UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL BIOLOGY
TUMOUR BIOLOGY RESEARCH GROUP

Expression and activity of NADPH oxidases (NOX) and the ROS-mediated effects on γ-Glutamyltransferase (GGT) expression in cancer cells

Chandra Sekhar. Ravuri

A dissertation for the degree of
Philosophiae Doctor

December 2010

Acknowledgements

The present work was carried out during the years 2006-2010 at the Tumour Biology Research group, Faculty of Health Sciences, University of Tromsø. This work was supported by the University of Tromsø as well as HelseNord, The Norwegian Cancer Foundation, Aakre fondet, Familien Blix' fond, and the AJP Hagens legat.

First I would like thank my supervisor Associate Professor Gunbjørg Svineng for giving me the opportunity and her great supervision during my PhD. Thank you so much for your endless patience and also always being available to answer my questions. Tusen takk for showing me the right direction when I am doing wrong and giving me the full freedom in the lab. Thank you so much for supporting me until the very of my PhD.

I always feel that I am not working "FOR" you but working "WITH" you.

Next I would like to express my sincere gratitude to my co-supervisor Professor Nils-Erik Huseby for his support and encouragement during the final years of my PhD. I would also like to thank you for your assistance in the preparation of my thesis and manuscripts. Without you I would not have been able to complete my PhD.

I am very grateful to Professor Joffe: correcting my calculations and also for introducing me to the Beer world.

A special thanks goes to my Swedish supervisor (in Masters) Professor Maria Fällman for introducing me to the research world.
A special thanks goes to Dr.Peter McCourt for critical reading and linguistic revision of manuscripts 1 and 2.

My sincere appreciation for the following people during my PhD life.

Eli for teaching me the cake preparation and correcting my Norwegian language skills, Bente for helping me in the lab, Synnøve for teaching me the lab techniques in my early years of PhD, Liv Tone for showing me how to ski and sharing my views in the office room, and also inviting me for dinners during the Christmas season. Thanks to Bodil, Elin, Hilde and all other current and former group members for helping me in many ways.

Rahul: for helping me in lot of ways in Tromsø.

Ketil and Johann; what can I say about them. They are my all time famous "BB". Thank you so much guys for our scary movie nights, UFC nights, cocktail nights, and curry nights. Without them I can't imagine my life in the northernmost university in the world.

Dear Polina thank you so much for your company during my PhD years and walking with me almost everyday to the university and listening and sharing all my thoughts during the walk. Thank you so much for introducing me to the exercise. There are no words to express my thanks to you. Dear Ira thank you so much for sharing my views with you, for the dinner parties and bringing presents every time you go somewhere. Thank you so much both of you for introducing me to the beautiful world of Russian culture and food.

Lastly, I would like to express my love to my parents and my brother and my sister who has been a source of my inspiration throughout my life especially during my PhD study. I have no words to say how much I thankful for them. Without their support and encouragement it's hard to picture my life outside India. **I dedicate this thesis to my parents**.

Tromsø, December 2010 Chandra Sekhar Ravuri

List of papers ... 5
1. Introduction ... 8
1.1 Reactive oxygen species (ROS) ... 8
1.2 Oxidative stress ... 10
1.3 Antioxidants ... 10
1.4 The NADPH oxidase (NOX) family ... 13
1.4.1 NOX2 ... 15
1.4.2 NOX1 ... 16
1.4.3 NOX3 ... 17
1.4.4 NOX4 ... 17
1.4.5 NOX5 ... 18
1.4.6 DUOX1 and DUOX2 subunits ... 18
1.5 Mitochondrial production of ROS ... 19
1.6 Gamma-glutamyltransferase (GGT) ... 20
1.7 Redox signalling in cancer ... 25
2. Aims of the study ... 28
3. Summary of papers ... 29
4. Discussion ... 31
5. Conclusions ... 36
6. Future studies ... 36
7. References ... 37
Paper I-III ... 47

List of papers

Paper I:

Chandra Ravuri[1], Elin Halder-Olsen[1], Synnøve Magnussen[1], Hilde Ljones Wetting[1,2], Reidar Grénman[3], Jan-Olof Winberg[1], Nils-Erik Huseby[1] and Gunbjørg Svineng[1]

NADPH oxidase isoforms and their regulatory subunits are differently expressed in human squamous cell carcinoma cells. Manuscript.

Paper II:

Chandra Ravuri, Gunbjørg Svineng, Serhiy Pankiv[*] and Nils-Erik Huseby

Endogenous production of reactive oxygen species by the NADPH oxidase complexes is a determinant of γ-glutamyltransferase expression. Manuscript submitted.

Paper III:

Chandra Ravuri[1], Gunbjørg Svineng[1] and Nils-Erik Huseby[1]

Upregulated expression of γ-glutamyltransferase as a consequence of mitochondrial uncoupling. Manuscript.

Abbreviations

AIR	Auto-inhibitory region
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CDPs	Cysteine dependent kinase
CGD	Chronic granulomatous disease
DUOX	Dual oxidase
EGF	Epidermal growth factor
ERK 1/2	Extracellular signal regulated kinase
ETC	Electron transport chain
FACS	Fluorescence Activated Cell Sorter
FAD	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
GCL	Glutamate cysteine ligase
GGT	γ-Glutamyltransferase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factor
HOCL	Hypochlorous acid
JNK	c-jun N-terminal kinase
Keap1	Kelch-like-ECH associating protein
LMW-PTP	Low molecular weight PTP
LTC	Leukotriene
MAPK	Miogen activated protein kinase
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NOX	NADPH oxidase
NO	Nitric oxide
Nrf2	NF-E2 related factor
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide dependent kinase 1

PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PTKs	Phospho tyrosine kinases
PTPs	Protein tyrosine phosphatases
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
SH3	Src homology 3
SOD	Superoxide dismutase
TPR	Tricodecapeptide repeat
uPA	Urokinase plasminogen activator
XO	Xanthane oxidase

1. Introduction

1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are a heterogeneous population of active biological molecules that is generated both as by-products in aerobic metabolism and also by specialized enzymes [1-3]. ROS are generated in all tissues and organs of multicellular organisms, including animals, plants and even microbes. These reactive species play different roles in biology including host defence, hormone biosynthesis, fertilization and redox signalling that regulate mitogenesis, apoptosis and oxygen sensing [4, 5]. They are also involved in several disease processes including aging and cancer [2, 4, 6].

The major ROS molecules include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid (HOCl) (Fig. 1) [2]. Cellular generation of ROS starts with the production of superoxide. Superoxide rapidly dismutases to hydrogen peroxide either spontaneously or through catalysis by superoxide dismutase (SOD). The formation of hydroxyl radical occurs in the presence of metals like iron and copper by the Fenton reaction or Haber-Weiss reaction [7, 8]. Peroxidases are the main source for the production of HOCl from H_2O_2 [9]. ROS are formed as by-products of mitochondrial respiration or from oxidases, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO) and arachidonic acid oxygenases [6, 10, 11].

ROS molecules are implicated in cellular redox signalling that regulate events such as proliferation, differentiation, apoptosis, angiogenesis and gene expression. They frequently act through redox sensitive cysteine residues on receptors, protein kinases, tyrosine phosphatases, as well as on transcription factors. H_2O_2 is continuously produced and maintained at low, nanomolar concentrations in all cells. The level of H_2O_2 is balanced through the action of several peroxidases and antioxidants (see chapter 1.3). This oxidant is generated in response to physiological stimuli and is in this context considered as a true second messenger. At such low levels, H_2O_2 can stimulate cell growth, which has led to the general recognition that redox signalling pathways have an important role in cellular growth [6, 12-14]. Recent studies show that both cellular and mitochondrial ROS have a role in the regulation of autophagy [15].

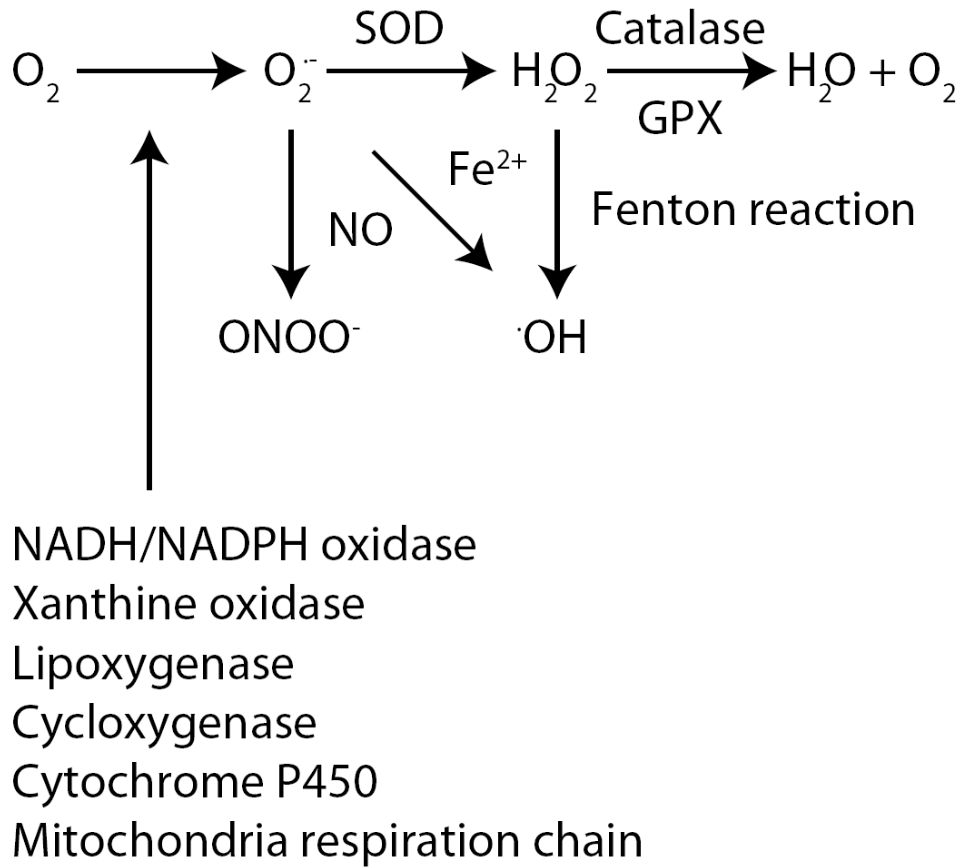


Figure 1: Generation of ROS

Superoxide ($O_2^{\cdot-}$) is generated from various sources such as NADPH oxidase, xanthine oxidase, mitochondrial respiratory chain. Two molecules of superoxide can react to generate hydrogen peroxide (H_2O_2) in a reaction called dismutation. This reaction is accelerated by the enzyme superoxide dismutase (SOD). In the presence of iron superoxide and hydrogen peroxide generate hydroxyl radicals ($OH^{\cdot}$). Superoxide can also be converted in to peroxynitrate ($ONOO^-$) in the presence of nitric oxide (NO). In the presence of catalase and glutathione peroxidise (GPX) hydrogen peroxide is converted to water and oxygen.

1.2 Oxidative stress

Oxidative stress is defined as an imbalance in pro-oxidants and antioxidants which results in macromolecular damage and dysfunction of redox signalling and control [16]. This imbalance of oxidants leads to overall excessive production of ROS and may result in damage of macromolecules as well as disruption of redox signalling [17]. When the ROS levels are raised above a certain threshold the defence system of antioxidants may become inhibited. This may then lead to DNA mutation or DNA damage, genome instability and cancer initiation and progression [6, 18]. Increased oxidative stress leads to variety of diseases such as cancer, neurodegenerative disorders, asthma, pulmonary fibrosis and aging [17, 19].

Data show that (a) some growth factors such as epidermal growth factor (EGF), insulin, and angioprotein-1 increase ROS production in the ovarian cancer cells in order to regulate cell migration and proliferation; (b) high levels of ROS are observed in some cancer cells, which may induce DNA damage leading to genomic instability and tumour initiation; (c) ROS induce the activation of mitogen activated protein (MAP) kinase, nuclear factor κB (NF-κB), and activator protein 1 (AP-1), which are all known to be associated with cancer development. The direct role of ROS in tumour growth and angiogenesis remains undefined [20, 21]. However, it has been shown that hypoxia and hypoglycemia may result in increased ROS production in mitochondria and lead to malignant transformation [6].

1.3 Antioxidants

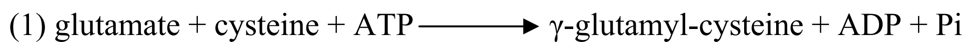
The cellular production of ROS is balanced by several antioxidant systems, including various enzymatic systems and antioxidant molecules. The enzymatic systems include among others SOD, catalase, GSH peroxidases, thioredoxin reductase and peroxiredoxin. The coordinated action of the antioxidant enzymes makes sure that ROS levels are balanced. Excessive production of ROS will lead to oxidative damage of many macromolecules and will also alter the intracellular redox homeostasis. Antioxidants are critical for life and they are mainly divided into two types; exogenous antioxidants supplied with the diet, and endogenous antioxidants as the enzymes mentioned above [6, 18, 22] The one described here, is the glutathione system.

