Final report for project K109843 entitled "Peroxidase function of peroxiredoxins in relation with cancer".

References to publications that emerged from this project are highlighted in red.

Prologue

The aim of this project was to provide deeper insight into the redox biology of cancer related physiological processes. We consider the project highly successful because it answered fundamental questions in the field and opened very exciting new directions to pursue. Since the starting date of the project we produced 28 publications and a large number of conference abstracts. 22 of our research publications are related to the proposed aims and acknowledge the project. The 22 publications consists of 18 original research articles, 2 invited reviews and 2 invited book chapters with an overall impact factor of 129,941. Most of these achievements were a result of national and international collaborative efforts. The PI serves as corresponding author in 9 of these publications.

What we consider to be one of the most exciting output of this project was the observation that a novel cysteine posttranslational modification (PTM), per/polysulfidation, can protect peroxiredoxin (Prx) functions under oxidative stress. We also have results showing that not only peroxiredoxins, but a number of other redox-regulated proteins are protected from oxidative stress via polysulfidation of their most redox sensitive thiol residues. In addition, this PTM is gaining exponentially increasing attention in the field as a major driving force of redox-signaling events. Based on these observations we obtained permission from the president of the study group to slightly change direction compared to what was originally proposed in the project, which resulted in a number of exciting new discoveries. Most notably, one of our publication within the scope of this endeavor described a novel methodology to detect per/polysulfidation on isolated proteins, inside cells and in tissue samples. Using this method we reported that the NADPH-driven reducing machineries, the thioredoxin (Trx) and glutathione (GSH) systems, play major roles in polysulfide homeostasis and specific components of the Trx-system regulate polysulfidation-based redox signaling events (Doka *et al.*, 2016). Based on these discoveries we obtained a grant from the National Research, Development and Innovation Office (KH 126766) within the framework of "Grant for Research Teams with Significant Achievements of Internationally Outstanding Impact".

Major findings

Cancer cells were shown to produce elevated levels of Reactive Oxygen Species (ROS) (Trachootham *et al.*, 2009). This play widespread roles in cancer biology including reprogramming of redox regulated cellular signaling processes. Among others it was previously demonstrated that epidermal growth factor receptor (EGFR) activation results in elevated extracellular ROS production (Paulsen *et al.*, 2012). The ROS response elicited by EGF is thought to amplify tyrosine-kinase signaling through the inhibition of protein tyrosine phosphatases that normally antagonize this signaling route, but not even the source of ROS in these processes were at the time fully elucidated. To this end in collaboration with Prof Miklós Geiszt's laboratory we demonstrated that in A431 epidermal carcinoma and HaCat aneuploid immortal human skin keratinocyte cell lines, EGF-induced peroxide is generated by DUOX1 in an agonist-initiated calcium signal dependent manner. We also demonstrated that the generated H_2O_2 is captured by Prx2 in the cytosol and Prx3 in the mitochondria of the cells (Sirokmany *et al.*, 2016).

Prx have major roles in the antioxidant defense mechanisms of cancer cells as well as normal cells, but it is also a major player in redox signaling events.

Regarding the antioxidant properties of Prx in relation to cancer therapy we demonstrated that Prx 2 in red blood cells is responsible for reducing the majority of doxorubicin (dox)-induced hydrogen peroxide hand in hand with *glutathione peroxidase* (Gpx). Both proteins rely on the NADPH-driven reducing systems to recycle them to their active reduced forms after oxidation by H_2O_2. We showed that using extracellular glucose, the *glucose-6-phosphate-dehydrogenase* enzyme (G6PD) can provide enough NADPH for the Trx-*thioredoxin reductase 1* (TrxR1) system to efficiently recycle oxidized Prx2 to its active reduced form and thereby protect red blood cells from dox-induced hemoglobin oxidation. These observations indicate that patients with G6PD deficiency (although rare in Hungary it represents the most prevalent enzyme deficiency in the human population) are more prone to dox-induced oxidative damage of their erythrocytes and likely to develop hemolytic side effects upon chemotherapy.

