine/single letter code C
DTT	Dithiothreitol
DUOX	Dual oxidase
EGF	Epidermal growth factor
eIF-4E	Eukaryotic initiation factor-4E
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FIH	Factor inhibiting HIF
Gly	Glycine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH/GSSG	Glutathione, reduced/ oxidized form
GST	Glutathione-S-transferase
HED	Hydroxyethyl disulphide (dithiodiethanol)
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
IκB	Inhibitors of κB
IKK	IκB kinase
IL-1	Interleukin-1
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MMP-9	Matrix metallopeptidase 9
Msr	Methionine sulfoxide reductase
Mst1/2	Mammalian sterile 20-like kinases 1 and 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NFκB essential modulator

NES	Nuclear export signal
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFκB-RE	NFκB response element
NLS	Nuclear localization signal
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NRF2	Nuclear factor (erythroid-derived 2)-like 2
ODDD	Oxygen-dependent degradation domain
p70S6K	p70S6 kinase
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PDI	Protein disulphide isomerase
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
pTRAF	Plasmid for transcription factor reporter activation based upon fluorescence
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
pVHL	Hippel-Lindau tumour suppressor protein
Ref-1	Redox effector factor 1
RHD	Rel homology domain
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulphur species
RTK	Receptor tyrosine kinase
Sec	Selenocysteine/ single letter code U
Ser	Serine
SecTRAP	Selenium **c**ompromised thioredoxin reductase-derived apoptotic protein
SHP-1/2	SH2-containing phosphatase 1/2
SOD	Superoxide dismutase
TAD	Transactivation domain
TGR	Thioredoxin glutathione reductase
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRP14	Thioredoxin related protein of 14 kDa, encoded by TXNDC17
Trx	Thioredoxin
TXNDC17	Thioredoxin Domain Containing 17, Human
TXNRD	Thioredoxin reductase human gene, encoding TrxR
TrxR	Thioredoxin reductase, encoded by TXNRD1
TXNIP	Thioredoxin interacting protein
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 Redox homeostasis

Reactive oxygen species (ROS) are a group of small reactive byproducts produced during incomplete oxygen reduction. Under control concentrations, ROS are essential as second messengers in the regulation of signaling cascades to mediate physiological responses (1-4). At distorted ROS concentrations, redox homeostasis will be disrupted inflicting damage to important organelles and biomolecules, causing fatal alterations implicated in carcinogenesis and neurodegenerative disorders (5-7).

In redox sensitive proteins, ROS induce reversible covalent modifications of specific cysteines also known as "thiol switches" (8). The post-transcriptional modifications exhibit by these cysteines usually reveal crucial roles in protein function as an ideal target for signal regulation and as vital players in redox homeostasis (9-12) (Fig. 1).

The cell has developed different mechanism to reverse these oxidative modifications, being the most distinguished systems the glutathione and thioredoxin system. These systems are essential to regulate the different redox signaling pathways and to sustain the redox homeostasis in the cell (12).

1.2 Reactive oxygen species

With a half-life of approximately 1 ms, H_2O_2 is one of the best candidates for redox signaling with its selective reactivity and its ability to circulate freely through membranes or through aquaporins (4, 13-15). Furthermore, H_2O_2 production is enabled by a number of oxidases (16-18) or by the quick reactions of superoxide ($O_2^{\bullet-}$) to H_2O_2 (19, 20).

Interestingly, the percentage of $O_2^{\bullet-}$ generated in the mitochondrial represents less than 2% of all the oxygen consume by the cell. During regular circumstances its production is strictly regulated by the antioxidant system (21-23), which regulates different redox sensitive pathways, like those involve in inflammation, degradation and transcriptional factors activation (24-27).

Furthermore, $O_2^{\bullet-}$ and thus H_2O_2 can be produced by NADPH oxidases (NOXs), for the activation of redox sensitive signaling pathways, including transcription factor activation (28, 29).

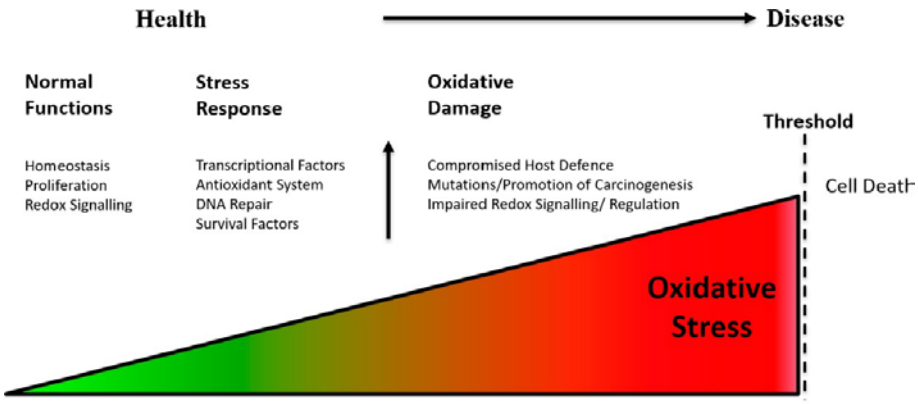

Figure 1. *Scheme of the effect of oxidative stress in health and disease. Redox homeostasis is crucial for the correct functioning of the cells. When an imbalance between ROS and the antioxidant system occur, oxidative stress takes places disrupting different procedures in the cell based on intensity.*

1.3 Antioxidants in redox signaling

All living organisms have developed an interacting network of small antioxidant molecules and enzymes to scavenge highly reactive radicals, being the most prominent the glutathione (GSH) and the thioredoxin (Trx) systems (11, 30-33). These systems play an important role not only protecting cells against oxidative damage but reversing oxidative modifications to maintain a tight regulation of the signaling pathways (34).

1.3.1 Glutathione system

The glutathione system is one of the two major NADPH-dependent redox regulatory systems in the cells. It regulates a broad number of cellular processes involved in redox signaling and antioxidant defense (35, 36). Its main function is to scavenge electrophilic or oxidizing compounds either directly by GSH or catalyzed by Glutathione-S-Transferases, which has been previously demonstrated to also have an important role modulating signaling pathways (37). Furthermore, GSH can act as a cofactor by Glutaredoxins (Grxs) and glutathione peroxidases (38).

1.3.1.1 Glutathione

GSH (Glu-Cys-Gly) in its reduced form, with a concentration range between 1 and 10 mM, is the most abundant low molecular weight antioxidant in cells (11, 39, 40). Localization wise, the vast majority of GSH is found in the cytosol, with a small percentage localized in the mitochondria and ER (41, 42).

GSH is synthesized by the consecutive reactions of 2 enzymes.

First, glutamate cysteine ligase (GCL) mediates the formation of γ-glutamylcysteine by the reaction between glutamate and cysteine. At this step, it is important to consider that cysteine is the reduced form of the semi-essential amino acid cystine. Cysteine can be generated de novo from methionine through the transsulfuration pathway or it can be incorporated into the cells as its oxidized form cystine (Cys) (43). Cys is imported via the alanine-serine-cysteine (ASC) and XAG- systems in a Na^+-dependent manner (44). Cystine, on the other hand, is imported in a Na^+ independent manner by system b^{0+} and by the cystine/glutamate antiporter Xc^- (45). Interestingly, Xc^- is highly inducible by O_2, electrophilic agents, TNFα, and the transcription factor NRF2, characteristics that would crucial for the development of paper IV of this thesis (45, 46). The capacity of the cell to obtain cysteine is crucial for GSH formation, and its availability representing the rate limiting step in GSH synthesis. Secondly, the reaction between glutamate and cysteine occurs, GSH synthetase catalyzes the condensation of glycine to form GSH (47).

Interestingly, the degradation of GSH can take place only extracellularly since γ-glutamyltranspeptidase (GGT), the only enzyme capable of degrading the γ-carboxylgroup in GHS, is localized on the external surface of some cell types. This procedure has been described in rats to last from 2 to 3 hours (47). In cir-circumstances of acute oxidative stress a large amount of GSSG will be present in the cells, this GSSG will be further transferred extracellularly to be use for de novo synthesis of GSH (48).

GSH also has an important role in redox signaling via glutathionylation. GSH can protect proteins from irreversible overoxidation via the formation of disulfide bonds with their reactive thiols in situation of elevated oxidative stress (49-51).

1.3.1.2 GSTs

GSTs are a family of enzymes responsible of detoxification, that in conjugation with GSH are capable of reducing a large number of endogenous and exogenous compounds (37, 52). Additionally, GSTs are involved in the reduction of lipid peroxidases and glutathionylation of proteins (53-55).