Glutathione (GSH)

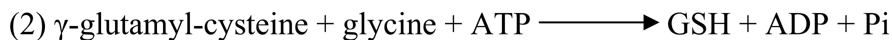
Glutathione (GSH) is a tripeptide (γ-glutamyl-cysteinyl-glycine) and is the most abundant non-protein thiol in cells being present in millimolar concentrations [23]. GSH has antioxidant functions and also multiple biological functions such as detoxification of xenobiotic compounds, and thiol-disulfide exchange reactions in the maintenance of normal cellular redox status [24]. It is also described as a short time reservoir of cysteine [14]. GSH is most abundant in the cytosol (85-90% of the cellular pool). Other pools of GSH are found in mitochondria, nuclear matrix and peroxisomes [25, 26]. GSH exits in two forms, one is the thiol reduced form (GSH), and the other is the oxidized form (GSSG). Under normal redox conditions it exits mostly in the reduced form.

GSH synthesis

GSH is synthesized from glutamate, cysteine and glycine in the cytosol by two sequential ATP-dependent reactions. These reactions are catalyzed by two cytosolic enzymes.



(1) glutamate + cysteine + ATP → γ-glutamyl-cysteine + ADP + Pi



(2) γ-glutamyl-cysteine + glycine + ATP → GSH + ADP + Pi

The first step is catalyzed by glutamate cysteine ligase (GCL), also known as γ-glutamylcysteine synthetase, which is both the rate limiting and the regulatory reaction. In this reaction a peptidic γ-linkage is formed by the reaction of γ-carboxyl group of glutamate and cysteine. This protects the GSH from hydrolysis by intracellular peptidases. This reaction requires Mg^{2+} or Mn^{2+}. The second step is catalysed by GSH synthase (GS), also known as GSH synthetase [26, 27]. One important factor that regulates the *de novo* GSH synthesis is the availability of cysteine. In fact, cysteine is rate-limiting in GSH biosynthesis. Extracelluar GSH cannot be taken up by most cells, and γ-glutamyltransferase (GGT) is the only enzyme that is able to hydrolyze the peptidic γ-linkage of GSH. Cells that express GGT activity will thus have an ability to obtain cysteine from extracellular hydrolysis of GSH [25, 27].
(See Fig. 4 the γ-glutamyl cycle)

GSH redox cycle

The antioxidant power of GSH is related to the GSH peroxidase reaction, in which H_2O_2 is reduced to H_2O and GSH is oxidised to GSSG (Figure 2). The GSH peroxidases are a family

of enzymes present in the cytosol or nucleus of most cells. They show different cellular levels and subcellular locations, as well as substrate specificities [28].

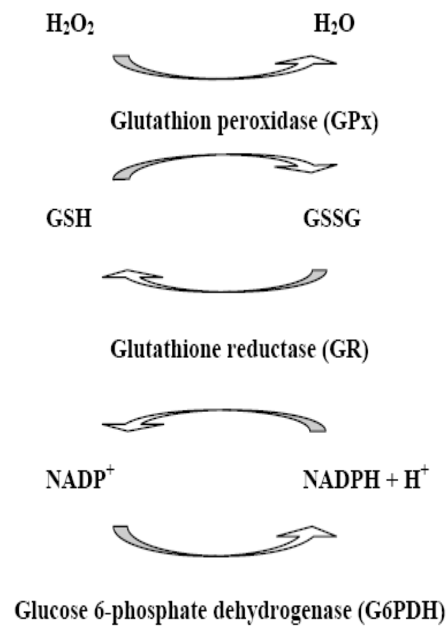


Figure 2. The GSH redox cycle

The reduction of H_2O_2 to H_2O is catalyzed by glutathione peroxidases (GPx) using GSH as reducing agent. The oxidised glutathione disulfide (GSSG) can be reduced back to GSH with glutathione reductase (GR) using NADPH as reducing agent. The oxidised $NADP^+$ can be reduced in the pentose phosphate shunt, using the oxidation of glucose 6-phosphate to 6-phosphogluconlactone catalysed by glucose 6-phosphate dehydrogenase.

1.4 The NADPH oxidase (NOX) family

NADPH oxidase was first identified in phagocytic cells such as neutrophils and macrophages. In phagocytic cells, ROS generation occurs during the oxidative burst by the plasma membrane NADPH oxidases [2]. It was for many years thought that ROS production was restricted to phagocytic cells. Recent findings however show that NADPH oxidases are found in different cells that have no role in host defence. For example, NOX components have been reported in fibroblasts, mesangial cells, endothelial cells, osteoclasts and chondrocytes [10]. The production of ROS is low in these cells compared to neutrophils. Altered production of ROS may lead to immunodeficiency, hearing loss and thyroid problems, cardiovascular pathologies, neurodegeneration and possibly cancer [2, 29]. Recently it was shown that ROS-producing NADPH oxidases, distantly related to animal Nox enzymes exist in a variety of eukaryotes including plants, fungi, and the myxomycete (Mycetozoa) Dictyostelium discoideum [5].

The human genome contains genes for 7 members of NADPH oxidase (NOX) family; NOX1, NOX2 (also known as gp91phox), NOX3, NOX4, NOX5, and the dual oxidases DUOX1 and DUOX2 [2, 5]. All members contain at least six transmembrane domains, motifs for NADPH and FAD binding, as well as conserved paired histidines that could ligate heme groups (Fig 3). NOX5, DUOX1 and 2 have additional structural features not shared by the other NOX proteins. All three have cytosolic EF-hands that are involved in calcium dependent regulation of their oxidase activity [4, 30].

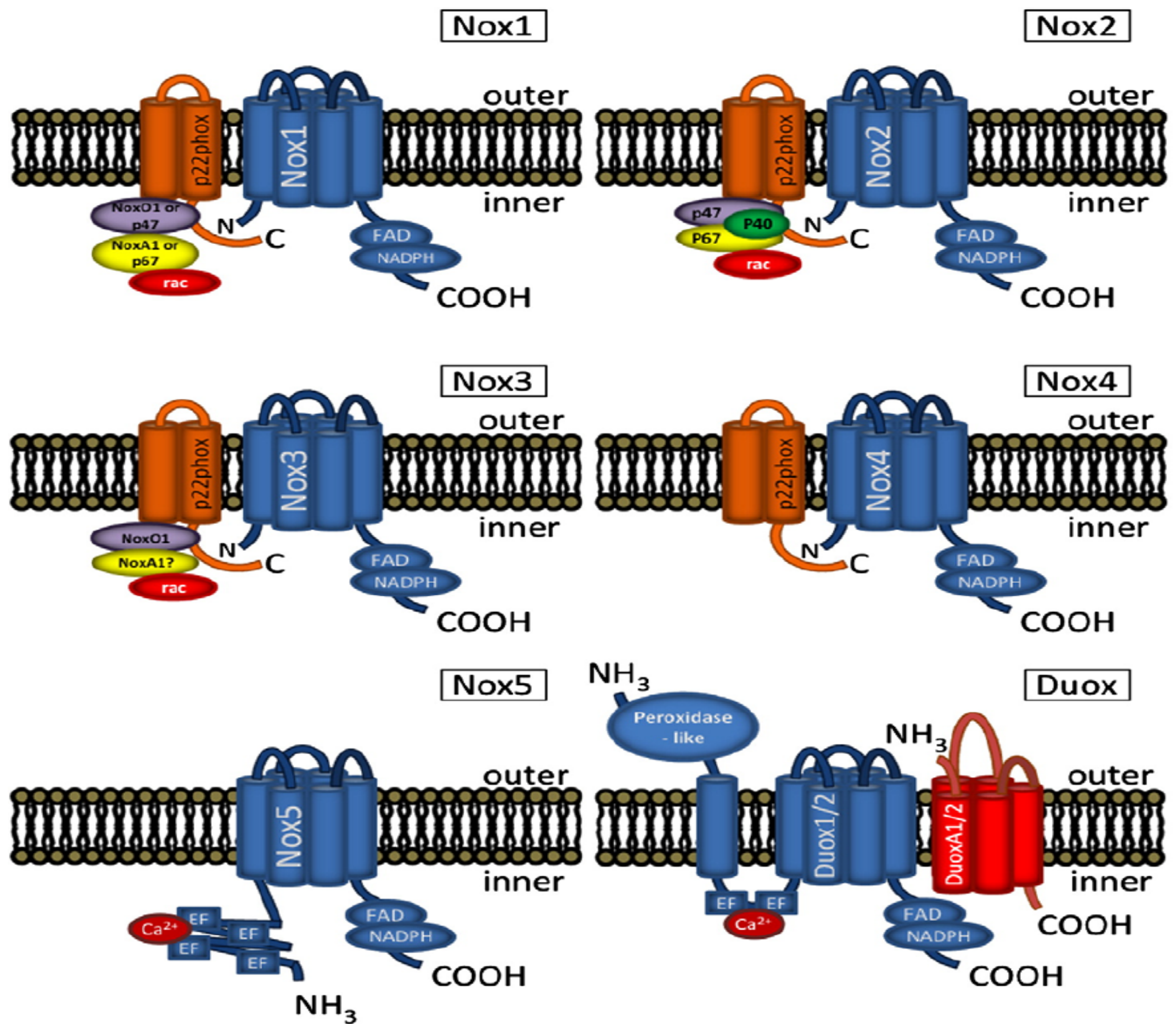


Figure 3: NOX family members and their regulatory subunits.

NOX1-4, the transmembrane subunit p22phox associates with active and inactive NOX. Ca^{2+} binding to EF-hand domains in the cytosol is involved in activation of NOX5 and DUOX1/2 [30] (with permission from publisher).

All NOX family members share common structural similarities, but the activation of each member is different. The enzymes have specific biological roles through a regulated superoxide generation in response to growth factors, cytokines and calcium signals [3]. Superoxide is rapidly converted to hydrogen peroxide, which may regulate target molecules through oxidation of sensitive cysteine-residues at low concentrations. Thus, the NOX enzymes produces second messengers effecting cellular signals and biochemical processes [31].

The NOX enzymes are classified into three main groups according to the presence of specific domains. The first group consists of NOX1, NOX3 and NOX4. They are nearly identical in size and structure to NOX2 and they contain the electron transfer centres that are required to pass electrons from NADPH to molecular oxygen in order to form superoxide. NOX5 belongs to the second group and builds on the basic structure of NOX2 but differs from other NOX isoforms by the presence of intracellular amino terminus containing four binding sites for calcium. NOX5 have in addition similarities with plant oxidases, such as the *Atrbohs* gene identified in Arabidopsis [32], with two calcium binding EF-hand motifs in the N-terminal elongation. The third group, the dual oxidases DUOX1 and DUOX2 (also known as Thox and Lnox) further extends the NOX5 structure by an N-terminal peroxidase domain that is separated from the dual EF-hands by an additional transmembrane segment. Duox homologs have been identified in *Caenorhabditis elegans (C.elegans)* and *Drosophila* [3, 33, 34].

1.4.1 NOX2

The catalytic subunit of the phagocytic NADPH oxidase is a membrane localized highly glycosylated protein [5]. It was earlier known as gp91phox, and is now called NOX2 and is the best studied protein of the NOX family members. It is highly expressed in phagocytic cells but it was also found in B lymphocytes, neurons, cardiomyocytes, skeletal and smooth muscle, hepatocytes, endothelium and haematopoietic stem cells [2]. The NOX2 protein contains six transmembrane α helices. The third and fifth helices contain two variant histidine residues which coordinate two hemes [2, 5, 11]. The C terminal part folds into a cytoplasmic domain containing the FAD and NADPH binding sites.

Activation of NOX2 is a complex process with several co-factors. The transmembrane p22phox protein plays a major role in the activation and has two major functions; one is to bind the NOX proteins leading to protein stabilization and the other is binding to the organizer subunits [2]. These are the small GTPase Rac1, p47phox or its equivalent NOX organizer 1 (NOXO1), p67phox or its equivalent NOX activator 1 (NOXA1), and p40phox. In phagocytic cells, NOX2 binds to p22phox in close association in both intracellular and plasma membranes to form cytochrome b_{558}, the catalytic component of the phagocytic NADPH oxidase [35]. In the resting conditions an auto-inhibitory region (AIR) prevents the binding of p47phox to p22phox [3]. Upon phagocytosis, or during stimulation of cells, phosphorylation of p47phox by protein kinase C is making the AIR inactive [2]. Phosphorylated p47phox

along with the other cytosolic subunits p67phox, p40phox and Rac1 translocate to the membrane and interact with the cytochrome b_{558} complex [36]. The active NOX2 complex transports electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen to generate superoxide [4, 5, 37]. NOX2 is reported to be mainly located in the submembranous phagosomes in neutrophils, endosomes [38], in the plasma membrane or perinuclear membranes [30, 39]. Deficiency of NOX2 has been reported in chronic granulomatous disease (CGD) patients, diabetic and some in oral diseases [2, 40].