Under severe oxidative stress conditions (which could be the case during chemotherapy), we found that the Prx pool is overwhelmed. In this case GAPDH, becomes the primary peroxide target. In collaboration with Prof. Tobias Dick's group we revealed a proton relay system that is responsible for the redox sensitivity of the active site Cys residue (Cys152) in GAPDH. We have shown that by introducing point mutations of the residues that constitute the proton relay

system, the peroxidase and glycolitic activities of the enzyme can be uncoupled. By replacing WT GAPDH with these point mutants in yeast we were able to show that this built in redox switch in GAPDH is a key component of the cellular adaptive response to increased peroxide stress. Briefly peroxide mediated inactivation of GAPDH reroutes the glycolytic flux into the pentose phosphate pathway. This results in elevated NADPH production, which is the primary fuel to reduce peroxiredoxins via NADPH/thioredoxin reductase/thioredoxin/Prx coupled pathways (Peralta *et al.*, 2015).

Under even more severe oxidative stress conditions, induced by for example the anticancer RITA compound, in a fruitful collaboration with Prof. Elias Arner's group, we have shown that the cytosolic TrxR1 enzyme, which plays a primary role in Prx reduction, has a redox sensitive solvent exposed Trp114 residue. Trp114 oxidation inactivates the enzyme by forming covalently linked dimers. The intracellular relevance of irreversible TrxR1 inactivation via Trp114 oxidation and dimerization was shown to induce oxidative stress mediated apoptosis of cancer cells upon treatment by RITA (Xu *et al.*, 2015).

Regarding the investigation of the roles of Prx in signal transduction we initially used A549 cell lines because we observed that in this cell line Prx2 is significantly less abundant compared to other Prx isoforms or the previously observed Prx2 levels in normal lung tissue (Lehtonen, 2005). In the meantime, Tobias Dick's laboratory provided evidence that NADPH oxidase-produced H_2O_2 plays a role in the inactivation of the transcription factor STAT3, which is mediated via a redox relay mechanism between Prx2 and STAT3 (Sobotta *et al.*, 2015). In addition, in A549, STAT3 was reported to be constitutively activated and its inhibition resulted in impaired cell proliferation and xenograft tumor growth (Zhang *et al.*, 2007). In light of these observations we hypothesized that the low expression of Prx2 could be involved in the constitutive activation of STAT3 in A549 cells. In order to get deeper insight in this, we engaged in elaborate experimentation to try to produce a model cell line, in which WT Prx2 and inactive point mutants are permanently overexpressed. A number of attempts using different methodologies failed to produce Prx2 overexpressed cell lines, including the modern and highly efficient sleeping beauty system (Ivics *et al.*, 1997). Recently, using proteasome inhibitors we discovered that this was not due to unsuccessful transfection of the plasmids, but due to preferential Prx2 degradation for which the mechanistic reasons are to be determined.

Both in its antioxidant and redox-relay functions, an important inactivation process of Prx proteins is the irreversible oxidation of their active site Cys residues to the corresponding

sulfinic (R-SO_2H) and sulfonic acid (R-SO_3H) derivatives. The sulfinic acid derivative of Prx (at the active site peroxidative cysteine) can be rescued by the sulfiredoxin system, but this is a very slow process and requires large quantities of ATP. Therefore, we hypothesized that a more efficient mechanism should exist to protect the active site peroxidative cysteine of these proteins (which is constantly engaged in reactions with peroxide species) from irreversible oxidation. To this end we discovered that a novel cysteine posttranslational modification, called per/polysulfidation, which is orchestrated via redox events (Nagy *et al.*, 2014; Ono *et al.*, 2014; Nagy, 2015; Vasas *et al.*, 2015; Fukuto *et al.*, 2018), is potentially involved in the protection of peroxiredoxins from oxidation of their active site cys to the sulfinic or sulfonic acid derivatives. Using human serum albumin as a model protein (which has only one cys residue that is not engaged in structural disulfide bonds) we showed that sulfide-mediated protection of protein cys residues from excessive oxidation is due to initial polysulfidation of cys thiols. The generated Cys-per/polysulfide species react further with oxidant molecules to produce perthiosulfenic (R-SSOH), perthiosulfinic (R-SSO_2H) or perthiosulfonic acid (R-SSO_3H) derivatives. We characterized these reactions and demonstrated that they are highly prevalent in cellular systems suggesting that Cys-SSH oxidation may represent a novel and yet uncharacterized, event in redox-regulation of cellular signaling. Among others, in collaboration with Prof. Albert van der Vliet we showed the formation of Cys-SSH on protein tyrosine kinases including EGFR, and their oxidation to Cys-SSOH upon NADPH oxidase activation (Heppner *et al.*, 2018). We obtained evidence that the H_2O_2 triggered oxidative modification on the Cys797 residue of EGFR -which is required for its activation- results in the formation of a Cys-perthiosulfenic acid residue (Cys-SSOH) as opposed to the previously proposed sulfenic acid (Cys-SOH). This was quantitatively demonstrated along with analyses of similar oxidative modifications on Src, both of which corroborated that persulfides and perthiosulfenic acids play pivotal roles in redox regulation of protein tyrosine phosphatases that are essential in onco-signaling. While thiol derived oxidation products mostly cause irreversible inactivation of enzyme activities, we demonstrated that a unique feature of these perthio- derivatives is that they are reducible by different components of the thioredoxin system in a protein thiol specific manner. Therefore, these modifications can protect functional and regulatory Cys residues from irreversible oxidation. We also fully characterized these modifications on Prx2 in isolated protein system as well as in HEK293 cells and demonstrated that they play a role in protecting Prx2 from accumulation of R-$SO_{2,3}H$ derivatives during their enzymatic reduction of H_2O_2 (coupled with the Trx-TrxR1-NADPH system). Furthermore, we have obtained evidence that these modifications serve as an important regulatory element of the redox-regulated