1.3.1.3 GPxs

GPxs are a group of glutathione-dependent peroxidases that catalyze the removal of different types of hydroperoxides leading to the generation of H_2O or alcohols (56). In humans, there are 8 variants that could be divided in two groups GPx1-4 and GPx6 are selenoproteins while the remaining isoforms contain a cysteine in their active site. This amino acid substitutions leads to a drop-in activity of two to three orders of magnitude having the selenium containing GPxs a high rate of reactivity with H_2O_2 (57, 58). Interestingly, only GPx4 knockout mice is embryonically lethal, it is the only isoform that directly can reduce lipid peroxidases and when inhibited leads to cell death through ferroptosis (56, 59, 60).

1.3.1.4 Grx

Grxs are a family of four small enzymes of approximately 100 amino acids that catalyze the reduction of mixed disulfides between protein thiols and GSH. Grxs can be further subdivided into diverse categories based in the number of thiols in their active site or their localization. Grx1 and Grx2 are dithiols containing the active site motif CXXC, while Grx3 and Grx5 are monothiols lacking the C-terminal Cys. Furthermore, Grx1 and Grx3 are mainly found in the cytosol while Grx2 and Grx5 are located in the mitochondria where they develop redox specific functions (35, 61, 62). Interestingly, Grxs play an important role in NFκB, AP-1 and NF-1 redox signaling regulation, as well as in apoptosis signaling binding to ASK1 and regulating the cleavage of caspase 3 (63-66).

1.3.2 Thioredoxin system

The thioredoxin system, together with the GSH system, is one of the key regulators in the defense against oxidative stress. It is comprised of isoenzymes of thioredoxin reductase (TrxR), thioredoxin (Trx) as main substrate and NADPH as electron donor. This system has key roles in redox regulation involved in the fight against oxidative stress, DNA synthesis and redox signaling (33, 67, 68).

1.3.2.1 Thioredoxin

Trx is 12-kDa ubiquitously expressed protein consisting of four β-sheets surrounded by three α-helix with a well conserve active site (CGPC) that confers Trx a robust disulfide reductase activity (69-72). In mammals, there are two distinct isoforms; Trx1 localized in the cytosol and Trx2 that is found in the mitochondria, both variants display distinct roles in redox regulation and antioxidant defense, however their main different resides in the three additional cysteines present in Trx1 outside its active site (C62, C69 C73) that are target of posttranscriptional modifications and have a regulatory function (73). C62 and C69 are capable of forming an intramolecular disulfide bond between them that can be reduce by the GSH system but not by TrxR1 (74, 75). Likewise, C73 is capable of forming a disulfide bond between homodimers that cannot be reduce by TrxR1 either (76, 77).

In the cytosol, Trx1 donates electrons to catalize the reduction of RNR, Prxs and Msrs (78, 79, 80). Additionally, reduced Trx1 can exert its function by direct binding to their target proteins modulating apoptosis, through its binding to PTEN, inhibiting its phosphatase activity and preventing the activation of the PI3K/Akt pathway (81). Trx1 can also bind to the apoptosis signal regulating kinase 1 (ASK1) inhibiting its activity (82). In situ ations of oxidative stress, Trx1 will get oxidized and it will be release from ASK1, however this release may be prompted by the Trx interacting protein (TXNIP), an endogenous inhibitor of Trx that competes with ASK1 in reducing conditions (83).

Despite lacking any nuclear translocation motif, Trx1 can translocate to the nucleus where it controls the activity of several redox regulated transcriptional factors including NRF2, NFκB, HIF that will be further discuss in this thesis (84-88).

1.3.2.2 Thioredoxin family of proteins

The Trx-fold family comprises a number of oxidoreductases with crucial regulatory roles in redox signaling. These proteins are characterized by the presence of a common structure named "Trx fold", constituted of four β-sheets and three α-helix. Many Trx-like proteins share an active site composed of CXXC indispensable for their catalytic activity, however several variants have been described with different number of Cys residues as well as an alternative composition of the XX dipeptide (38, 89, 90). Unfortunately, due to the sparsely characterization of the Trx superfamily, there is a discordance in the nomenclature of its members varying between the classical representatives and the new thioredoxin-domain-containing-(TXNDC) (38).

This superfamily of proteins has members present in all the cellular compartments, even in some circumstances some proteins can be secreted extracellularly. Most of these proteins are localized in the ER corresponding to members of the protein disulfide isomerase (PDI) family with important roles in protein folding. TMX1-TMX4 are thioredoxin-like transmembrane proteins that have an ER targeting signal, being the only member displaying oxidoreductase activity TMX1, whereas TMX2-TMX4 behaves like PDIs (91-95). In the cytosol, together with Trx, Grx and Prxs there are some other members that are more scarcely characterizes like TRP14 which will be described in depth in the following section or thioredoxin related protein of 32 kDa which appears to have unique role in proteolysis (96-99).

Finally, nucleoredoxins (Nxn), the members of the family localized in the nucleus which best describe function is to act as negative regulators in transcription (100-106).

1.3.2.3 Thioredoxin Related Protein of 14 kDa (TRP14)

TRP14 is a123 amino acids protein encoded by *TXNDC17*. It was initially detected searching for proteins containing redox-sensitive cysteine residues with low pKa in rat brains (107). TRP14 is a cytosolic protein expressed in most cells and tissues and it can be reduced by TrxR1. Interestingly, despite some similarities with Trx1, TRP14 is incapable of reducing classical Trx1 substrates such as RNR, Prx1 or Msrs (107, 108).

Like Trx1, human TRP14 has a well conserved active site motif containing two Cys residues (WCPDC) and despite being similar topologically speaking, TRP14 only shares 20% sequence identity with Trx1. The presence of an extended loop, an

additional α-helix, near the active site of TRP14, together with a different distribution of charged residues compared to Trx1, may be the culprits of the differences in substrate specificity between these two oxidoreductases (108).

From the evolutionary point of view, TRP14 orthologues are found in a broad number of organisms from bacteria until mammals (107). However, a detail analysis comparing their active site revealed that the WCPDC characteristic active site was not completely conserved among species during evolution. In some cases, there are some variations in the WCPYC sequence in the active site. The change from "D" to "Y", turns the TRP14 active site into the typical glutaredoxin active site. This controversy makes us to rethink if those species are really TRP14 orthologous, an observation that needs to be further scrutinize (35, 109-111). Nevertheless, studies performed in different TRP14 orthologues species highlight some important roles of TRP14 protecting against oxidative stress challenges, toxic compounds, and viral infections. The fact that TRP14 is so well-preserved during evolution points out towards an unrecognize role of TRP14 in cell survival that need to be further explore (107).

Previously we have mentioned the inability of TRP14 to reduce classical Trx1 substrates. However, when coupled to cystine reduction, TRP14 becomes an even better substrate for TrxR1 than Trx1, with a catalytic efficiency (k_{cat}/K_m) of TRP14 (2217 $min^{-1} \cdot \mu M^{-1}$) being fivefold higher than that of Trx1 (418 $min^{-1} \cdot \mu M^{-1}$). These observations suggest that TRP14 has a more dedicated role towards the catalysis of cystine reduction (112).

Another interesting role of TRP14 is as a suppressor of NF-κB signaling pathway (113, 114). It has been previously described the role of Trx1 in NF-κB regulation (84, 115). However, TRP14 seems to be a more potent regulator of NF-κB signaling than Trx1, despite being present at lower concentrations in the cells. TRP14 via the activation of LC8 through the reduction of a disulfide bond keeps NF-kB inhibited, since reduced LC8 prevents IκB phosphorylation (116).

TRP14 can additionally control redox signaling via NO and nitrosylation (112). The nitrosylation state of cysteine residue can be modulated by Trx1 either by direct reduction of cysteine S-nitrosolyated groups or by transnitrosylation (78, 117, 118). Interestingly, TRP14 is also efficient at reducing nitrosylated cysteine residues (112). However, whether Trx1 and TRP14 have different nitrosylated target proteins remains unknown.

Recently, hydrogen sulfide has emerged as a "hot molecule" in signaling by its ability to form persulfate cysteine residues. Our group, has previously revealed TRP14 as an efficient protein at reducing persulfide moieties on cysteine residues as well

as inorganic polysulfides (119). The reduction of protein persulfides by TRP14 is also interesting because TRP14 can reactivate oxidized forms of PTP1B and regulate the persulfidation status as control of growth factor responsiveness (120, 121).