1.4.2 NOX1

NOX1 was the first recognised homologue of NOX2. NOX1 contains 564 amino acids and shows 56% identity at protein level to NOX2 [41]. It is highly expressed in colon epithelial cells, and also present in several other tissues including smooth muscle, uterus, prostate, kidney, stomach and cells such as osteoclasts. NOX1 contains six transmembrane domains and conserved motifs corresponding to binding sites of heme, flavin and NADPH [29]. Activation of NOX1 requires the cytosolic NOX Organizer1 (NOXO1), a homologue of p47phox, and NOX Activator1 (NOXA1) a homologue of p67phox [2]. NOXO1 contains the functional domains of p47phox in the same configuration: an amino terminal phox homology (PX) domain, two tandem Src homology 3 (SH3) domains and a carboxy terminal, proline rich motif serving as an SH3 domain binding site. NOXO1 has domain architecture similar to p47phox, except the absence of the AIR. The absence of AIR makes NOXO1 prelocalized at membranes together with NOX1 and p22phox. This may be responsible for some constitutive activity of NOX1. Similar to p67phox, NOXA1 contains four amino-terminal tricodecapeptide repeat (TPR) domains that bind Rac1, a phox and Bem1 (PBI) domain, and a single, carboxy terminal SH3 domain that binds NOXO1. It has been shown that the transmembrane protein p22phox is also required for NOX1 activity. The best studied activation of NOX1 is occurs via angiotensisn-II (Ang II) in vascular smooth musle cells by the activation of protein kinase C (PKC) [42]. Invitro studies showing that cell stimulants such as phorbol esters, increases the NOX1 activity by up to 2-fold in many cell systems [43]. Rho GTPases also play a role in NOX1 activity. Currently, only guanine nucleotide exchange on Rac1 is documented as a regulatory factor for NOX1. In vascular smooth muscle NOX1 localizes to the cell surface, possibly corresponding to caveolar localization [29, 44]. In keratinocytes a weak cytoplasmic and a strong nuclear staining has been demonstrated [2].

The physiological role of NOX1 is currently unknown. The many reported roles of NOX1 may reflect that it has various roles in different cells. May be the role of NOX1 depends where the enzyme is expressed and the cell lines studied. NOX1 expression is upregulated by growth factors and growth related agonists like angiotensin. It has been shown that NOX1 is involved in the development of angiotensin II-induced hypertension in NOX1 deficient mouse [45]. In addition, several factors have been reported to up regulate the NOX1 expression such as urokinase plasminogen activator (uPA), platelet derived growth factor, bone morphogenic protein 4, prostaglandin F2, keratinocyte growth factor-α and activated K-Ras [43].

1.4.3 NOX3

NOX3 is a 568 amino acid protein which shows 58% amino acid sequence identity to NOX2 [41, 46]. The major site for NOX3 expression is the inner ear, but it has also been found in several fetal tissues including kidney, liver, lung and spleen [29]. NOX3 forms a complex with p22phox to produce ROS. NOX3 is constitutively active compared to NOX1 and NOX2; and there is a limited role of the cytosolic co-factors in its activation. NOX3 dependency on NOXO1 is species specific. Human NOX3 depends on NOXO1 whereas murine NOX3 depends on both NOXO1 and NOXA1 [4]. Several studies show a possible role of Rac1 in the NOX3 activation, but it is not well documented [43]. The intracellular localization of NOX3 is currently not known. It has been shown that NOX3 along with p22phox localized to the plasma membrane in HEK293 cells [29, 30]. Deficiency of NOX3 leads to severe balance problems in mouse and also NOX3 derived ROS may contribute in hearing loss [47, 48].

1.4.4 NOX4

NOX4 was originally called Renox (renal oxidase) since NOX4 is most expressed in the kidney. NOX4 is a 578 amino acid protein with 39% homology to NOX2 [29]. NOX4 mRNA has been found in many tissues and cells including fetal liver, vascular endothelial cells, smooth muscle cells, murine osteoclasts, hematopoietic stem cells, adipocytes and also in cancer cells including various prostate cancers [49, 50]. NOX4 requires p22phox for its activity and it does not require any other regulatory subunits [43]. The complex of NOX4 and p22phox is constitutively active. It was reported that NOX4 can have effects on insulin signalling cascade via the production of H_2O_2 in adipose cells. This increased ROS production was due to the effect on tyrosine phosphorylation through inhibition of protein tyrosine

phosphatases [51]. NOX4 may be localized to focal adhesions, the perinuclear endoplasmic reticulum, or the nucleus [52-54].

1.4.5 NOX5

NOX5 is a 737 amino acid protein and has a 27% identity at the protein level to NOX2 [41]. NOX5 is mainly expressed in spleen, testis and in fetal tissues. RT-qPCR experiments showed that NOX5 mRNA was detected in several other tissues and cells including ovary, placenta, pancreas, vascular smooth muscle, bone marrow and uterus [29]. The enzymatic activity of NOX5 dependent on calcium and NOX5 has a N-terminal domain containing four calcium-binding EF-hand motifs [43]. Calcium ions activate NOX5 by binding to the EF-hands and this binding change the conformation of this domain. This enables an intramolecular interaction between the N-terminal and C-terminal domains [29].

1.4.6 DUOX1 and DUOX2 subunits

Dual oxidases (DUOX) were previously called thyroid oxidase because they were initially described in the thyroid gland. The name DUOX derived from the protein structure analysis of *C.elegans* NADPH oxidase family [29, 55, 56]. Human DUOX1 protein contains 1551 amino acids whereas DUOX2 contains 1548 amino acids. Both show 83% DNA sequence similarity to each other. DUOX1 shows 53% similarity to NOX2 and DUOX2 shows 47% resemblance to NOX2 but there promoters are different [55-57]. Human DUOX1 is highly expressed in pancreas, lung, placenta, prostate, testis and salivary glands. Human DUOX2 is highly expressed in the trachea, stomach, colon and rectum [43].

The DUOXs are glycoproteins containing seven transmembrane helices, an extracellular peroxidase-like domain, a long intracellular loop with two calcium binding EF-hand motifs like NOX5, a FAD binding domain and four NADPH binding sites. The presence of EF-hand motifs suggests an important role of calcium ions in regulating the enzymatic activity of DUOX in thyroid cells and also in human bronchial epithelial cells. These cells show a high expression of DUOX proteins [43, 56]. Characterization of the function and regulation of DUOXs had not been fully understood until the discovery of DUOX maturation proteins DUOXA1 and DUOXA2 [58]. Partially glycosylated DUOX2 remains in the endoplasmic reticulum (ER) to generate superoxide whereas fully glycosylated DUOX2 is transported to

the plasma membrane to generate hydrogen peroxide. DUOX maturation proteins help DUOXs to exit from the ER and move to their appropriate location at the plasma membrane [43, 56]. A physiological role of DUOX2 has been identified by studying patients who have hypothyroidism. These patients have mutations in the DUOX2 gene, but not in the DUOX1 gene. In addition, it has been shown that ROS generated by DUOX2 have an important role in controlling gut epithelial infection [29, 30, 59].

1.5 Mitochondrial production of ROS

Mitochondria is a major source for the production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Mitochondria consume 90% of cellular oxygen and of that 2-4% is converted into ROS [60-63]. The primary production of ROS in mitochondria is $O_2^{\bullet-}$ which rapidly is converted into the more stable H_2O_2 by the action of MnSOD and Cu/Zn-SOD in the intermembrane space [63]. Many mitochondrial enzymes are known to generate ROS, for example the Electron Transport Chain (ETC) complexes I, II and III, the tricarboxylic acid cycle enzymes aconitase 2 and α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and glycerol-3-phosphate dehydrogenase and cytochrome *b*5 reductase [60]. Production of $O_2^{\bullet-}$ varies from organ to organ. In heart and lung $O_2^{\bullet-}$ is mainly produced from complex III and in brain pyruvate and α-ketoglutarate dehydrogenase are important ROS sources through complex I [64, 65].

Mitochondria contain various antioxidant systems to detoxify the effects of ROS. Enzymatic sources include MnSOD, catalase, GPX and non-enzymatic sources including ascorbic acid, reduced coenzyme Q10 and GSH. The MnSOD eliminates the $O_2^{\bullet-}$ formed in the matrix or on the inner side of the inner membrane. GPX helps to detoxify the H_2O_2 and in heart mitochondria catalase plays the important role in detoxification of H_2O_2 [60, 65, 66].

Mitochondrial $O_2^{\bullet-}$ and H_2O_2 can modulate several signalling proteins such as c-jun N-terminal kinase (JNK) in primary cortical neurons and PKC in primary cultured hepatocytes and they can activate apoptotic and necrotic pathways [67, 68]. Many oncogenic proteins like p53, MYC, STAT-3 and Ras can modulate the mitochondrial function by changing the expression of genes in the mitochondria [6]. Hypoxia and glucose deprivation may lead to elevated ROS production in the mitochondria which, over time, may stabilize cells via elevated HIF-2α expression and thus ability to survive. Furthermore, increased ROS may lead

to malignant transformation in the mitochondria, resulting in escape from senescence and facilitating growth [6]. In normal fibroblasts mutant Ras can induce the senescent cell cycle arrest by increasing the mitochondrial mass, the mitochondrial DNA, and the mitochondrial production of ROS. These studies showed that mitochondrial dysfunction leads to low ATP levels and activation of AMP-activated kinase (AMPK) [69]. In another study on p53 knockdown, human primary fibroblasts decreased superoxide production in both mitochondrial and cellular level and disrupted cellular ROS homeostasis were demonstrated. This indicates the possible effect by p53 on mitochondria and the production ROS [70].

Several studies have shown that there is a link or crosstalk between NOX system and the mitochondria mediated ROS (for reviews see [6, 71]). In human osteosarcoma cells downregulation of NOX1 was observed when the mitochondria genes were inactivated. This resulted in low $O_2^{\bullet-}$ production by NOX1. In this study the authors claimed that NOX1 is localized in the mitochondria and when mitochondria lose control on the NOX1 activity this may lead to carcinogenesis in breast and ovarian cells [61]. Mitochondrial ROS activate PKC which triggers a vicious cycle on NOX activation [71]. Studies on human 293T cells showed that production of ROS is high when these cells are grown in serum depleted medium through stimulation of both mitochondria and NOX1. In this study the authors showed that ROS produced by mitochondria was not enough to promote the cell death which requires further action of NOX1[72].

1.6 Gamma-glutamyltransferase (GGT)

γ-Glutamyltransferase (GGT) (E.C. 2.3.2.2; (5-L-glutamyl)-peptide:amino acid 5-glutamyltransferase) is found expressed in bacteria, plants and in the animal kingdom including humans [73]. GGT is an important enzyme for GSH homeostasis and is the only known enzyme that catalyses the hydrolysis of the unique γ-glutamyl group of GSH. By initiating the breakdown of extracellular GSH, GGT provides the cells with cysteine, the rate-limiting substrate for GSH biosynthesis [74].

Structure

GGT is a heterodimeric plasma membrane bound glycoprotein located on the outer surface of the membrane [75]. The apparent molecular weight of the heavy chain is 50 to 62 kDa and the light chain 22 to 30 kDa. The variation in molecular weight is due to the degree of

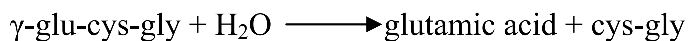
glycosylation of the chains that differ in various tissues [75, 76]. The light chain includes the active site while the heavy chain is transmembraneous and anchors the protein to the plasma membrane [77, 78]. The enzyme is translated as one polypeptide chain, which is catalytically inactive, and is further processed into the heterodimeric form after glycosylation in the Golgi [75]. The heavy glycosylation (about 30% of the enzyme isolated from liver is carbohydrate) prevents cleavage and inactivation by proteases [79, 80].

Function

GGT catalyzes the transfer of the γ-glutamyl moiety from GSH. The enzyme acts both as a glutathionase that is hydrolysing GSH to glutamate and cys-gly and as a transpeptidase by transferring the γ-glutamyl group to acceptors such as amino acids and dipeptides including cystine. These two types of catalytic reactions can be written as [81]:

1) The transfer or transpeptidase reaction: the γ-glutamyl-group is transferred to accepting aminoacids or dipeptides.

2) Hydrolysis: in this reaction water will accept the γ-glutamyl residue and GGT acts as a glutathionase.



The cysteinylglycine product is further cleaved to cysteine and glycine by plasma membrane dipeptidases [24, 82]. The resulting amino acids are taken up by the cells and used for intracellular biosynthesis of GSH. GGT is part of the γ-glutamyl cycle suggested by Meister in 1983 [83]. Fig. 4 is a simplified version of the cycle.