activation/inactivation of the phosphatase functions of PTP1B in isolated protein systems (based on enzyme kinetic assays and mass spectrometry based determination of these modifications) as well as in A431 cell (Doka *et al.*, 2018).

We considered the most exciting discoveries in our laboratory are related to our contributions to the recognition that redox signaling pathways are orchestrated by per/polysulfidation on functional or regulatory Cys residues (reviewed in (Ono *et al.*, 2014; Nagy, 2015; Fukuto *et al.*, 2018)). A role of cys persulfidation in relation to lung cancer biology was highlighted in our recent publication in which we reported that elevated persulfidation of the EXOG protein stimulates mitochondrial DNA repair in cancer cells by promoting mitochondrial DNA repair complex assembly (Szczesny *et al.*, 2016). Therefore, based on our proposal in the original application "*the proposed proteomic analyses in relation to Prx oxidation might give us insight into these mechanisms and could set the stage for more focused studies*" we concentrated more efforts to investigate how these processes are involved in cancer biology. This new direction proved more important than the initially proposed objective 4 of our proposal to test the roles of Prx in neutralizing UV-radiation-induced peroxides in melanocytes. Therefore, we asked for permission to follow up on these leads in the interim report of our project, which was officially approved by the president of the study section and resulted in a number of important observations.

First we developed a novel protocol to detect protein persulfides inside the cell and in vivo, which we named ProPerDP (Doka *et al.*, 2016). This method was validated from several angles and we demonstrated that it was the first method that can detect cellular persulfides by preventing artificial oxidation upon cell lysis (Doka *et al.*, 2017). Using ProPerDP, we confirmed that polysulfidation of protein Cys residues is a prevalent modification, but also raised awareness of the caveats that are associated with Cys PTM detection protocols. In this regard we carried out a systematic analysis on how alkylation (which is a prerequisite process in all current detection protocols) affect sulfur species speciation. We unambiguously demonstrated on inorganic-, cys- GSH- and protein polysulfides that alkylation with the conventionally used agents is under Curtin Hammett control. This means that the final alkylated mixture mostly represent the reactivities of the different nucleophiles (thiols and polysulfide species) with the alkylating agent rather than true speciation. We demonstrated that this has very important implications in redox biology not only in polysulfide species detection protocols, but for all Cys PTM measurements (Bianco *et al.*, 2018; Bogdandi *et al.*, 2018).

Furthermore, for the first time we presented strong evidence for the thioredoxin and glutathione systems to be major players not only in recycling peroxiredoxins, but also in regenerating other Cys residues from persulfide species. Importantly, we discovered that the most potent reducing pathway is via the *thioredoxin like protein of 14 kDa* (TRP14), which reveals a novel function for this new member of the Trx system. TRP14 is recycled by NADPH and TrxR1. We managed to show that these systems are fundamental regulators of intracellular sulfane sulfur homeostasis and redox signaling using TrxR1 and TRP14 knockdown HEK293 cells and novel mouse models that lack either *glutathione reductase* (GR) or TrxR1 and GR in the hepatocytes of their livers (Doka *et al.*, 2016). As a result of a systematic *in vivo* analysis we recently obtained evidence that the GSH-system is mostly responsible for intracellular maintenance of sulfane sulfur homeostasis and TRP14 together with the Trx-system is involved in cellular signaling processes by reducing only a subset of protein persulfides on functional Cys thiols.