Furthermore, TRP14 it has been shown to be upregulated in many tumors. TRP14 supports the induction of autophagy as a mechanism of resistance of cancer cells to paclitaxel supported by BECN1 (122). Autophagy as well as BECN1 expression are known to be intimately linked to oxidative stress and redox signaling pathways. However, how TRP14 directly regulates BECN1 remains unknown (123).

1.3.2.4 Thioredoxin reductases

TrxRs are dimeric flavoenzymes which in higher species, such as mammal, are selenoproteins containing a selenocysteine in their active site. However, there are some species like some parasites that contain a cysteine in their active site (124, 125).

The catalytic mechanism of mammalian TrxR demand the transfer of electrons from NADPH to the N-terminal disulfide through FAD. These electrons with the selenenylsulfide in the C-terminal active site of the other subunit form a reduced selenolthiol motif that is in charge of reducing the majority of substrates (Fig. 2) (126, 127). However, there are several quinones that can be directly reduced through the N-terminal dithiol motif (128).

There are several characteristics that are essential for the mechanism of action of mammalian TrxR:

- A selenocysteine in their active site (129-132)
- A flexible C-terminal tail (132, 133)
- Key amino acids in the TrxR/substrate connection or in the vicinity of the active site
- An exact electron flow during catalysis (132, 134)

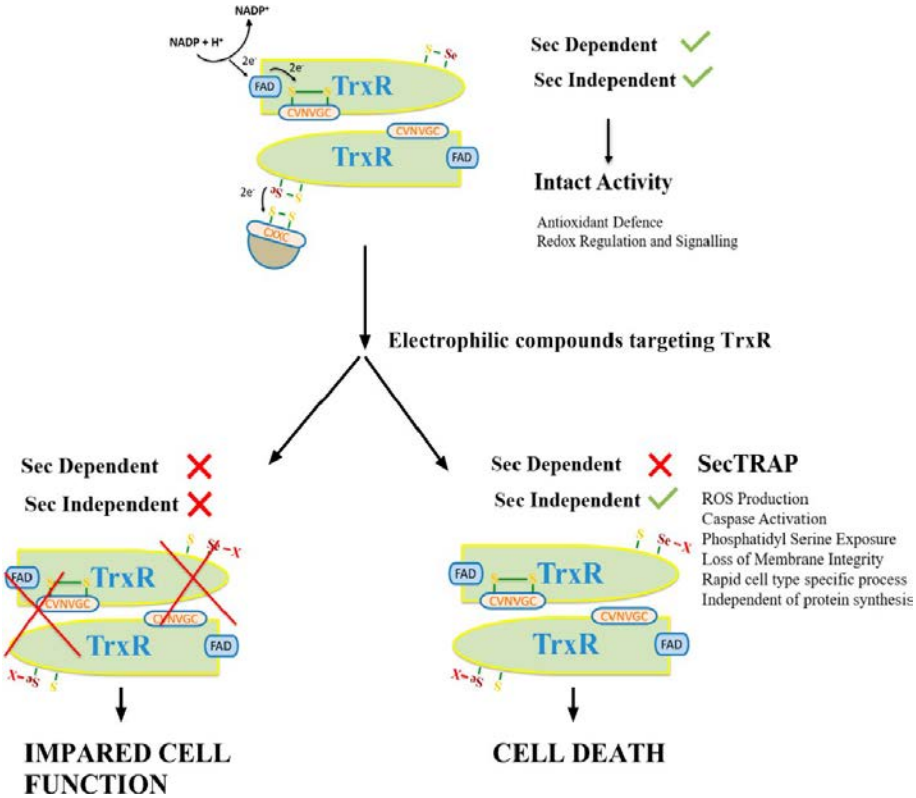

Figure 2. *Scheme of mammalian TrxR electron flow and its inhibition mechanism. This is a scheme of the configuration of the mammalian TrxR homodimer, its electron flow and how different compounds can lead to a complete inactivation or its transformation into pro-oxidant SecTRAP.*

1.3.2.5 TrxR inhibition and SecTRAP formation

The Sec residue in the C-terminal active site of TrxR1 has two striking characteristics. Its nucleophilicity makes TrxR highly reactive but simultaneously its reduced selenolate can be an easy target of electrophilic compounds (129). Now a days there are a broad number of known inhibitors of TrxR both naturally occurring as well as some specifically constructed that now a days are used clinically (135-139). Some examples include auranofin, cisplatin, arsenic oxide or dinitrohalobenzenes (140-143).

These TrxR1 inhibitors can provoke diverse effects on the activity of TrxR. They can be divided between those that completely inhibit its enzymatic activity and the ones that transform TrxR into a pro-oxidant forming what is known as a SecTRAP (selenium compromised thioredoxin reductase-derived apoptotic protein) (144, 145) (Fig. 2).

SecTRAPs are formed when the differnt compounds derivatize the Sec residue. In this case, TrxR can no longer catalyze the reactions dependent on Sec but in turn it gains a potent NADPH oxidase activity. In these circumstances, TrxR is capable of redox cycle with certain substrates via the intact FAD and N-terminus active inducing different mechanism of cell death potentiating the cytotoxic effect of many TrxR inhibitors (144, 146-148).

1.3.2.6 Thioredoxin reductases as targets for anticancer therapy

ROS has been involved in the development and progression of tumor cells (149, 150). As a counteractive mechanism, the malignant cells also enhance their antioxidant systems to avoid oxidative damage that could be a consequence of an hyperactive metabolism (151) (Fig. 7). Since cancer cells seem to rely more in the antioxidant systems than normal cells, its inhibition could be used as a therapeutic approach to promote cell death in cancer cells via oxidative stress with a minimal impact in normal healthy cells (152, 153).

Based in these principles, TrxR1 has become an interesting candidate to target for cancer therapy, atacking on of the hallmarks of cancer (154). It was shown that several cancer course with upregulated levels of TrxR1 and Trx1, correlating directly with aggressiveness, poor prognosis and treatment resistances (155, 156). As described before, TrxR1 can be inhibited by electrophilic compound as well as transformed into a SecTRAP enhancing the accumulation of ROS in the tumor cells therefore committing the cells to death. In fact, our group has successfully characterized two potent TrxR1 inhibitors, TRi-1 and TRi-2, with a minimal effect on mitochondrial function showing cytotoxicity to cancer cells, in comparison to unaffected normal cells (161).

1.4 Thiols in redox regulation

There are several amino acids such that can be modify by ROS. However, tryptophan, histidine and tyrosine oxidation are less favored being less important in physiological processes (162, 163). Methionine oxidation is reversed via methionine sulfoxide reductases and its contribution to redox signaling it is not clear (164). Cysteine, in the other hand, it is considered the prime residue involved in redox signaling with an important role in the diverse papers of this thesis.

1.4.1.1 Cysteine

Cysteine is a sulfur containing amino acid and one of the least abundant, although often it is directly involved in catalysis, protein binding and stabilization (165).

Cysteine has a low pKa which promotes the formation of reactive deprotonated thiolate at physiologic pH. However, this is not the only characteristic that determines its oxidative susceptibility. It is important to notice, the wide range of oxidation states of sulfur (-2 to +6) which allows different modifications (166).

1.4.1.2 Selenocysteine

Selenocysteine is an analogue of cysteine. It is the 21st naturally occurring amino acid showing a higher reactive when compared to cysteine. The key characteristic that differentiate selonocysteine from cysteine is the selenol group in place of the sulfur which grants a higher nucleophilicity (167, 168).The importance of this aminoacid was evidenced in a experiment where the Sec residue was replace for Cys in GPx and TrxR1. Those mutated proteins containing the Cys variant saw their reactivity drastically reduced (169, 170). Nevertheless, this phenomenon does not impl that selenoproteins have a higher capacity regulating redox processes than the Cys containing ones but perhaps the following characteristics may provide some advantages (171-175):

- An increase flexibility towards substrates
- An efficient reaction for one electron transfer
- Higher nucleophilicity increasing the reaction rates

Interestingly, it was also shown that the presence of the Sec residue it is not indispensable for the protein function. Several studies using TxR1 showed that under selenium deficient conditions the Cys variant can take over as a backup mechanism sustaining TrxR1 activity (176, 177).

1.5 Redox sensitive transcription factors

Transcriptional factors regulate gene transcription regulatory binding to specific DNA sequences in order to generate rapid responses.

These redox procedures occur at different points (178, 179):

- mRNA stability and translation
- Stability/ degradation
- Transport between the cytoplasm and the nucleus
- DNA binding
- Activators vs repressors

Now a days it is accepted that oxidants act as mediators and modulators of signaling pathways and protein function, however how they are redox regulated remain poorly characterized (178, 180).