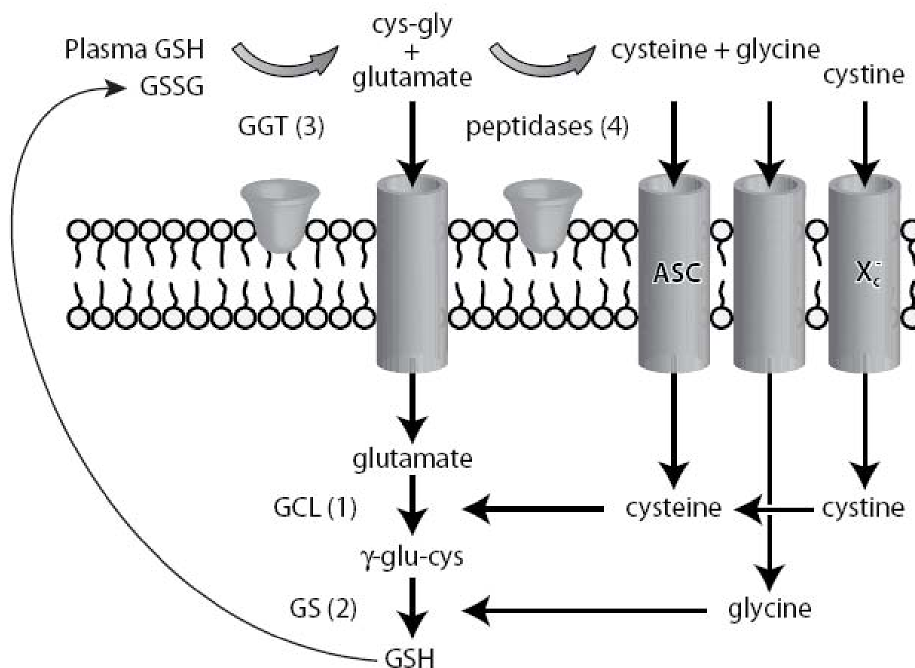


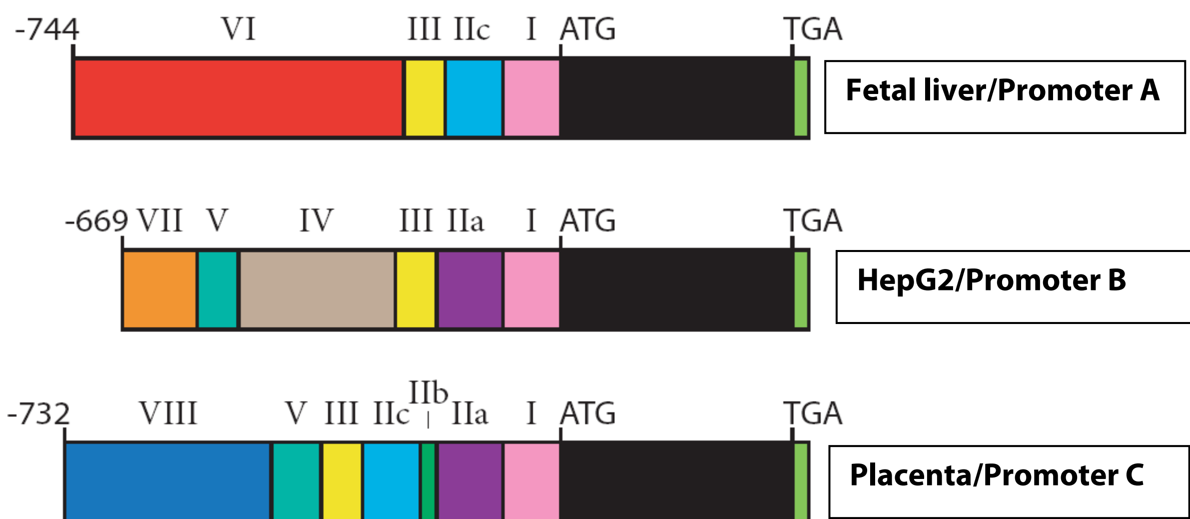
Figure 4. The γ-glutamyl cycle: GSH biosynthesis and salvage

GSH is synthesised from the constituent amino acids in two reactions, which are catalysed by glutamate cysteine ligase (GCL, reaction 1) and glutathione synthase (GS, reaction 2). Each reaction needs ATP. For synthesis, cysteine is obtained through uptake of extracellular cysteine or cystine through the ASC and the x_c- transporters, respectively. Extracellular GSH is also a cysteine reservoir, being degraded by γ-glutamyltransferase, GGT (reaction 3) and the action of various peptidases (reaction 4). Figure is modified from [84].

GGT acts both on reduced and oxidised glutathione, and on S-conjugated GSH compounds including xenobiotics and leukotriene B4. Leukotrienes are biologically active lipid derivatives synthesized in hematopoietic cells which are involved in inflammation. GGT cleaves and removes the γ-glutamyl moiety from leukotriene LTC4 to give LTD4 [85]. Lieberman et al. have shown that GGT deficient mice have highly elevated plasma and urine GSH levels and growth retardation due to low levels of cysteine. They also found that deficiency of GGT may cause dysfunction of mouse reproductive system and severe cataract development [86-88]. Therefore, GGT is a critical enzyme in maintaining GSH and cysteine homeostasis and for cellular antioxidant function, as it acts as a glutathionase that helps the cells to use extracellular glutathione as a source of cysteine. Studies on the colon carcinoma cell line HT29 have shown that cysteine deficiency leads to imbalance between GSH/GSSG redox state and that this effects cellular redox signalling [89, 90].

Human GGT gene structure

The GGT gene is present as a single copy in mice and rats. In humans, GGT belongs to a multigene family that contains at least seven different genes. Among these, only 4 are expressed and only GGT1 and 5 have been found to encode proteins exhibiting GGT activity [85]. Among all GGT genes the GGT1 is well studied and is translated into a single protein that can be cleaved into a light and a heavy chain. All GGT genes are assigned to chromosome 22 on q111-q112 close to the BCR and Ig-λ loci [91]. It has been shown by southern blot analysis that human and rat GGT sequences are almost 90% homologous. Several human GGT1 cDNAs have been cloned from hepatoma cells (HepG2), placenta, lung and fetal liver. These GGT transcripts exhibits different 5´-UTRs but share the same coding sequence (Fig. 6) [92]. This difference may reflect tissue specific splicing or the presence of multiple tissue specific promoters [93, 94].



100bp

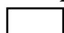


Figure 6: Structure of the human GGT mRNAs

The reading frame of type 1 mRNAs is represented by black boxes. The pink colour boxes represent 5'-UTRs common to the mRNAs. Different colours represent specific 5'UTRs. The mRNAs are designated by the tissue from which they were cloned. Nucleotides are numbered from A (+1) in the initiation codon. Roman numerals refer to the 5'-non coding exons. Figure is modified from [92]. (With permission from publisher)

Regulation of expression

Acute oxidative stress will reduce the cellular GSH level, and it is well documented that cells can respond to lower amounts of oxidants to increase the GSH synthesis [95-97]. This synthesis appears to be a concerted action involving GCL, GGT and other enzymes of the γ-glu cycle [98, 99]. The expression of GGT is upregulated after exposure of lung cells and colon carcinoma cells to menadione, a redox cycling quinone [100], hyperoxia [101] and nitric acid [102]. GGT is also upregulated by other agents that may induce oxidative stress, such as hormones, carcinogens, and inflammatory cytokines [103-105]. ERK1/2 and p38MAPK signaling pathways are involved in the rat GGT P5 promoter activation mediated by 4-Hydroxynonenal (HNE). It was shown that Ras and its downstream targets P13K/Akt, ERK1/2, and p38MAPK were involved in rat GGT P2 promoter activation by menadione in CC531 cells [106, 107]. In V79 cell line the production of ROS by GGT activates the NF-κB signaling [108].

Subcellular localisation and organ distribution

In normal human tissues GGT is located to the luminal surface of secretory and absorptive cells throughout the body, including sweat glands, breast ducts, prostate, salivary gland ducts and the proximal tubules of the kidney [109]. In addition, the GGT protein is expressed at high levels in biliary epithelium and brain capillaries [92] . GGT expression may vary during organ development; thus GGT levels are high in fetal liver and low in adult liver. On the other hand the kidney GGT levels are low at birth but increases with age [93]. High GGT activities are also measured in pancreas and prostate [77, 78, 110].

Abnormal levels of GGT are often observed in tumors of a variety of tissues including hepatocellular carcinomas, malignant squamous carcinomas of the skin, squamous cell carcinomas of the buccal pounch epithelium and adenocarcinomas of the lungs [74]. In melanoma cells it was shown that overexpression of GGT increased the metastatic growth of the cells in mouse liver [111].

Clinical applications

GGT is normally found in serum and has been used as a marker for liver diseases for more than 70 years [73, 81]. GGT activity measurements in blood was originally suggested to be a diagnostic marker of liver alcoholic damage, but is now used more widely in relation to inflammation, jaundice and liver cancer. GGT serum levels will increase with age and is higher in males than females. Alcohol consumption, drug intake and excessive weight influence the enzyme activity in serum. Population studies have shown that increased serum

GGT is associated with higher risk of cancer in both men and women [112, 113], and recently GGT is reported to be a cardiovascular risk factor [114].

GGT deficiency in humans is very rare and only 7-8 patients have been reported worldwide. None of the patients had complete absence of GGT activity and it is not clear which GGT gene was mutated in these patients. The symptoms are mental retardation and central nervous system deficiencies, but there is no clear evidence for the direct role of GGT [73, 85] .

1.7 Redox signalling in cancer

Oxidants, in particular H_2O_2, acts as second messenger and play central roles in the regulation of cellular functions such as proliferation, differentiation and migration. The term redox signalling is now an accepted description of how ROS function in signal transduction. At normal redox status, cells will respond properly to endogenous and exogenous stimuli, and ROS and Reactive Nitrogen Species (RNS) are participating in cellular redox signalling. When redox homeostasis is disturbed it may lead to disease development such as cancer and degenerative disorders. Many enzymatic and non-enzymatic antioxidants such as SOD, catalase and GSH will eliminate excessive ROS and help maintaining the redox homeostasis [14, 17]. H_2O_2 acts mainly through reversible cysteine oxidation on formation of cysteine oxidative adduct. Protein tyrosine phosphatases (PTPs), protein tyrsosine kinases (PTKs), signalling adaptors and transcription factors are the main targets for the oxidants. In this process protein kinases are known to be activated and protein phosphatases inhibited through oxidants [6, 115].

Protein tyrosine phosphatases (PTPs)

Protein tyrosine phosphatases (PTPs) are well documented targets for ROS. PTPs contain cysteine in their active site and the oxidation of cysteine to cysteine sulfenic derivatives leads to their enzymatic inactivation which is done by various oxidants including H_2O_2 [22, 116]. PTPs are divided into two groups; tyrosine specific phosphatases and dual–specificity phosphatases. Tyrosine specific phosphatases are PTP1B, Low molecular weight PTP (LMW-PTP) and Src homology 2 domain containing PTPs (SHP2) and dual specific phosphatases include mitogen activated protein kinase (MAPK) phosphatases, tumour suppressor PTEN [116, 117]. These phosphatases can be inactivated by H_2O_2 through cytokines, growth factor- and integrin signalling [115]. It has been shown that PTP1B activation is regulated by H_2O_2 in

insulin signalling pathway. It was shown that insulin stimulated H_2O_2 production reversibly inhibits PTP1B and thus enhanced the early insulin cascade [118].

The phosphoinositide 3-kinase (PI3K) pathway is important in maintaining a number of cellular processes including cell proliferation, survival, growth and motility [17, 119]. The PI3K pathway can be activated by growth factor receptors such as tyrosine kinase receptor and integrin ligand binding [120-122]. It has been shown that TonB.210 cells shows high production of ROS. Elevated levels of ROS can activate PI3-K/Akt pathway. They found that ROS can increase survival signalling through redox inhibition of PP1 alpha [123].

Many of the components in the PI3K/Akt pathway are redox sensitive; including cys-dependent phosphatases (CDPs) and protein tyrosine kinases. ROS can oxidize cysteine residues of protein tyrosine phosphatases (PTPs) resulting in their inactivation [124]. PTEN is a well studied target molecule for ROS in the PI3K/Akt pathway. It has been found that hydrogen peroxide inactivates PTEN and activates the PI3-kinase signaling in RAW264.7 macrophages [125]. Mutations in Tumour suppressor gene PTEN can trigger the tumour angiogenesis. Over expression of PI3K active forms can induce the angiogenesis invivo [126]. It was shown that ROS will play a role in tumour induced angiogenesis via PI3K/Akt signalling in ovarian cancer cells [127]. Mitochondrial ROS oxidize the PTEN which enhance the activation of PI3K signaling. This signaling increases the vascular endothelial growth factor which is a key regulator in the regulation of angiogenesis.[128]. The consequences of inhibiting these molecules leads to irregular Akt signaling which can cause increased cell proliferation, enhanced survival and growth [119].