Most publications in the field acknowledge that the majority of endogenous sulfide is produced via reverse transsulfuration pathways via moonlighting functions of *cystathionine beta synthase* (CBS) and *cystathionine gamma lyase* (CSE). However, our comprehensive analyses of human urine and blood samples from patients with CBS deficiency failed to provide evidence for retarded sulfide production. Although the metabolome demonstrated that these pathways indeed are reprogrammed, which may affect intracellular sulfide levels, these were not reflected in blood and urine samples (Kozich *et al.*, 2018).

On the other hand, in collaboration with Prof. Takaaki Akaike we discovered a novel function for the mitochondrial *cysteinyl-tRNA synthetases* (CARS2) to produce cysteine per/polysulfide using Cys as substrate (Akaike *et al.*, 2017). This enzyme activity was shown to be independent of the t-RNA synthetase activity of the enzyme. In addition, we demonstrated that CARS2 uses Cys per/polysulfides more favorably than Cys for t-RNA synthesis and most of the Cys is incorporated in nascent polypeptides in a per/polysulfidated form, representing a translational production of protein Cys-per/polysulfides. Using a new quantitative mass spectrometry method we proposed that a large fraction of the intracellular persulfide pool is produced via this pathway and not via previously proposed posttranslational reverse transsulfuration pathways. Prof Akaike's group found *in vivo* evidence to corroborate this proposal using newly developed CARS2 KO mice. Importantly, they also demonstrated that the per/polysulfide producing activity of CARS2 is critical for mitochondrial biogenesis and bioenergetics. The most important message of these findings in light of our K109843 project is that if most of the Cys is incorporated into newly synthetized proteins in a per/polysulfidised state, than the Trx or

glutathione systems need to cleave these (potentially protecting) persulfides off in order to trigger protein thiol functions. Since our Science Advances paper (in which we first demonstrated the protein Cys per/polysulfide reducing functions of the Trx and GSH systems) was well received by the scientific committee (represented by 51 independent citations since its 2016 publication date), we obtained a KH17 126766 grant to follow up on this hypothesis.

Metalloproteins play essential roles in redox processes. One of the key peroxidase enzymes in human neutrophil white blood cells is called myeloperoxidase (MPO). This enzyme is not only involved in the antimicrobial clearance stratagem of the neutrophils, but it is also implicated in a plethora of pathophysiological processes including carcinogenesis. MPO can convert H_2O_2 into highly reactive molecules and radical species including HOCl, HOSCN, HOBr and phenoxyl radicals. By developing novel analytical techniques (Garai *et al.*, 2017a), we have obtained evidence that the small signaling molecule hydrogen sulfide efficiently inhibit these MPO activities in a reversible manner (Palinkas *et al.*, 2015). Reactions of MPO with sulfide produce inorganic polysulfides that can contribute to protein thiol protection by polysulfidation of Cys residues via the above described mechanism (Garai *et al.*, 2017b). We developed new methods to investigate these processes using neutrophils, isolated from human blood (Garai *et al.*, 2017a), and recently obtained a K18 grant to further investigate their roles in MPO-ANCA-vasculitis.

Although not strictly related to the current project, we also contributed to our understanding how per/polysulfides and hydrogen sulfide regulate NO and nitrosothiol biology. On these grounds two comprehensive chemical/biochemical studies and a book chapter were recently published in collaboration with Prof. Martin Feelisch's group (Berenyiova *et al.*, 2015; Cortese-Krott *et al.*, 2015; Kevil *et al.*, 2017). One of the key finding of these investigations was the characterization of a hybrid S/N molecule $SSNO^-$ with potential physiological implications. Recently we demonstrated that this molecule can escape thiol mediated reduction events and has the potential to serve as an agent which can induce sustained protein cys persulfidation on target proteins in cellular systems (Bogdándi *et al.*, manuscript in preparation).

With internationally renowned experts we recently published a comprehensive review article in which we outlined our perspective how redox-, sulfur- and NO-based biological events are intimately intervened (Cortese-Krott *et al.*, 2017).

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