The Trx and GSH systems regulate the transcriptional activity of NFκB AP-1, NRF2, HIF and p53, which activities are intertwined. Interestingly, these specific transcriptional factors are involved in cell survival, stress response and cell death leading to pathological conditions when dysregulated (181-185).

1.5.1 NRF2

NRF2 (Nuclear factor (erythroid-derived 2)-like 2) is the most important transcription factors involved in detoxification and response against oxidative stress. Its activation is mediated by ROS (184, 185). It binds to the antioxidant responsive element (ARE) in the promoter region of genes of detoxifying enzymes and antioxidant proteins (Trx, TrxR, Prx1, GPx2 and transcription factors (37, 129, 178, 186, 187).

Under normal conditions, NRF2 binds to its inhibitor Keap1, which constantly targets NRF2 for proteasomal degradation. Keap1 is responsible of acting as sensor for NRF2 activation. It is subject of conformational changes upon oxidation on critical cysteines residues (188, 189). Upon oxidation, the NRF2-Keap1 binding is disturbed and NRF2 degradation is terminated. However, NRF2 is not completely released from Keap1, instead is the newly synthesized NRF2 that translocate into the nucleus forming heterodimers with bZIP transcription factors such as Mafs (predominantly), c-Jun or ATF4 prior binding to ARE (190) (Fig. 4). It is currently a source of debate, if NRF2 dissociates from Keap1, however the most convincing results suggest that NRF2 is not completely released from Keap1 (178, 191).

Keap1 is an adaptor of the Cullin-3-based E3 ligase. Each subunit of Keap1 contains 27 residues of which only 9 are believed to be reactive, depending their specific reactivity on the type of electrophile that targets the different cysteines residues (192, 193). For example, Cys151 is important NRF2 activation mediated by H_2O_2 (194, 195).

Furthermore, NRF2 is also redox regulated. In the nucleus NRF2 remains bind to Crm1 (chromosome region maintenance 1; exportin) via Cys183 which is regulated GSH or Trx systems. Moreover, Trx1 it is involved in the exportation of NRF2 from the nucleus and via Trx1/Ref is capable of reducing Cys506 in the NLS crucial for the interaction with the coactivators CBP/p300 (Fig. 3) (85).

NRF2 is also regulated by phosphorylation. Phosphorylation of Ser40 by protein kinase C (PKC) prevents its binding to Keap1 and supports its translocation to the nucleus (196). Also, NRF2 can be phosphorylated by Fyn at Tyr568 in the nucleus, promoting Crm1 interaction and thus nuclear export (Fig. 3).

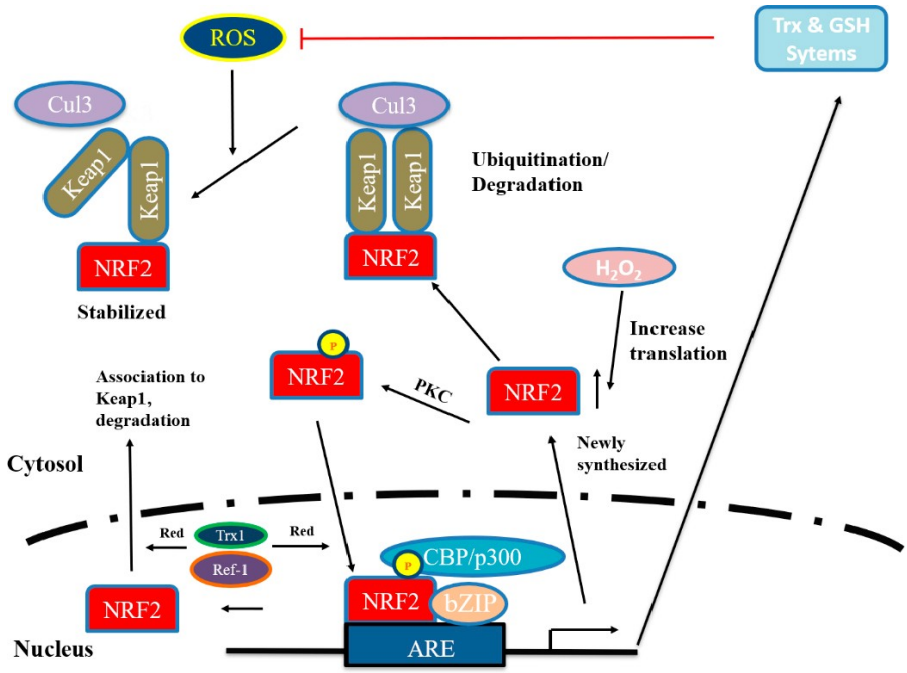

Figure 3. *Schematic overview of NRF2 regulation. During normal conditions, the NRF2 inhibitor Keap1 binds to NRF2 for its proteosomal degradation. Upon oxidative stress newly synthesize NRF2 bypasses Keap1 and it translocates it to the nucleus where it binds to the antioxidant response element.*

1.5.2 NFκB

NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) comprise a family of dimeric transcription factors containing several subunits: p65 (RelA), RelB, c-Rel, p50 and p52, being p50 and p52 generated from their precursors p105 and p100 (197). These proteins have a nuclear localization signal (NLS) responsible for their DNA and protein binding. p50 and p52 are unable to activate transcription, however they act as repressors binding to the DNA (198).

The NFκB system is mainly responsible for the immune and inflammatory response however it is also involved in cellular growth and apoptosis. The activation of NFκB is mediated by diverse cytokines, antigens and growth factors that stimulate different receptor families triggering a series of phosphorylation events that ends in the activation of NFκB (178). The incorrect regulation of NFκB is furthermore associated with many diseases such cancer, diabetes and atherosclerosis (199).

NFκB, in a similar manner as NRF2, remains on an inactive state in the cytosol. Once stimulated NFκB gets rid of its inhibitor IκB and translocates into the nucleus where it binds to promoter regions of its target genes (200) (Fig. 4).

Under normal circumstances, NFκB dimers are targeted by members of the IκB family (183, 201). Upon induction, IκB gets phosphorylated by the IκB kinase (IKK) (a complex conform of IKKα, IKKβ and two IKKγ subunits that form the NFκB essential modulator (NEMO)) and subsequently IκB gets ubiquitinated for proteasomal degradation (202, 203) (Fig. 4).

Additionally, phosphatase can be inactivated by oxidations, which in turn leads to increased phosphorylation, NFκB activation and enhanced signaling (204, 181, 205).

In the nucleus, as opposed to its preventive role in the cytosol, Trx1 is essential for DNA binding of the p65/p50 complex by reducing Cys62 in the p50 subunit, (206, 207).

As previously described, TRP14 has also been implied in the prevention of NFκB activation by keeping reduced LC8 binding to IκB and preventing IKK phosphorylation and degradation (113). Also, the Grx system modulate NFκB activation through deglutathionylation of critical cysteines in the p65 and p50 (114, 208).

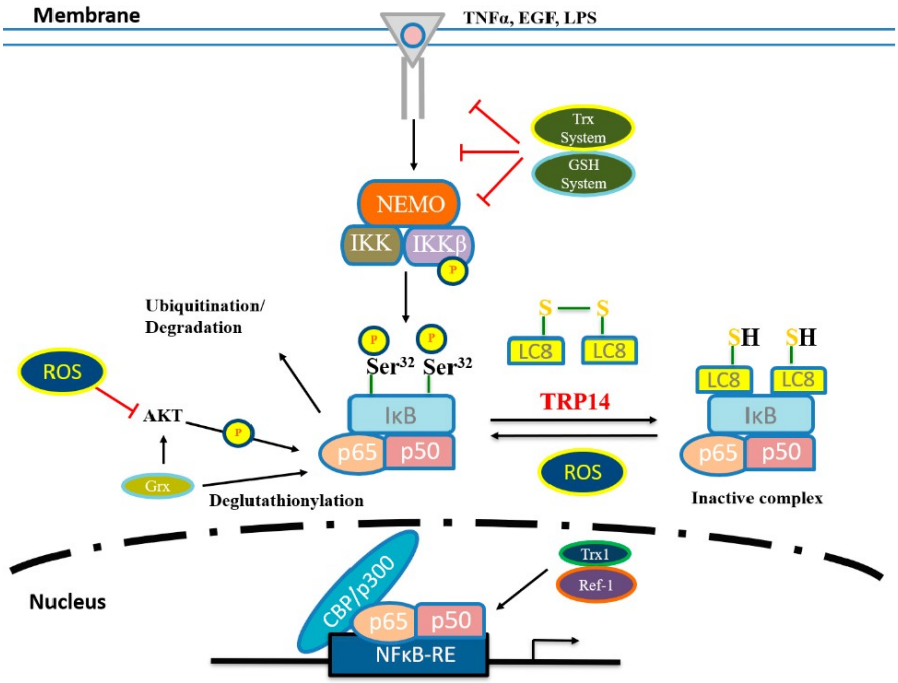

Figure 4. *Schematic overview of the regulation of NFκB canonical pathway modulated by the Trx and GSH systems.*

1.5.3 HIF

Hypoxia inducible factors (HIFs) are key transcription factors that mediate the response to in oxygen deficiency with an implication in angiogenesis, erythropoiesis and glycolysis (209).