Protein tyrosine kinases (PTKs)

PTKs are divided into receptor and non-receptor families and both are important in cellular signaling pathways that regulate the cell growth, differentiation, migration and metabolism [116]. The receptor tyrosine family includes epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor and the non-receptor family includes Src, Focal adhesion kinase (FAK) and others [129]. ROS can activate PTKs in three different mechanisms. First, by altering protein-protein interactions depending on sulfhydryl groups and second, inhibiting cysteine residue of the PTPs. This can lead to tyrosine phosphorylation of the kinases and thus affect the kinase activities. Third, oxidation stimulates proteolysis of regulatory proteins that may inhibit tyrosine kinase activity [130]. Hydrogen peroxide can activate several protein kinases including extracellular signal-regulated kinase (ERK)1/2 and

protein kinase B (PKB) [131]. It was shown that UV-light induced ROS activates and dimerizes the Ret tyrosine kinase [132]. This effect was due to the presence of Cys residues in Ret tyrosine kinase. Block and colleagues found that NOX4 and ROS production was upregulated in angiotensin II treated mesangial cells due to the oxidation of Src. They claim this step is important for angiotensin II induced fibronectin accumulation [133].

Transcriptional factors

A number of transcriptional factors can be regulated by redox signaling including AP-1, Hypoxia inducible factor-1(HIF-1α), p53, Nuclear factor kappa B (NF-κB) and NF-E2-related factor (Nrf2)[134]. These contains cysteine residues at their binding sites [17]. NF-κB is a redox sensitive transcriptional factor that regulates the expression of genes involved in immune and inflammatory responses [8, 19]. ROS has different effects on inhibition and activation of NF-κB and the activation depends on the level of ROS production, cell types and type of stimuli [135, 136]. In colon carcinoma cell lines mitochondrial ROS production during hypoxia plays a dual role by promoting the cell survival by NF-κB activation via c-Src or cell death by over production of ROS [137]. Hypoxia activates a number of transcriptional factors including HIF-1 and HIF-2. HIF-1 is composed of two subunits HIF1-α and β. The levels of HIF-1α is elevated under hypoxic conditions which allows translocation to the nucleus and subsequent dimerization with HIF1-β and binding to promoter elements [119]. Under normoxic conditions HIF will be stabilized by different stimuli by the increased production of ROS [17]. In addition, it has been shown that mitochondrial production of ROS, specially by complex III, was necessary for stabilization of HIF-1α during hypoxia [138]. Hypoxia induces the early epithelial-mesenchymal transition (EMT) in cancer cells by the production of mitochondrial ROS and HIF-1α plays a role in increased invasiveness and migration [139]. In murine neuroblastoma cell line HIF-1α activation by mitochondrial ROS mediates the cyanide induced mitochondrial cell death through upregulation of BNIP3 (Bcl_2/E1B 19 kDa interacting protein, member of the Bcl_2 family) [140]. Under normal redox conditions Nrf2 localizes with Kelch-like-ECH-associating protein 1(Keap1) in the cytoplasm. Keap1 contains Cys residues that are the targets of ROS. Oxidation of Cys triggers the dissociation of Keap1 from Nrf2 allowing Nrf2 translocation into the nucleus and activation of stress-response genes [17, 22].

2. Aims of the study

The overall aim of this study was to analyse the expression of NOX family members in cancer cells and to address the link between intracellular ROS levels and the regulation of GGT expression and activity. More particularly we wanted to study:

- the distribution pattern of NOX isoforms and their regulatory subunits in human cancer cells
- the knockdown effect of p22phox on ROS production in Ishikawa cells
- role of NOX produced ROS in the regulation of GGT activity
- role of mitochondrial ROS production for GGT regulation
- effects of mitochondrial uncouplers and protein kinase Cs on ROS production and GGT expression and activity

3. Summary of papers

Paper I:

This paper presents a RT-qPCR screen on the expression levels of NOX family members and their regulatory subunits in 19 human cancer cell lines and in xenograft tumours. The results of the study showed that:

- all human cancer cell lines tested express a subset of NOX family members and regulatory subunits. However, no apparent correlation between expression pattern and cancer type could be identified, suggesting that each cancer cell lines needs to be tested individually.
- *NOX1, NOX2* and *NOX5* were the NOX core proteins that were most frequently upregulated compared to the expression level in fibroblasts.
- expression of *NOX3* and *NOX4* was found at very low levels or absent from most cell lines tested.
- of the regulatory subunits tested, p67phox was most frequently upregulated compared to fibroblasts.
- in xenograft tumors of two of these three cell lines, the *NOX5* mRNA level was significantly increased compared to the expression level when the cell lines were grown in vitro. Only minor changes were found in expression level of p22phox.

Paper II:

In this paper the effect of endogenouse ROS levels on GGT expression and activity was investigated. Downregulation of NOX activity through stable knockdown of the NOX regulatory subunit p22phox was investigated in Ishikawa cells, and the effects of the NOX inhibitor apocynin and the NOX stimulator PMA was studied in HT-29 cells. The results of the study showed that:

- knockdown of p22phox expression resulted in reduced production of ROS.
- downregulation of p22phox levels reduced the expression of GGT.
- activation of NOX with phorbol ester increases ROS production and induces GGT expression.
- Apocynin treatment reduces both the ROS production and the GGT expression.

Paper III:

In this study the role of mitochondrial derived ROS on GGT expression and activity was investigated. Two mitochondrial uncouplers and one electron transport modulator were used in HT-29 cells in order to alter the ROS levels. Then the combined effect of mitochondrial uncouplers together with PMA was investigated with regards to ROS production and GGT expression. The results obtained showed that:

- Rottlerin and FCCP, two mitochondrial uncouplers, increased the ROS levels and resulted in elevated GGT mRNA transcription.
- Rotenone which is a mitochondrial electron chain inhibitor, increased ROS levels but not significantly *GGT* mRNA expression and activity.
- Incubations with uncouplers together with PMA resulted in a significantly high ROS levels, as well as increased GGT mRNA expression.
- These combined incubations resulted also in increased GGT activity, but after a delayed period of time (8 days).

4. Discussion

This thesis is based on my studies of the analysis of the ROS producing NOX complexes in cancer cells (paper I), the effect of NOX generated ROS on human *GGT1* expression (paper II), and the role of mitochondrial ROS production together with NOX activation for the expression of GGT (paper III).

In the first study (paper I) we have investigated the expression profile of the five members of the NOX gene family and their regulatory subunits in various cancer cell lines and tumour samples. The cell lines tested were human oral and skin squamous cell carcinoma (SCC) cell lines, as well as one adenocarcinoma and one melanoma cell line. Two normal human fibroblast cell lines were used as references. We also analyzed the expression in xenograft tumour of three of the cell lines.

NOX core proteins and their regulatory subunits have been described to have cell type specific expression, localization and distribution [30]. It has been clearly shown that cancer tumours have elevated levels of ROS. Many signalling pathways are ROS sensitive, and alterations in the ROS levels may influence on these signalling pathways and potentially lead to cancer cell proliferation, prevention of apoptosis, survival and metastasis [13, 119, 141]. In cancer cells, high levels of ROS can result from increased metabolic activity, mitochondrial dysfunction, increased cellular receptor signaling and increased activity of oxidases such as the NOX family of oxidases [13]. Several studies have shown that oxidative stress may lead to tumour growth and metastasis [31, 142], and NOX1 has been reported to be highly expressed in several cancers including breast, ovarian, prostate and colon cancers [61, 143, 144].

Over 90% of the oral cancers are squamous cell carcinomas (SCCs) and they are the seventh most frequent cause of cancer death worldwide [145]. The overall survival rate for oral cancer patients is estimated to be 50% and this has not improved over the past two decades [146]. ROS production plays a major role in oral cancers, as well as in periodontitis, and it has also been shown that production of ROS can be induced through HIF-1α by chemotherapeutic drugs and gamma-rays in oral cancer [2, 40, 147-149]. It was found that blood and tumour tissues of oral SCC (OSCC) patients have increased levels of NO and decreased levels of catalase and SOD. This oxidant-antioxidant imbalance can leads to oxidative stress, one of the causes for cancer [150].

Currently there are no studies showing the distribution of NOX isoforms in head and neck cancer cell lines derived from patient tumours. Our results shows that the expression varies considerably between the cell lines, however all cell lines express two or more of the NOX core proteins and several of the regulatory subunits. Thus, all cell lines in our set have the potential to produce ROS via the NOX system if they are adequately stimulated. We also found that cell lines derived from the primary tumour and corresponding metastasis from same patient showed different expression patterns. This indicates that the expression pattern of *NOX* genes may change during the various stages of cancer progression. We also analysed the mRNA expression of *NOX5* and *p22phox* in xenograft tumours of three of the cell lines. The results showed that for two of these three cell lines the *NOX5* mRNA level was significantly increased in the tumor tissues compared to the expression level when the cell lines were grown *in vitro*. Only minor changes were found in the expression level of p22phox. This indicates that the expression of NOX5 may be influenced by the growth conditions and in particular by the microenvironment. However, more studies are needed before concluding whether the expression levels found in the cell lines are changed from when the cells were part of the original tumor in the patient.

In paper II we studied the effect of NOX produced ROS on the expression of GGT. GGT is one of the key enzymes in the regeneration of the anti-oxidant glutathione (GSH), thus its expression levels and activity has major influence on the total oxidative stress burden of the cell. In order to study the relationship between NOX enzymes and GGT we used the Ishikawa and the HT-29 cell lines.

Being able to measure intracellular ROS levels were essential for this study and we were able to establish a robust flow cytometric method for ROS quantification that could be used to monitor the changes in ROS levels as a consequence of the manipulations of the NOX enzymes. Phagocytic cells produce high levels of ROS compared to nonphagocytic cells, and many commercially available kits are available for detection of such high ROS levels. In our hands, these kits were not sensitive enough to be able to detect the low ROS levels in nonphagocytic cells. However they reliably measured ROS levels in phagocytic cell such as the THP-1 cells (data not shown). We chose to use the fluorescently labelled probe (CM-H_2DCFDA) that is redox sensitive (details on materials and methods paper II) and succeeded to quantifying the ROS levels in nonphagocytic cells by Fluorescence Activated Cell Sorter (FACS) analysis.

Ishikawa is an endometrial adenocarcinoma cell line derived from an asian woman. These cells are well-differentiated and display estrogen and progesterone receptors in culture and in induced tumours [151]. We showed in paper I that this cell line expressed mRNA for all NOX core proteins and regulatory subunits including p22phox. Although it had less p67phox mRNA compared to the fibroblast cell lines used as references, we were able to show by flow cytometry that they produced detectable levels of intracellular ROS. The production of ROS through NOX 1-4 activity is regulated by different cytosolic subunits and p22phox plays a critical role for the activity of all these enzymes. p22phox binds with the other cytosolic subunits of the NOX proteins upon activation (only NOX 1-3) and also stabilises the NOX core protein [2, 3, 34, 43]. With the importance of p22phox for the activity of NOX1-4 enzymes we had reasons to assume that knockdown of p22phox would effect the total level of ROS in the cell. We generated stable knockdown of p22phox Ishikawa cells using shRNA targeting p22phox and could show by flow cytometery that the ROS levels were lower than in clones stably tranfected with the control shRNA construct. We obtained the same results in transiently transfected cell, excluding the possibility that the results were due to clonal variations.

Several studies have shown that GGT expression can be upregulated after acute oxidative stress [26, 109, 152, 153]. However, it remains unclear whether changes in intracellular ROS levels following variation in NOX activity can alter GGT expression. To address this question we used Ishikawa p22phox knockdown cells to follow the GGT expression. The results showed that knockdown of p22phox reduces the mRNA expression of *GGT-1* as well as GGT protein level and activity in Ishikawa cells (paper II). This indicates that the NOX system have a potentially important role in the regulation of GGT. To support our finding we used the colon carcinoma cell line HT-29. This cell line is known to express high levels of NOX1 [154], which was also confirmed by our RT-qPCR results of HT-29 cells (data not shown). Treatment with apocynin, a NOX inhibitor, reduced the production of ROS and decreased the expression of GGT (paper II). In addition, treatment with PMA, a stimulator of NOX activity [155], increased both the ROS levels and the expression of GGT (paper II).

Taken together, our results show that variations in the endogenous level of ROS generated by NOX are a significant determinant for basal GGT expression. Higher levels of oxidative stress are correlated with cancer malignancy and poor prognosis [103] which indicates that tumour cells are able to compensate and benefit from such increased oxidative stress situations [6,

20]. GGT may act as one of several adaptive responses to protect against severe oxidative stress and, as was suggested for melanoma therapy, GGT enzyme could thus be a target for cancer treatment.

In paper III we investigated the role of mitochondrial derived ROS on GGT and also whether the combined effect of mitochondrial and NOX production of ROS had additional influence on GGT expression.