HIFs are dimeric proteins conform of a constitutive HIF-1β subunit and an inducible HIFα subunit. HIF-1β is always express in exceed amounts making the levels of HIF-1α the accountable of its activity (210).

HIF-1α has an oxygen-dependent degradation domain with two conserved proline residues. At physiological oxygen levels, at least one proline residue is hydroxylated by PHDs which are recognized by pVHL, which targets HIF-1α for degradation (211).

PHDs activity dependent on oxygen availability, therefore at lower oxygen levels PHDs get inhibited, which leads to a HIF-1α dependent transcriptional activation (212). Also, in an oxygen dependent manner HIF-1α gets hydroxylated at the

asparaginyl residue by the factor inhibiting HIF (FIH). Its association with the p300 coactivator gets blocked and therefore, HIF dependent transactivation is repressed (213, 214). Upon stabilization, HIF-1α dimerizes with HIF-1β. Subsequently, it binds to HRE in the promoter region to initiate transactivation (215).

Several studies elucidate a crosstalk mechanism between NFκB and HIF-1α, suggesting that NFκB promotes HIF-1α activation upon binding to HIF-1α promoter region (217-219), the cooperative effect of these two transcriptional factors may be a protective mechanism of organs exposed to ischemia (220).

HIF-1α has been described as redox sensitive. ROS were described to aid HIF-1α stabilization. Interestingly, TrxR1 studies showed that it played no effect on the stabilization of HIF nor in its function when TrxR1 was overexpressed or depleted (221, 222). In the other hand, studies on Trx1 suggest that it has an important role in translocation and binding of HIF-1α (222-229). The reduction of cysteine 800 in HIF-1α required for its activation takes places via Trx1/Ref-1 (223, 225) as well as Trx1 is involved in the detachment of pVHL from HIF-1α (226).
Furthermore, when Trx1 was overexpressed it played a crucial for HIF-1α transactivation by activating p70S6K and eIF-4E which are essential for translation initiation (81, 229, 230) (Fig. 5).

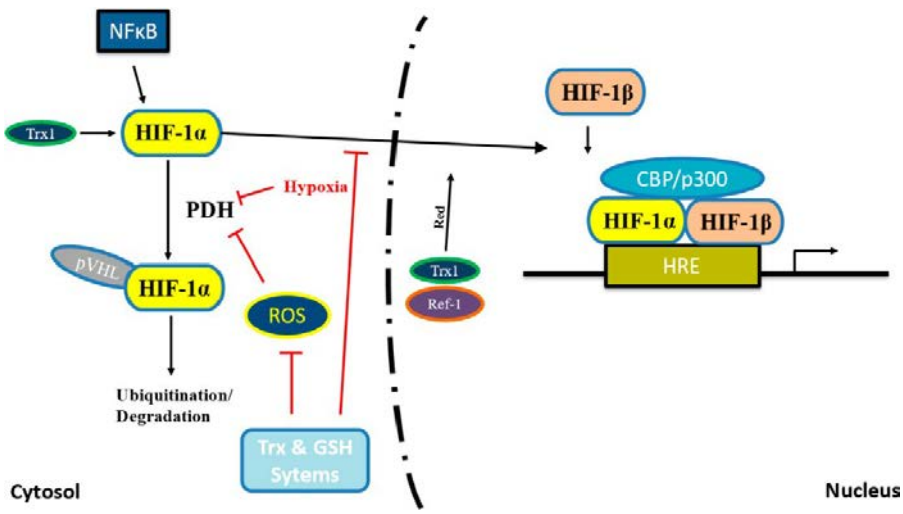

Figure 5. *Schematic overview of HIF regulation by the GSH and Trx Systems.*

1.6 Protein homeostasis and redox control

1.6.1 Protein synthesis

Translation consumes up to 40% of the cellular energy (231). Hence, suppressing translation can save up for cellular defense or repair processes. Even Though translation can be controlled virtually at any step, most of the translational regulation happens during the initiation step (232).

1.6.1.1 The eIF2α pathway

Many different types of insults cause translational suppression by phosphorylation of eIF2 at Ser51 (233). The resulting impaired nucleotide exchange blocks protein synthesis which is required for binding of the initiator Met-tRNAi to the 40S subunit during initiation (234).

In mammals, there are several conserved Ser/Thr kinases responds to different to the different stresses. PKR is very important in ribotoxic stress (235). PKR-like endoplasmic reticulum kinase (PERK) is activated in response to ER stress (236) and HRI) which is activated under heme deficiency. This kinase is also activated by arsenite-induced oxidative stress and reduction of global translation under arsenite stress (237).

1.6.1.2 HRI kinase

Translation suppression under arsenite stress and heme deficiency exclusively depends on HRI (238). While heme has a binding site in HRI, arsenite treatment in vitro does not lead to eIF2α phosphorylation (239). Interestingly. increased levels ROS are required for arsenite induced activation of HRI (240). This is consistent with cells experiencing oxidative stress upon arsenite treatment (241). ROS might act directly on one or more of the cysteine residues in HRI.

2 METHODOLOGY

This section provides a short introduction into some of the methods used in paper I-V. For more detailed information see the Materials and Methods part of each study.

2.1 Activity Assays for TrxR1, Trx1 and TRP14 (paper I-III)

In this thesis, we developed an assay for examining the ability of Trx1, TRP14 and TrxR1to reduce HRI utilizing the fluorescence characteristics of NADPH. (Fig 6).

Furthermore, TrxR1 activity has been studied with purified enzyme and the DTNB assay (246). TrxR1 directly reduces DTNB to two TNB- molecules, which absorb at 412 nm. In this thesis, we used this method to examine how different proteosomal inhibitors affected TrxR1 activity based on the knowledge that one unit (U) of TrxR1 is defined as the amount of enzyme catalyzing the reduction of 1 mol DTNB (formation of 2 mol TNB-) per minute (246).

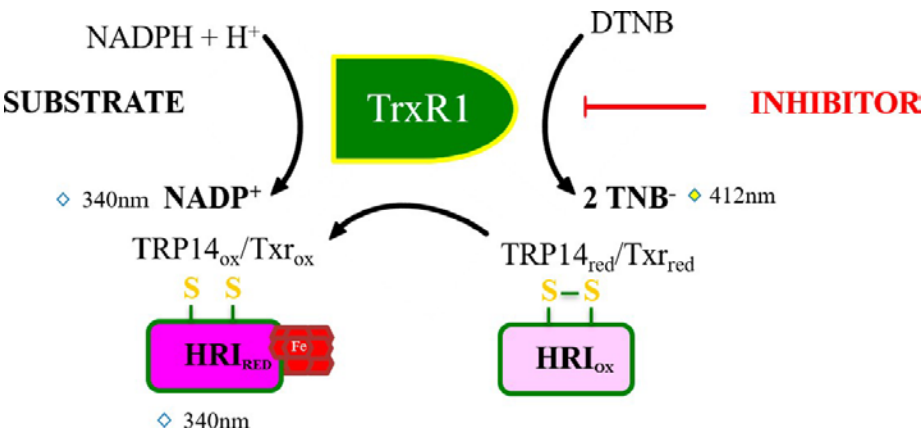

Figure 6. *Principles of the activity assays used in this thesis*

2.2 pTRAF (Paper IV-V)

With our developed tool pTRAF we could study the activation of 3 different transcriptional factors; NRF2, NFκB and HIF by locating all the cassettes on the same plasmid. Thus, by coupling mCherry to NRF2, YPet to HIF and CFP to NFκB we could compared the different intensities to each other, making it is possible to study the relative activation of all three transcription factors in single cells with high resolution in a high throughput manner (Fig. 7)

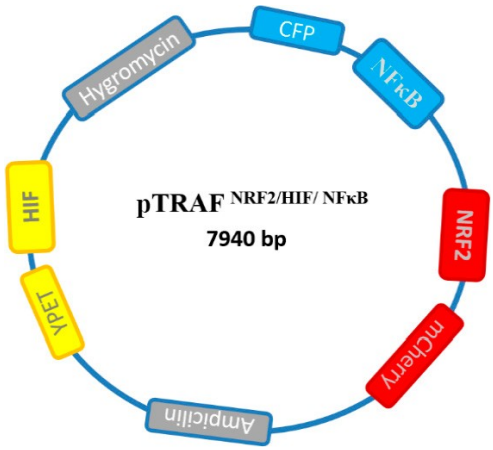

Figure 7. *Schematic overview of the pTRAF plasmid*

3 AIMS OF THIS THESIS

3.1 Aims

The thioredoxin system has been discussed in a variety of different contexts and many of its important roles in health and disease have been discovered. However, its involvement in cellular pathways is versatile and countless aspects that contribute to the final outcome of the cell are still unknown. Taking this into account we studied several separate aspects of the Trx system that had the following specific aims:

Paper I

- Determine the mechanisms of redox generation in cells treated with b-AP15 and the potential implication the Trx system.