Mitochondrial electron transport chain is one of the major sources for ROS production. It is well known that complex I and III are responsible for the ROS production in mitochondrial ETC [62, 65, 156]. Recent studies showing that there is a possible crosstalk between mitochondrial ROS and NOX for amplifying ROS signalling (per reviews [71, 157]). In paper II we showed that GGT expression and activity can be regulated by cellular ROS production by NOX enzymes and in paper III we used different modulators to address whether mitochondrial ROS can also have a role in GGT expression and activity.

First we treated the HT29 cells with mitochondrial uncouplers rottlerin and FCCP. Rottlerin is originally reported as a protein kinase C inhibitor but it also works as mitochondria uncoupler. It reduces the ATP levels and depolarized the mitochondrial membrane potential. Recent data show that rottlerin upregulates the production of ROS in HT-29 cells and induces the heme oxygenase-1 through a PKCδ independent pathway [158-160]. We found that HT29 cells produce high levels of ROS after treatment with rottlerin alone. To confirm this result we used FCCP, which is a classical and potent uncoupler of oxidative phosphorylation, and similar results were obtained. Both uncouplers also upregulated GGT mRNA transcription, but we failed to detect increases in GGT activity when measured after 4 days. The results indicates that mitochondrial ROS play a role in GGT regulation.

Significantly higher ROS levels were found when cells were treated simultaneously with PMA and uncoupler. This increase was apparently higher than the summation of the levels of either PMA or uncoupler alone, indicating a crosstalk in ROS production between mitochondria and NOX activity [71]. In a study by Desouki *et al* it was shown that mitochondria can crosstalk with NOX1 mediated by ROS produced in the mitochondria [61]. As we know HT29 cells have a high expression of NOX1 we hypothesized that the cellular ROS production was from NOX1. The GGT mRNA expression was increased in these cells indicating that mitochondrial ROS can have a role in GGT regulation.

We were however, unable to verify that this amplification of ROS level was due to crosstalk, and also whether PKC activation takes part in the PMA effect. More studies will be needed for this, and also more selective inhibitors. The PKC inhibitors used, Ro 31-8220 and GF 109203X (Gö6850) are not specific or selective for PKC isotypes. Ro 31-8220 was originally known as a PKC inhibitor but it also inhibits other kinases such as MAPK and ERK1/2, and activates JNK and glycogen synthase [161,162]. It was well known that H_2O_2 can activate several protein kinases including ERK1/2 [131]. In our experiments we found that Ro 31-8220 showed decreased production of ROS and reduced *GGT* mRNA expression and activity. The other protein kinase inhibitor used, GF 109203X (Gö6850) may be selective for PKC α and β1 isoforms. This inhibitor showed significantly low levels of ROS production, GGT expression and activity when used with the HT29 cells. When we stimulated the cells with PMA together with GF 109203X high levels of ROS was detected along with increased GGT expression and activity levels. This may be due to the PMA, as we know that PMA is a potent activator of PKC.

We were unable to detect increased GGT enzyme activity the first 4 days after rottlerin and FCCP incubations. This may be caused by endoplasmic reticulum (ER) stress. Lim *et al* have shown that in HT-29 cells rottlerin induce a number of ER markers [163]. In another study, Reuter *et al* demonstrated a similar time course of GGT activity increases following TNF-α incubation of leukemia cells that was caused by ER stress [105]. Similar results were obtained in our study following the FCCP treatment.

Rotenone is a natural hydrophobic pesticide, which acts as a mitochondrial ETC1 inhibitor. It was shown that MCF-7 cells will produce high levels of ROS when treated with rotenone and can induce the apoptosis through JNK and p38 signalling [161]. We found that rotenone increases the ROS and GGT levels but less compare to FCCP.

5. Conclusions

The main conclusions that can be drawn from this study are:

- Of 22 human cancer cell lines tested, all expressed transcripts encoding two or more NOX core proteins and several of the regulatory subunits

- the p67phox expression levels were higher in the metastatic tumour cells compare to primary tumour cells

- ROS produced by NOX complexes are involved in regulating the expression of GGT

- Release of ROS from the mitochondria can effect GGT expression

6. Future studies

Although the function of all NOX isoforms is to catalyze the reduction of molelular oxygen and by that produce ROS; they differ in their expression (paper I), subcellular localization, and mechanism for activation. Due to these differences it is hard to make conclusions about which NOX isoform is playing a role in cancer progression. Experiments such as knockdown studies will be an interesting approach in order to study these differences and to determine their role in cancer progression. In follow up studies of paper II and III it is important to determine which signalling pathways are responsible for the induction of GGT expression, both by the NOX systems and also mitochondrial ETC. In order to answers these questions it will be interesting to develop new cell lines with either up regulated or down regulated NOX isoforms and to test these cell lines in both *in vitro* and *in vivo*. These cell lines will be helpful to understand the role of NOX isoforms in disease progression. Now it is increasingly clear that inhibition of NOX enzymes is a promising pharmacologic concept for oxidative stress. To date there is no specific NOX inhibitors in clinical use. The new knowledge how ROS influences on cancer progression and studies on both oxidant systems like NOX and mitochondria and also the role of antioxidants on these systems will be useful to design inhibitors that could be used in cancer therapy.

7. References

[1] Miyano, K.; Sumimoto, H. Role of the small GTPase Rac in p22phox-dependent NADPH oxidases. *Biochimie* **89:**1133-1144; 2007.

[2] Bedard, K.; Krause, K. H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* **87:**245-313; 2007.

[3] Lambeth, J. D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4:**181-189; 2004.

[4] Nauseef, W. M. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* **283:**16961-16965; 2008.

[5] Sumimoto, H.; Miyano, K.; Takeya, R. Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem Biophys Res Commun* **338:**677-686; 2005.

[6] Ralph, S. J.; Rodriguez-Enriquez, S.; Neuzil, J.; Saavedra, E.; Moreno-Sanchez, R. The causes of cancer revisited: "mitochondrial malignancy" and ROS-induced oncogenic transformation - why mitochondria are targets for cancer therapy. *Mol Aspects Med* **31:**145-170; 2010.

[7] Kehrer, J. P. The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology* **149:**43-50; 2000.

[8] Thannickal, V. J.; Fanburg, B. L. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* **279:**L1005-1028; 2000.

[9] Fu, X.; Kassim, S. Y.; Parks, W. C.; Heinecke, J. W. Hypochlorous acid generated by myeloperoxidase modifies adjacent tryptophan and glycine residues in the catalytic domain of matrix metalloproteinase-7 (matrilysin): an oxidative mechanism for restraining proteolytic activity during inflammation. *J Biol Chem* **278:**28403-28409; 2003.

[10] Hancock, J. T.; Desikan, R.; Neill, S. J. Role of reactive oxygen species in cell signalling pathways. *Biochem Soc Trans* **29:**345-350; 2001.

[11] von Lohneysen, K.; Noack, D.; Jesaitis, A. J.; Dinauer, M. C.; Knaus, U. G. Mutational analysis reveals distinct features of the Nox4-p22 phox complex. *J Biol Chem* **283:**35273-35282; 2008.

[12] Tonks, N. K. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121:**667-670; 2005.

[13] Liou, G. Y.; Storz, P. Reactive oxygen species in cancer. *Free Radic Res* **44:**479-496; 2010.

[14] Jones, D. P. Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol* **295:**C849-868; 2008.

[15] Azad, M. B.; Chen, Y.; Gibson, S. B. Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. *Antioxid Redox Signal* **11:**777-790; 2009.

[16] Sies H, J. D. *Oxidative stress In; Encyclopedia of stress (2nd ed.)* Elsevier AP; 2007.

[17] Trachootham, D.; Lu, W.; Ogasawara, M. A.; Nilsa, R. D.; Huang, P. Redox regulation of cell survival. *Antioxid Redox Signal* **10:**1343-1374; 2008.

[18] Visconti, R.; Grieco, D. New insights on oxidative stress in cancer. *Curr Opin Drug Discov Devel* **12:**240-245; 2009.

[19] Yao, H.; Yang, S. R.; Kode, A.; Rajendrasozhan, S.; Caito, S.; Adenuga, D.; Henry, R.; Edirisinghe, I.; Rahman, I. Redox regulation of lung inflammation: role of NADPH oxidase and NF-kappaB signalling. *Biochem Soc Trans* **35:**1151-1155; 2007.

[20] Xia, C.; Meng, Q.; Liu, L. Z.; Rojanasakul, Y.; Wang, X. R.; Jiang, B. H. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res* **67:**10823-10830; 2007.

[21] Puri, P. L.; Avantaggiati, M. L.; Burgio, V. L.; Chirillo, P.; Collepardo, D.; Natoli, G.; Balsano, C.; Levrero, M. Reactive oxygen intermediates mediate angiotensin II-induced c-Jun.c-Fos heterodimer DNA binding activity and proliferative hypertrophic responses in myogenic cells. *J Biol Chem* **270:**22129-22134; 1995.
[22] Chen, K.; Craige, S. E.; Keaney, J. F., Jr. Downstream targets and intracellular compartmentalization in Nox signaling. *Antioxid Redox Signal* **11:**2467-2480; 2009.
[23] Sies, H. Glutathione and its role in cellular functions. *Free Radic Biol Med* **27:**916-921; 1999.
[24] Zhang, H.; Forman, H. J.; Choi, J. Gamma-glutamyl transpeptidase in glutathione biosynthesis. *Methods Enzymol* **401:**468-483; 2005.
[25] Masella, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; Giovannini, C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J Nutr Biochem* **16:**577-586; 2005.
[26] Wu, G.; Fang, Y. Z.; Yang, S.; Lupton, J. R.; Turner, N. D. Glutathione metabolism and its implications for health. *J Nutr* **134:**489-492; 2004.
[27] Lu, S. C. Regulation of glutathione synthesis. *Mol Aspects Med* **30:**42-59; 2009.
[28] Brigelius-Flohe, R. Glutathione peroxidases and redox-regulated transcription factors. *Biol Chem* **387:**1329-1335; 2006.
[29] Geiszt, M. NADPH oxidases: new kids on the block. *Cardiovasc Res* **71:**289-299; 2006.
[30] Brown, D. I.; Griendling, K. K. Nox proteins in signal transduction. *Free Radic Biol Med* **47:**1239-1253; 2009.
[31] Kamata, T. Roles of Nox1 and other Nox isoforms in cancer development. *Cancer Sci* **100:**1382-1388; 2009.
[32] Keller, T.; Damude, H. G.; Werner, D.; Doerner, P.; Dixon, R. A.; Lamb, C. A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca2+ binding motifs. *Plant Cell* **10:**255-266; 1998.
[33] Bokoch, G. M.; Knaus, U. G. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem Sci* **28:**502-508; 2003.
[34] Lambeth, J. D. Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidases. *Curr Opin Hematol* **9:**11-17; 2002.
[35] Bokoch, G. M.; Diebold, B.; Kim, J. S.; Gianni, D. Emerging evidence for the importance of phosphorylation in the regulation of NADPH oxidases. *Antioxid Redox Signal* **11:**2429-2441; 2009.
[36] Okada, F.; Kobayashi, M.; Tanaka, H.; Kobayashi, T.; Tazawa, H.; Iuchi, Y.; Onuma, K.; Hosokawa, M.; Dinauer, M. C.; Hunt, N. H. The role of nicotinamide adenine dinucleotide phosphate oxidase-derived reactive oxygen species in the acquisition of metastatic ability of tumor cells. *Am J Pathol* **169:**294-302; 2006.
[37] Wang, Y.; Lou, M. F. The regulation of NADPH oxidase and its association with cell proliferation in human lens epithelial cells. *Invest Ophthalmol Vis Sci* **50:**2291-2300; 2009.
[38] Li, Q.; Harraz, M. M.; Zhou, W.; Zhang, L. N.; Ding, W.; Zhang, Y.; Eggleston, T.; Yeaman, C.; Banfi, B.; Engelhardt, J. F. Nox2 and Rac1 regulate H2O2-dependent recruitment of TRAF6 to endosomal interleukin-1 receptor complexes. *Mol Cell Biol* **26:**140-154; 2006.
[39] Anilkumar, N.; Weber, R.; Zhang, M.; Brewer, A.; Shah, A. M. Nox4 and nox2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation. *Arterioscler Thromb Vasc Biol* **28:**1347-1354; 2008.
[40] Giannopoulou, C.; Krause, K. H.; Muller, F. The NADPH oxidase NOX2 plays a role in periodontal pathologies. *Semin Immunopathol* **30:**273-278; 2008.
[41] Geiszt, M.; Leto, T. L. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* **279:**51715-51718; 2004.

[42] Ushio-Fukai, M.; Alexander, R. W.; Akers, M.; Yin, Q.; Fujio, Y.; Walsh, K.; Griendling, K. K. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* **274:**22699-22704; 1999.
[43] Lambeth, J. D.; Kawahara, T.; Diebold, B. Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med* **43:**319-331; 2007.
[44] Ushio-Fukai, M. Localizing NADPH oxidase-derived ROS. *Sci STKE* **2006:**re8; 2006.
[45] Matsuno, K.; Yamada, H.; Iwata, K.; Jin, D.; Katsuyama, M.; Matsuki, M.; Takai, S.; Yamanishi, K.; Miyazaki, M.; Matsubara, H.; Yabe-Nishimura, C. Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. *Circulation* **112:**2677-2685; 2005.
[46] Kikuchi, H.; Hikage, M.; Miyashita, H.; Fukumoto, M. NADPH oxidase subunit, gp91(phox) homologue, preferentially expressed in human colon epithelial cells. *Gene* **254:**237-243; 2000.
[47] Paffenholz, R.; Bergstrom, R. A.; Pasutto, F.; Wabnitz, P.; Munroe, R. J.; Jagla, W.; Heinzmann, U.; Marquardt, A.; Bareiss, A.; Laufs, J.; Russ, A.; Stumm, G.; Schimenti, J. C.; Bergstrom, D. E. Vestibular defects in head-tilt mice result from mutations in Nox3, encoding an NADPH oxidase. *Genes Dev* **18:**486-491; 2004.
[48] Banfi, B.; Malgrange, B.; Knisz, J.; Steger, K.; Dubois-Dauphin, M.; Krause, K. H. NOX3, a superoxide-generating NADPH oxidase of the inner ear. *J Biol Chem* **279:**46065-46072; 2004.
[49] Cheng, G.; Cao, Z.; Xu, X.; van Meir, E. G.; Lambeth, J. D. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* **269:**131-140; 2001.
[50] Yang, S.; Zhang, Y.; Ries, W.; Key, L. Expression of Nox4 in osteoclasts. *J Cell Biochem* **92:**238-248; 2004.
[51] Goldstein, B. J.; Mahadev, K.; Wu, X.; Zhu, L.; Motoshima, H. Role of insulin-induced reactive oxygen species in the insulin signaling pathway. *Antioxid Redox Signal* **7:**1021-1031; 2005.
[52] Chen, K.; Kirber, M. T.; Xiao, H.; Yang, Y.; Keaney, J. F., Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* **181:**1129-1139; 2008.
[53] Kuroda, J.; Nakagawa, K.; Yamasaki, T.; Nakamura, K.; Takeya, R.; Kuribayashi, F.; Imajoh-Ohmi, S.; Igarashi, K.; Shibata, Y.; Sueishi, K.; Sumimoto, H. The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of human vascular endothelial cells. *Genes Cells* **10:**1139-1151; 2005.
[54] Hilenski, L. L.; Clempus, R. E.; Quinn, M. T.; Lambeth, J. D.; Griendling, K. K. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24:**677-683; 2004.
[55] Donko, A.; Peterfi, Z.; Sum, A.; Leto, T.; Geiszt, M. Dual oxidases. *Philos Trans R Soc Lond B Biol Sci* **360:**2301-2308; 2005.
[56] Ohye, H.; Sugawara, M. Dual oxidase, hydrogen peroxide and thyroid diseases. *Exp Biol Med (Maywood)* **235:**424-433; 2010.
[57] De Deken, X.; Wang, D.; Many, M. C.; Costagliola, S.; Libert, F.; Vassart, G.; Dumont, J. E.; Miot, F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem* **275:**23227-23233; 2000.
[58] Grasberger, H.; Refetoff, S. Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent. *J Biol Chem* **281:**18269-18272; 2006.
[59] Ris-Stalpers, C. Physiology and pathophysiology of the DUOXes. *Antioxid Redox Signal* **8:**1563-1572; 2006.
[60] Addabbo, F.; Montagnani, M.; Goligorsky, M. S. Mitochondria and reactive oxygen species. *Hypertension* **53:**885-892; 2009.
[61] Desouki, M. M.; Kulawiec, M.; Bansal, S.; Das, G. M.; Singh, K. K. Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. *Cancer Biol Ther* **4:**1367-1373; 2005.

[62] Kowaltowski, A. J.; de Souza-Pinto, N. C.; Castilho, R. F.; Vercesi, A. E. Mitochondria and reactive oxygen species. *Free Radic Biol Med* **47:**333-343; 2009.
[63] Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem J* **417:**1-13; 2009.
[64] Tretter, L.; Adam-Vizi, V. Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase. *J Neurosci* **24:**7771-7778; 2004.
[65] Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J Physiol* **552:**335-344; 2003.
[66] Radi, R.; Turrens, J. F.; Chang, L. Y.; Bush, K. M.; Crapo, J. D.; Freeman, B. A. Detection of catalase in rat heart mitochondria. *J Biol Chem* **266:**22028-22034; 1991.
[67] Saberi, B.; Shinohara, M.; Ybanez, M. D.; Hanawa, N.; Gaarde, W. A.; Kaplowitz, N.; Han, D. Regulation of H(2)O(2)-induced necrosis by PKC and AMP-activated kinase signaling in primary cultured hepatocytes. *Am J Physiol Cell Physiol* **295:**C50-63; 2008.
[68] Zhou, Q.; Lam, P. Y.; Han, D.; Cadenas, E. c-Jun N-terminal kinase regulates mitochondrial bioenergetics by modulating pyruvate dehydrogenase activity in primary cortical neurons. *J Neurochem* **104:**325-335; 2008.
[69] Moiseeva, O.; Bourdeau, V.; Roux, A.; Deschenes-Simard, X.; Ferbeyre, G. Mitochondrial dysfunction contributes to oncogene-induced senescence. *Mol Cell Biol* **29:**4495-4507; 2009.
[70] Lebedeva, M. A.; Eaton, J. S.; Shadel, G. S. Loss of p53 causes mitochondrial DNA depletion and altered mitochondrial reactive oxygen species homeostasis. *Biochim Biophys Acta* **1787:**328-334; 2009.
[71] Daiber, A. Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochim Biophys Acta* **1797:**897-906; 2010.
[72] Lee, S. B.; Bae, I. H.; Bae, Y. S.; Um, H. D. Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death. *J Biol Chem* **281:**36228-36235; 2006.
[73] Whitfield, J. B. Gamma glutamyl transferase. *Crit Rev Clin Lab Sci* **38:**263-355; 2001.
[74] Hanigan, M. H.; Pitot, H. C. Gamma-glutamyl transpeptidase--its role in hepatocarcinogenesis. *Carcinogenesis* **6:**165-172; 1985.
[75] Tate, S. S. Single-chain precursor of renal gamma-glutamyl transpeptidase. *FEBS Lett* **194:**33-38; 1986.
[76] Huseby, N. E. Separation and characterization of human gamma-glutamyltransferases. *Clin Chim Acta* **111:**39-45; 1981.
[77] Huseby, N. E. Subcellular localization of gamma-glutamyltransferase activity in guinea pig liver. Effect of phenobarbital on the enzyme activity levels. *Clin Chim Acta* **94:**163-171; 1979.
[78] Tate, S. S.; Meister, A. gamma-Glutamyl transpeptidase: catalytic, structural and functional aspects. *Mol Cell Biochem* **39:**357-368; 1981.
[79] Smith, T. K.; Meister, A. Active deglycosylated mammalian gamma-glutamyl transpeptidase. *FASEB J* **8:**661-664; 1994.
[80] Huseby, N. E. Hydrophilic form of gamma-glutamyltransferase: proteolytic formation in liver homogenates and its estimation in serum. *Clin Chim Acta* **124:**113-121; 1982.
[81] Goldberg, D. M. Structural, functional, and clinical aspects of gamma-glutamyltransferase. *CRC Crit Rev Clin Lab Sci* **12:**1-58; 1980.
[82] Pompella, A.; De Tata, V.; Paolicchi, A.; Zunino, F. Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochem Pharmacol* **71:**231-238; 2006.
[83] Meister, A.; Anderson, M. E. Glutathione. *Annu Rev Biochem* **52:**711-760; 1983.
[84] Huseby, N.-E. *GLUTATHIONE AND SULFUR AMINO ACIDS IN HUMAN HEALTH AND DISEASE.* WILEY; 2009.

[85] Heisterkamp, N.; Groffen, J.; Warburton, D.; Sneddon, T. P. The human gamma-glutamyltransferase gene family. *Hum Genet* **123:**321-332; 2008.
[86] Lieberman, M. W.; Wiseman, A. L.; Shi, Z. Z.; Carter, B. Z.; Barrios, R.; Ou, C. N.; Chevez-Barrios, P.; Wang, Y.; Habib, G. M.; Goodman, J. C.; Huang, S. L.; Lebovitz, R. M.; Matzuk, M. M. Growth retardation and cysteine deficiency in gamma-glutamyl transpeptidase-deficient mice. *Proc Natl Acad Sci U S A* **93:**7923-7926; 1996.
[87] Kumar, T. R.; Wiseman, A. L.; Kala, G.; Kala, S. V.; Matzuk, M. M.; Lieberman, M. W. Reproductive defects in gamma-glutamyl transpeptidase-deficient mice. *Endocrinology* **141:**4270-4277; 2000.
[88] Chevez-Barrios, P.; Wiseman, A. L.; Rojas, E.; Ou, C. N.; Lieberman, M. W. Cataract development in gamma-glutamyl transpeptidase-deficient mice. *Exp Eye Res* **71:**575-582; 2000.
[89] Miller, L. T.; Watson, W. H.; Kirlin, W. G.; Ziegler, T. R.; Jones, D. P. Oxidation of the glutathione/glutathione disulfide redox state is induced by cysteine deficiency in human colon carcinoma HT29 cells. *J Nutr* **132:**2303-2306; 2002.
[90] Hanigan MH, R. W. Extracellular glutathione is a source of cycteine for cells that express gamma-glutamyl transpeptidase. *Biochemistry* **32(24):**6302-6306; 1993.
[91] Pawlak, A.; Lahuna, O.; Bulle, F.; Suzuki, A.; Ferry, N.; Siegrist, S.; Chikhi, N.; Chobert, M. N.; Guellaen, G.; Laperche, Y. gamma-Glutamyl transpeptidase: a single copy gene in the rat and a multigene family in the human genome. *J Biol Chem* **263:**9913-9916; 1988.
[92] Chikhi, N.; Holic, N.; Guellaen, G.; Laperche, Y. Gamma-glutamyl transpeptidase gene organization and expression: a comparative analysis in rat, mouse, pig and human species. *Comp Biochem Physiol B Biochem Mol Biol* **122:**367-380; 1999.
[93] Diederich, M.; Wellman, M.; Visvikis, A.; Puga, A.; Siest, G. The 5' untranslated region of the human gamma-glutamyl transferase mRNA contains a tissue-specific active translational enhancer. *FEBS Lett* **332:**88-92; 1993.
[94] Visvikis, A.; Pawlak, A.; Accaoui, M. J.; Ichino, K.; Leh, H.; Guellaen, G.; Wellman, M. Structure of the 5' sequences of the human gamma-glutamyltransferase gene. *Eur J Biochem* **268:**317-325; 2001.
[95] Kugelman, A.; Choy, H. A.; Liu, R.; Shi, M. M.; Gozal, E.; Forman, H. J. gamma-Glutamyl transpeptidase is increased by oxidative stress in rat alveolar L2 epithelial cells. *Am J Respir Cell Mol Biol* **11:**586-592; 1994.
[96] Deneke, S. M.; Fanburg, B. L. Regulation of cellular glutathione. *Am J Physiol* **257:**L163-173; 1989.
[97] Borud, O.; Mortensen, B.; Mikkelsen, I. M.; Leroy, P.; Wellman, M.; Huseby, N. E. Regulation of gamma-glutamyltransferase in cisplatin-resistant and -sensitive colon carcinoma cells after acute cisplatin and oxidative stress exposures. *Int J Cancer* **88:**464-468; 2000.
[98] Dickinson, D. A.; Levonen, A. L.; Moellering, D. R.; Arnold, E. K.; Zhang, H.; Darley-Usmar, V. M.; Forman, H. J. Human glutamate cysteine ligase gene regulation through the electrophile response element. *Free Radic Biol Med* **37:**1152-1159; 2004.
[99] Yamane, Y.; Furuichi, M.; Song, R.; Van, N. T.; Mulcahy, R. T.; Ishikawa, T.; Kuo, M. T. Expression of multidrug resistance protein/GS-X pump and gamma-glutamylcysteine synthetase genes is regulated by oxidative stress. *J Biol Chem* **273:**31075-31085; 1998.
[100] Liu, R. M.; Hu, H.; Robison, T. W.; Forman, H. J. Increased gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase activities enhance resistance of rat lung epithelial L2 cells to quinone toxicity. *Am J Respir Cell Mol Biol* **14:**192-197; 1996.
[101] Knickelbein, R. G.; Ingbar, D. H.; Seres, T.; Snow, K.; Johnston, R. B., Jr.; Fayemi, O.; Gumkowski, F.; Jamieson, J. D.; Warshaw, J. B. Hyperoxia enhances expression of gamma-glutamyl transpeptidase and increases protein S-glutathiolation in rat lung. *Am J Physiol* **270:**L115-122; 1996.