Paper II

- Show the potential pharmacological applications of enone containing compounds, relative selectivity for USP14, specifically inhibit the UPS and evaluate their effect in the Txr system.

Paper III

- Evaluate the role of the Trx system as a redox cellular sensor of arsenic compounds triggering the translational stress response.

Paper IV

- Discern the role of the Trx system member, TRP14, in the activation of redox regulated transcriptional factors NRF2, NFκB and HIF.
- Understand the importance of TRP14 for intracellular reduction of cystine.

Paper V

- Apply the pTRAF method to evaluate the activation NRF2, NFκB and HIF, in drug development.

4 PROJECTS

4.1 Paper I

Oxidative Stress Induced by the Deubiquitinase Inhibitor b-AP15 Is Associated with Mitochondrial Impairment

Xiaonan Zhang, **Belén Espinosa**, Amir Ata Saei, Padraig D'Arcy, Roman A Zubarev, and Stig Linder

Background

b-AP15 is a small molecule that blocks proteasome activity at the level of the proteasome -associated deubiquitinases (DUB). Excitingly, b-AP15 has provided a new mechanism to overcome intrinsic and acquired resistance that limited the therapeutic efficacy of other anti-cancer therapies. It is well described that b-AP15 boosts proteotoxic and oxidative stress in tumors cells, however its efficacy is hindered by antioxidants (247). In this paper we address the mechanisms by which b-AP15 induces oxidative stress.

Main Findings

- **b-AP15 oxidative stress depends on having functional mitochondria**

We exposed cells to low doses of ethidium bromide until reaching non detectable expression of mitochondrial genome. We treated those cells with b-AP15, and we could observe a remarkable abrogation of NRF2 induction compared to normal cells, with no changes in the levels of proteotoxic stress.

- **Dubs inhibitors do not required inhibition of Thioredoxin Reductase activity**

Thioredoxin Reductase activity was previously shown to be inhibited by b-AP15 (247). In order to evaluate this possibility, we tested some newly identified DUB inhibitors that did not inhibit TrxR and all induced NFR2 activation

- **b-AP15 decreases the expression of COX5b**

We found that 3 proteins were significantly downregulated TOMM34 (translocase of outer mitochondrial membrane 34), CHDH (Choline dehydrogenase) and COX5B (Cytochrome C subunit 5B). COX5B is a component of the electron transport chain and its decrease may explain reduce oxidative phosphorylation.

Conclusions

Despite its strong cytotoxicity to tumor cells, b-AP15 and similar compounds show limited activity against normal cells. We here found weaker induction of the NRF2 targeted protein HO-1 and decreased elevation of GSSG/GSH ratios in $\rho 0$ cells exposed to b-AP15, consistent with a mitochondrial involvement in b-AP15-induced oxidative stress. We also found that increasing the level of proteotoxic

stress by inhibiting anterograde ER translocation resulted in increased induction of expression of HO-1 and without affecting the selenoprotein TrxR1. These findings suggest cells exposed to b-AP15 will have pursue its effect due to an increase ROS level of mitochondrial origin.

4.2 Paper II

Cytotoxic unsaturated electrophilic compounds commonly target the ubiquitin proteasome system

Karthik Selvaraju, Arjan Mofers, Paola Pellegrini, Johannes Salomonsson, Alexandra Ahlner, Vivian Morad, Ellin-Kristina Hillert, **Belén Espinosa**, Elias S. J. Arnér, Lasse Jensen, Jonas Malmström, Maria V. Turkina, Padraig D'Arcy, Michael A. Walters, Maria Sunnerhagen & Stig Linder

Background

Drug resistance remains the principal limiting factor in the treatment of patients suffering from cancer. In this regard, the ubiquitin proteasome system has emerged as a promising area for the development of new drugs since they can overcome other type of proteosomal inhibitor resistance (248). Cysteine deubiquitinases (DUBs) are druggable by α,β-unsaturated ketones (Enones) by Michael addition (249). Unfortunately, Michael acceptors are often avoided by drug developers due to their reputation of displaying general reactivity.

Many natural products with antineoplastic activity usually contain Michael acceptors like those contained in b-AP15. The objective of this paper was to perform a drug screen of ≈ 5000 compounds selected for unsaturated ketone motifs and determine their usefulness as deubiquitinase inhibitors despite their proclaimed off-target activity. We also analyzed the propensity of these compounds to inhibit TrxR1, as this selenoprotein is typically considered to be a prime target of electrophilic compounds (250).

Main findings

- **Only 3% of the enone-containing compounds where cytotoxic to tumor cells**

Using a library of enone containing compounds based on the assumption that enone electrophiles will inhibit cysteine DUBS we found that only 3% were cytotoxic to tumor cells in the micromolar range.

- **Hit compounds inhibit the proteasome deubiquitinase activity**

Of the 141 antiproliferative compounds we found that 25% inhibited the UPS. We further characterized the selected hit compounds and consistent with inhibition of the proteasome, they showed increased levels of proteotoxic stress, ER stress and apoptotic markers, together with elevated levels of HMXO1as an indication

of oxidative stress. A glycerol gradient cell fractionation demonstrated that poly-ubiquitin chains that accumulate in cells co-sedimented with proteasomes, this finding excluded the possibility that those chains were further processed. We were able to identify that those compounds inhibited USP14/UCHL5, however none of the compounds inhibited total cellular DUBs. We confirmed these find- ings using Isothermal Calorimetry (ITC) and Cellular Extract Thermostabilizing Assay (CETSA). A molecular Mass shifts of USP14 was observed with mass spectrometry and binding of the compounds to the UPS14 catalytic domain with Tryptophan fluorescence.

- **USP14 and TrxR are not necessarily targeted by the same compounds**

Enones are known to inhibit TrxR1 (251). We found that surprisingly only 20 of the 141 cytotoxic compounds could directly inhibit TrxR1 activity and none of them belonged to our hit compounds.

- **The selected compounds showed anti-neoplastic activity in Zebrafish**

Some of the hit compounds inhibited the proliferation and dissemination of human melanoma cells in zebrafish embryo model at a concentration of 5μM and did not affect embryo development at 20μM.

Discussion

Many of the most used chemotherapeutical drugs are natural products. Many natural products contain α,β-unsaturated ketones and are expected to be associated with widespread cysteine reactivity. Several such thiol-reactive natural products have been reported to affect the ubiquitin-proteasome system (UPS) (252). Whether this type of biological activity is evolutionary selected or an intrinsic property of α, β-unsaturated compounds was not known.

In this paper, we screened ~5000 synthetic α, β-unsaturated compounds for cytotoxicity to tumor cells and inhibition of proteasome degradative activity. Interestingly, of 141 cytotoxic compounds, 28 compounds (20%) increased the levels of proteasome substrates in cells. We found evidence of inhibition of proteasome-associated cysteine deubiquitinases, USP14. Structural analysis suggested that the compounds bind to a crevice close to the USP14 active site with modest affinity, followed by covalent binding. Counter screening performed using non-proteasome associated cysteine deubiquitinases and thioredoxin reductase suggested a degree of selectivity to proteasome deubiquitinases. We finally demonstrate limited developmental toxicity and significant antineoplastic activity in zebrafish embryos.

These findings suggest that proteasome inhibition is a common mode of cytotoxicity by natural products containing α, β-unsaturated carbonyl functionalities. The combination of high sensitivity of tumor cells to UPS inhibition and the potential for high druggability of components of the UPS may explain the common pharmacological response of proteasome inhibition to this class of drugs. (Fig. 8)

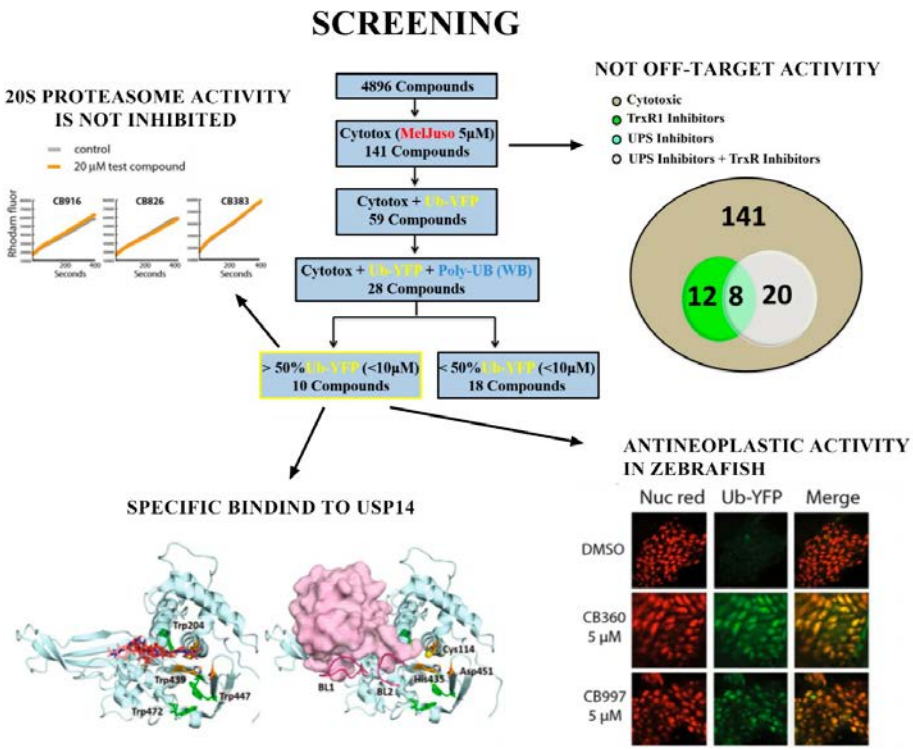

Figure 8. *Schematic overview of paper II*

4.3 Paper III

Cellular protection from arsenic compounds through control of global protein synthesis by TrxR1-mediated HRI kinase activation

Bogdan Jovanovic, **Belén Espinosa**, Shawn M. Lyons, Nga Ly-Hartig, Tobias Dick, Elias Arnér and Georg Stoecklin

Background

Arsenic compounds are a potent health hazard associated with the development of many diseases including cancer. Paradoxically, some of these compounds are approved for the treatment of different types of tumor malignancies (253). These compounds induce their toxicity by oxidizing thiol groups (253). To try to overcome this type of insults, cells have developed a protective stress response diminishing their level of protein translation by activating HRI and therefore phosphorylating the translation initiation factor eIF2α (254). In this study we hypothesized that this protective mechanism is redox regulated.

Main findings

- **Trx system keeps HRI in its reduced state**

In this paper we showed that HRI redox status is controlled by TRP14, Trx and TrxR keeping HRI in its reduced form. Under exposure to arsenic compounds all 3 enzymes lost their activity and reduction of HRI was inhibited which can activate the HRI kinase activity.

- **TrxR is required for the ASN-induced translational stress response**

We could demonstrate that TrxR inhibitors led to HRI oxidation, eIF2α phosphorylation, translation inhibition and stress granules formation. We could show for the first time that through its prooxidative function, TrxR1 regulates the activity of a substrate.

Discussion

In this study, we could confirm a direct link between the thioredoxin system and HRI activation. Our results show that several inhibitors of TrxR1 lead to HRI-dependent eIF2α phosphorylation, translation inhibition and stress granule formation. Furthermore, we found that TrxR1 co-immunoprecipitates with HRI, and that TrxR1 reduces HRI in vitro. Interestingly, TRP14 and Trx1 in vitro enhance the reducing activity of TrxR1 towards HRI indicating that several thioredoxin family proteins may participate in controlling the redox state of HRI. Given that the deregulation of protein synthesis is increasingly recognized as a mechanism that drives cancer progression, this redox-driven translational stress responses strategies to inhibit protein synthesis might be of use in anticancer therapy (Fig. 9).

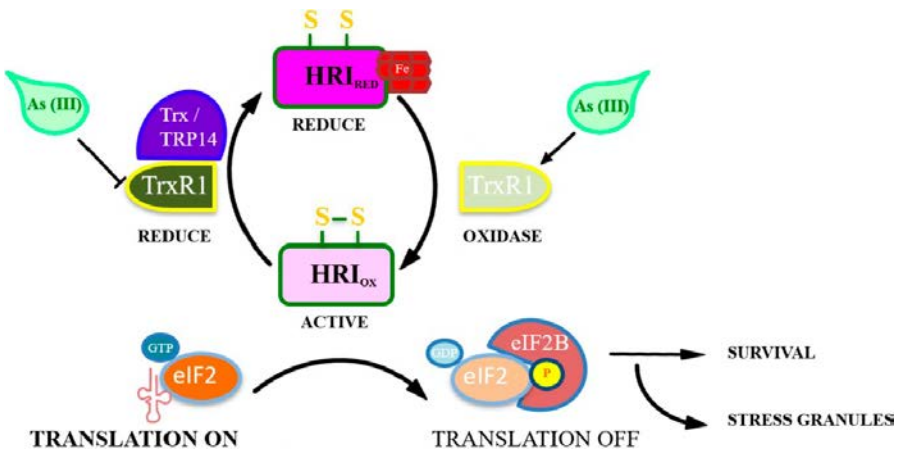

Figure 9. *Schematic overview of paper III*

4.4 Paper IV

Thioredoxin related protein of 14 kDa (TRP14, TXNDC17) regulates NRF2, NFκB and HIF activities, and is essential for intracellular cystine reduction

Belén Espinosa, Irina Pader, Marcus Cebula, Katarina Johansson, Elias S. J. Arnér.

Background

Cellular responses to diverse stimuli are often controlled by redox regulatory events. Thioredoxin-related protein of 14 kDa is a sparsely characterized member of the Trx system and a good substrate of TrxR1 (107). TRP14 has the ability of efficiently reducing L-cystine, nitrosothiols and persulfide moieties of proteins (112). TRP14 is also known to support PTP1B activities and to indirectly counteract NFκB (113, 120). Taking into consideration that TRP14 is an efficient redox active protein, here we asked if TRP14 is also functionally linked to NRF2, the main transcriptional regulator of the antioxidant system and to HIF, the oxygen dependent transcription factor that is also redox regulated and indispensable during hypoxic metabolism.

Through the generation of a stable knockdown of TRP14 in HEK239 cells and using our previously developed pTRAF reporter plasmid.

Main findings

- **Knockdown of TRP14 increases the activation of NRF2 and NFκB in normoxic and hypoxic conditions**

After transfecting theTRP14 knockdown cells with our developed reporter plasmid we could observe the effect of TRP14 in the activation of the different transcriptional factors. Indeed, we could confirm the repression that TRP14 exerts in NFκB upon TNFα stimulation previously described but here we could see that effect remains in hypoxic conditions at a lesser extent. Furthermore, we observed that similarly to NFκB, TRP14 represses the activation of NRF2 after treating the cells for 24 hours with auranofin, both in normoxic and hypoxic conditions.

- **TRP14 is crucial for HIF activation upon TNFα stimulation during hypoxic conditions**

We furthermore tested the role of TRP14 in HIF activation. In normoxic conditions was unfeasible to determine the role of TRP14 in the regulation of HIF due its low activation at high oxygen concentrations. However, under hypoxic conditions TRP14 proved to be indispensable specially after treating the cells with TNFα for 24 hours.

- **TRP14 levels increased under hypoxia or TNFα treatment**

Here, in order to identify potential transcriptional factors that may modulate the expression of TRP14 we performed a bioinformatic analysis of the proximal TXNDC17 promoter region, we could find several potential binding sites for the transcriptional factors study here NFκB and HIF but none for NRF2 the main regulator of the antioxidant system. Interestingly, the levels of TRP14 expression increased after treating cells with TNFα and under hypoxia, however no changes were observed after treating the cells with auranofin, correlating with the initial promoter predictions and suggesting that TRP14 is a downstream target of NFκB and HIF but not of NRF2.

- **TRP14 knockout in HEK293 cells diminished global protein translation**

In order to determine if the complete abolition of TRP14 would further increase the effects of TRP14 on the transcriptional factors, we made a TRP14 knockout cell line. Surprisingly, when transfecting those cells with the pTRAF, all signals diminished significantly. These observations suggested that the lack of signal in these pTRAF experiments was related to global effects on protein translation rather than an effect in redox regulated transcription factors.

To confirm this hypothesis, we performed a puromycin assay where we could see that the puromycinylated polypeptides in the TRP14 knockout cells decreased, showing inhibited protein translation.

- **Supplementation of the cellular media with methionine or N-acetylcysteine**

Based on previous findings in our group that TRP14 efficiently reduces cystine we speculated that perhaps a complete depletion of TRP14 leads to a major deficiency of cysteine inside the cell, which would be essential for protein synthesis. Since cells can alternatively obtain cysteine from methionine through the transsulfuration pathway we thus supplemented the cells with either methionine or NAC and we could observe that the effect of TRP14 knockout cells in protein translation was reverted

- **TRP14 knockout cells are highly sensitive to PPG in comparison to control cells**

To demonstrate that TRP14 knockout cells would be entirely dependent on the transsulfuration pathway as a cysteine source, we treated cells with propargylglycine (PPG), a cystathionase inhibitor to complete prevent the utilization of methionine as a source of cysteine. As expected TRP14 knockout cells showed signs of strong toxicity by PPG where normal cells were unaffected (Fig.10)

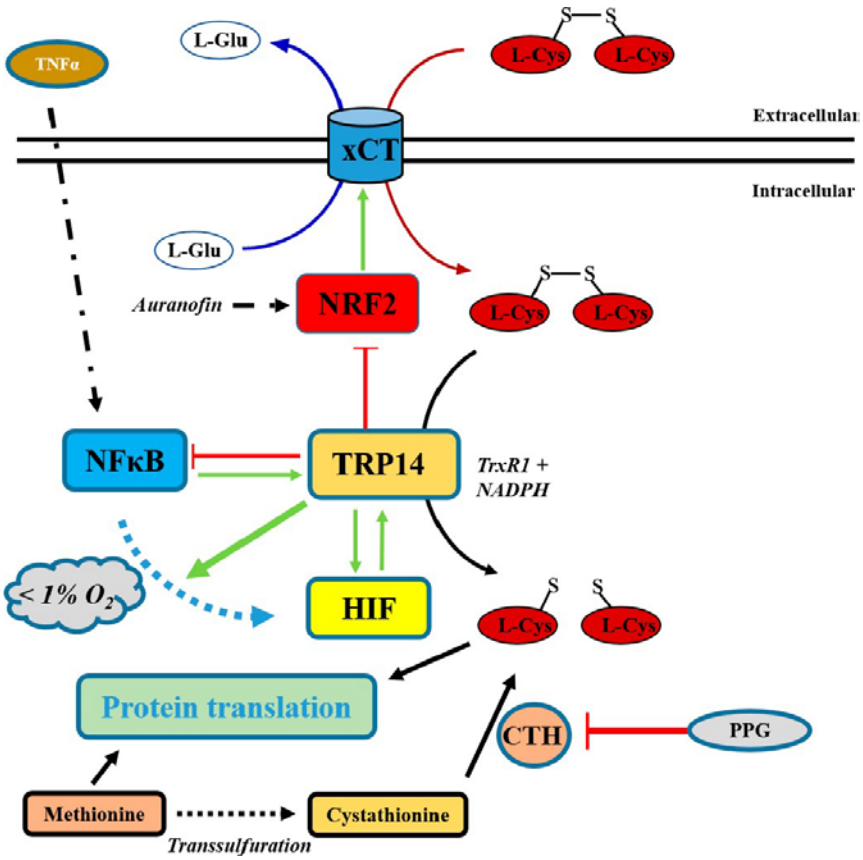

Figure 10. *Schematic overview of paper IV*

Discussion

In this study we have characterized the role of TRP14 in the regulation of NRF2, NFκB and HIF activities. We found that TRP14 can suppress NRF2 as well as NFκB activation, and that under hypoxic conditions TRP14 seems to be required for HIF activation as prompted by TNF-α stimulation. While TRP14 may be upregulated by both NFκB and HIF, no evidence was found of TRP14 being a NRF2 target. Most importantly, TRP14 seems to be an essential enzyme for utilization of intracellular cystine as a source of cysteine, suggesting that TRP14 has important roles in redox homeostasis.

4.5 Paper V

Novel vinyl sulfone compounds are more specific NRF2 activators in the in vitro and in the central nervous system than dimethyl fumarate

Karl E Carlström, Praveen K Chinthakindi, **Belén Espinosa**, Faiez Al Nimer, Katarina Johansson, Elias S J Arnér, Per I Arvidsson, Fredrik Piehl

Background

Dimethyl fumarate (DMF) is the first line of treatment for patients suffering from Multiple Sclerosis (MS) worldwide (255). It is known that NRF2 is a target of DMF, however its precise mechanism of action remains elusive. Due to the significant off-target effects of DMF and its poor ability to penetrate the blood-brain barrier, we hypothesized that by modifying certain moieties we could improve NRF2 specificity and increase the penetrance to the central nervous system. In this paper we evaluated the capacity of eight de novo synthesized vinyl sulfone compounds (CH-1to CH8) to activate NRF2 and other transcriptional factors in comparison to DMF.

Main findings

- **CH3 is more specific to NRF2 compared to DMF as evaluated using pTRAF transfected cells**

Out of the eight de novo synthesized compounds that we evaluated using pTRAF, CH3 was the most promising candidate in comparison to DMF showing similar NRF2 activation but less off-target effects when measuring the activation of NFκB and HIF.

- **Transcriptional changes observed in human cells produced by CH3 and DMF are observed in rat glial cultures**

We treated oligodendrocytes with CH3 and DMF showing in both cases and increase in NRF2 expression in line like with previously observe results using pTRAF. However, this response varied in microglia cells when comparing CH3 and DMF.

- **CH3 and DMF affects numbers of pre-OLs and neurons after TBI**

Following TBI, DMF (but not CH-3) lowered systemic CD45+ cells. In addition, DMF also limited axonal degeneration following TBI compared to vehicle.

In turn, CH-3 preserved or facilitated proliferation and differentiation of OLs following TBI compared to DMF and vehicle.

Discussion

Here, we tested a series of novel vinyl sulfone compounds as compared to DMF through in vitro and in vivo models. We demonstrated that the best candidate, CH-3, was more specific activating NRF2 in contrast to DMF. The NRF2 specific effect is suggested to influence oligodendrocyte, specifically promoting proliferation of pre-myelinating cells. In contrast, CH-3 cannot reduce the concentrations of CSF, a nerve cell death biomarker in, suggesting that DMF has a neuroprotective off-target effect independent of NRF2. These observations suggest a potential therapeutic role of NRF2 inducing compounds in conditions such of demyelination or brain trauma.

5 SUMMARY AND CONCLUSIONS

Within this thesis we investigated different aspects of the Trx system in the context of redox signaling.

In **Paper I** and **Paper II** we investigated the potential use of proteasomal inhibitors in anticancer therapy focusing with special interest in b-AP15 and other compounds containing an enone motif. These compounds are characterized by an inhibition of the 19S proteasome ubiquitinase, becoming a great alternative to those inhibitors of the 20S subunit, like bortezomib, that have developed high level of resistance. In **Paper I** we showed how b-AP15 increased the level of oxidative stress in the cells by inflicting direct damage in the mitochondrial and not being that phenomenon affected by the status of TrxR1. In **Paper II** we performed a large screening of compounds containing the previously mentioned reactive enone motif. Despite their claim high reactivity, we demonstrated that these compounds had a high selectivity towards the 19S DUB USP14.

In **Paper III,** we showed how the Trx system can regulate cellular translation by redox regulating HRI. Furthermore, we showed that TrxR1 is required for Asn translational inhibition and for the first time we could show how TrxR1 regulates the activity of a substrate through its prooxidative function.

In **Paper IV**, we elucidated the role of TRP14 as a specialized member of the Trx system with important roles in the regulation of several redox regulated transcriptional factors. Furthermore, we demonstrated the crucial role that TRP14 plays in the incorporation of cysteine into the cells, becoming the cells dependent on the transsulfuration pathway in its absence.

In **Paper V**, we used our unique tool, pTRAF, to understand how different transcriptional factors intertwined when testing different drugs, helping us to design more specific drugs minimizing the off-target effects.

In conclusion, with this thesis we have expanded the knowledge about the Trx System in redox signaling. We showed how TrxR1 could be an interesting target in anticancer therapy with specific activities despite its nucleophilicity. And we disclosed TRP14 as a dedicated modulator of several transcriptional pathways and how its presence in the cells is crucial for the intracellular reduction of cysteine.

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