[102] Huseby, N. E.; Asare, N.; Wetting, S.; Mikkelsen, I. M.; Mortensen, B.; Sveinbjornsson, B.; Wellman, M. Nitric oxide exposure of CC531 rat colon carcinoma cells induces gamma-glutamyltransferase which may counteract glutathione depletion and cell death. *Free Radic Res* **37:**99-107; 2003.
[103] Ripple, M. O.; Pickhardt, P. A.; Wilding, G. Alteration in gamma-glutamyl transpeptidase activity and messenger RNA of human prostate carcinoma cells by androgen. *Cancer Res* **57:**2428-2433; 1997.
[104] Griffiths, S. A.; Good, V. M.; Gordon, L. A.; Hudson, E. A.; Barrett, M. C.; Munks, R. J.; Manson, M. M. Characterization of a promoter for gamma-glutamyl transpeptidase activated in rat liver in response to aflatoxin B1 and ethoxyquin. *Mol Carcinog* **14:**251-262; 1995.
[105] Reuter, S.; Schnekenburger, M.; Cristofanon, S.; Buck, I.; Teiten, M. H.; Daubeuf, S.; Eifes, S.; Dicato, M.; Aggarwal, B. B.; Visvikis, A.; Diederich, M. Tumor necrosis factor alpha induces gamma-glutamyltransferase expression via nuclear factor-kappaB in cooperation with Sp1. *Biochem Pharmacol* **77:**397-411; 2009.
[106] Zhang, H.; Forman, H. J. Redox regulation of gamma-glutamyl transpeptidase. *Am J Respir Cell Mol Biol* **41:**509-515; 2009.
[107] Pandur, S.; Pankiv, S.; Johannessen, M.; Moens, U.; Huseby, N. E. Gamma-glutamyltransferase is upregulated after oxidative stress through the Ras signal transduction pathway in rat colon carcinoma cells. *Free Radic Res* **41:**1376-1384; 2007.
[108] Accaoui, M. J.; Enoiu, M.; Mergny, M.; Masson, C.; Dominici, S.; Wellman, M.; Visvikis, A. Gamma-glutamyltranspeptidase-dependent glutathione catabolism results in activation of NF-kB. *Biochem Biophys Res Commun* **276:**1062-1067; 2000.
[109] Hanigan, M. H.; Frierson, H. F., Jr.; Swanson, P. E.; De Young, B. R. Altered expression of gamma-glutamyl transpeptidase in human tumors. *Hum Pathol* **30:**300-305; 1999.
[110] Frierson, H. F., Jr.; Theodorescu, D.; Mills, S. E.; Hanigan, M. H. gamma-Glutamyl transpeptidase in normal and neoplastic prostate glands. *Mod Pathol* **10:**1-6; 1997.
[111] Obrador, E.; Carretero, J.; Ortega, A.; Medina, I.; Rodilla, V.; Pellicer, J. A.; Estrela, J. M. gamma-Glutamyl transpeptidase overexpression increases metastatic growth of B16 melanoma cells in the mouse liver. *Hepatology* **35:**74-81; 2002.
[112] Strasak, A. M.; Pfeiffer, R. M.; Klenk, J.; Hilbe, W.; Oberaigner, W.; Gregory, M.; Concin, H.; Diem, G.; Pfeiffer, K. P.; Ruttmann, E.; Ulmer, H. Prospective study of the association of gamma-glutamyltransferase with cancer incidence in women. *Int J Cancer* **123:**1902-1906; 2008.
[113] Strasak, A. M.; Rapp, K.; Brant, L. J.; Hilbe, W.; Gregory, M.; Oberaigner, W.; Ruttmann, E.; Concin, H.; Diem, G.; Pfeiffer, K. P.; Ulmer, H. Association of gamma-glutamyltransferase and risk of cancer incidence in men: a prospective study. *Cancer Res* **68:**3970-3977; 2008.
[114] Mason, J. E.; Starke, R. D.; Van Kirk, J. E. Gamma-glutamyl transferase: a novel cardiovascular risk biomarker. *Prev Cardiol* **13:**36-41; 2010.
[115] Pani, G.; Galeotti, T.; Chiarugi, P. Metastasis: cancer cell's escape from oxidative stress. *Cancer Metastasis Rev* **29:**351-378; 2010.
[116] Chiarugi, P. PTPs versus PTKs: the redox side of the coin. *Free Radic Res* **39:**353-364; 2005.
[117] Gates, K. S.; Tanner, J. J.; Parsons, Z. D.; Cummings, A. H.; Zhou, H. Redox Regulation of Protein Tyrosine Phosphatases: Structural and Chemical Aspects. *Antioxid Redox Signal*; 2010.
[118] Mahadev, K.; Zilbering, A.; Zhu, L.; Goldstein, B. J. Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *J Biol Chem* **276:**21938-21942; 2001.

[119] Weinberg, F.; Chandel, N. S. Reactive oxygen species-dependent signaling regulates cancer. *Cell Mol Life Sci* **66:**3663-3673; 2009.
[120] Vanhaesebroeck, B.; Alessi, D. R. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* **346 Pt 3:**561-576; 2000.
[121] Lawlor, M. A.; Mora, A.; Ashby, P. R.; Williams, M. R.; Murray-Tait, V.; Malone, L.; Prescott, A. R.; Lucocq, J. M.; Alessi, D. R. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J* **21:**3728-3738; 2002.
[122] Sarbassov, D. D.; Guertin, D. A.; Ali, S. M.; Sabatini, D. M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307:**1098-1101; 2005.
[123] Naughton, R.; Quiney, C.; Turner, S. D.; Cotter, T. G. Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. *Leukemia* **23:**1432-1440; 2009.
[124] Meng, T. C.; Fukada, T.; Tonks, N. K. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* **9:**387-399; 2002.
[125] Leslie, N. R.; Bennett, D.; Lindsay, Y. E.; Stewart, H.; Gray, A.; Downes, C. P. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J* **22:**5501-5510; 2003.
[126] Jiang, B. H.; Liu, L. Z. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta* **1784:**150-158; 2008.
[127] Liu, L. Z.; Hu, X. W.; Xia, C.; He, J.; Zhou, Q.; Shi, X.; Fang, J.; Jiang, B. H. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. *Free Radic Biol Med* **41:**1521-1533; 2006.
[128] Connor, K. M.; Subbaram, S.; Regan, K. J.; Nelson, K. K.; Mazurkiewicz, J. E.; Bartholomew, P. J.; Aplin, A. E.; Tai, Y. T.; Aguirre-Ghiso, J.; Flores, S. C.; Melendez, J. A. Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. *J Biol Chem* **280:**16916-16924; 2005.
[129] Chiarugi, P.; Buricchi, F. Protein tyrosine phosphorylation and reversible oxidation: two cross-talking posttranslation modifications. *Antioxid Redox Signal* **9:**1-24; 2007.
[130] Frank, G. D.; Eguchi, S. Activation of tyrosine kinases by reactive oxygen species in vascular smooth muscle cells: significance and involvement of EGF receptor transactivation by angiotensin II. *Antioxid Redox Signal* **5:**771-780; 2003.
[131] Mehdi, M. Z.; Azar, Z. M.; Srivastava, A. K. Role of receptor and nonreceptor protein tyrosine kinases in H2O2-induced PKB and ERK1/2 signaling. *Cell Biochem Biophys* **47:**1-10; 2007.
[132] Kato, M.; Iwashita, T.; Akhand, A. A.; Liu, W.; Takeda, K.; Takeuchi, K.; Yoshihara, M.; Hossain, K.; Wu, J.; Du, J.; Oh, C.; Kawamoto, Y.; Suzuki, H.; Takahashi, M.; Nakashima, I. Molecular mechanism of activation and superactivation of Ret tyrosine kinases by ultraviolet light irradiation. *Antioxid Redox Signal* **2:**841-849; 2000.
[133] Block, K.; Eid, A.; Griendling, K. K.; Lee, D. Y.; Wittrant, Y.; Gorin, Y. Nox4 NAD(P)H oxidase mediates Src-dependent tyrosine phosphorylation of PDK-1 in response to angiotensin II: role in mesangial cell hypertrophy and fibronectin expression. *J Biol Chem* **283:**24061-24076; 2008.
[134] Reuter, S.; Gupta, S. C.; Chaturvedi, M. M.; Aggarwal, B. B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic Biol Med*; 2010.
[135] Kabe, Y.; Ando, K.; Hirao, S.; Yoshida, M.; Handa, H. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal* **7:**395-403; 2005.
[136] Pantano, C.; Reynaert, N. L.; van der Vliet, A.; Janssen-Heininger, Y. M. Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway. *Antioxid Redox Signal* **8:**1791-1806; 2006.
[137] Lluis, J. M.; Buricchi, F.; Chiarugi, P.; Morales, A.; Fernandez-Checa, J. C. Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of nuclear factor-{kappa}B via c-SRC and oxidant-dependent cell death. *Cancer Res* **67:**7368-7377; 2007.

[138] Bell, E. L.; Klimova, T. A.; Eisenbart, J.; Moraes, C. T.; Murphy, M. P.; Budinger, G. R.; Chandel, N. S. The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. *J Cell Biol* **177:**1029-1036; 2007.
[139] Cannito, S.; Novo, E.; Compagnone, A.; Valfre di Bonzo, L.; Busletta, C.; Zamara, E.; Paternostro, C.; Povero, D.; Bandino, A.; Bozzo, F.; Cravanzola, C.; Bravoco, V.; Colombatto, S.; Parola, M. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. *Carcinogenesis* **29:**2267-2278; 2008.
[140] Zhang, L.; Li, L.; Liu, H.; Prabhakaran, K.; Zhang, X.; Borowitz, J. L.; Isom, G. E. HIF-1alpha activation by a redox-sensitive pathway mediates cyanide-induced BNIP3 upregulation and mitochondrial-dependent cell death. *Free Radic Biol Med* **43:**117-127; 2007.
[141] Lambeth, J. D.; Krause, K. H.; Clark, R. A. NOX enzymes as novel targets for drug development. *Semin Immunopathol* **30:**339-363; 2008.
[142] Nishikawa, M. Reactive oxygen species in tumor metastasis. *Cancer Lett* **266:**53-59; 2008.
[143] Lim, S. D.; Sun, C.; Lambeth, J. D.; Marshall, F.; Amin, M.; Chung, L.; Petros, J. A.; Arnold, R. S. Increased Nox1 and hydrogen peroxide in prostate cancer. *Prostate* **62:**200-207; 2005.
[144] Szanto, I.; Rubbia-Brandt, L.; Kiss, P.; Steger, K.; Banfi, B.; Kovari, E.; Herrmann, F.; Hadengue, A.; Krause, K. H. Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease. *J Pathol* **207:**164-176; 2005.
[145] Czesnikiewicz-Guzik, M.; Lorkowska, B.; Zapala, J.; Czajka, M.; Szuta, M.; Loster, B.; Guzik, T. J.; Korbut, R. NADPH oxidase and uncoupled nitric oxide synthase are major sources of reactive oxygen species in oral squamous cell carcinoma. Potential implications for immune regulation in high oxidative stress conditions. *J Physiol Pharmacol* **59:**139-152; 2008.
[146] Arora, S.; Matta, A.; Shukla, N. K.; Deo, S. V.; Ralhan, R. Identification of differentially expressed genes in oral squamous cell carcinoma. *Mol Carcinog* **42:**97-108; 2005.
[147] Sasabe, E.; Yang, Z.; Ohno, S.; Yamamoto, T. Reactive oxygen species produced by the knockdown of manganese-superoxide dismutase up-regulate hypoxia-inducible factor-1alpha expression in oral squamous cell carcinoma cells. *Free Radic Biol Med* **48:**1321-1329; 2010.
[148] Ding, H.; Han, C.; Guo, D.; Chin, Y. W.; Ding, Y.; Kinghorn, A. D.; D'Ambrosio, S. M. Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS-mediated mechanism. *Nutr Cancer* **61:**348-356; 2009.
[149] Sasabe, E.; Zhou, X.; Li, D.; Oku, N.; Yamamoto, T.; Osaki, T. The involvement of hypoxia-inducible factor-1alpha in the susceptibility to gamma-rays and chemotherapeutic drugs of oral squamous cell carcinoma cells. *Int J Cancer* **120:**268-277; 2007.
[150] Gokul, S.; Patil, V. S.; Jailkhani, R.; Hallikeri, K.; Kattappagari, K. K. Oxidant-antioxidant status in blood and tumor tissue of oral squamous cell carcinoma patients. *Oral Dis* **16:**29-33; 2010.
[151] Nishida, M. The Ishikawa cells from birth to the present. *Hum Cell* **15:**104-117; 2002.
[152] Forman, H. J.; Zhang, H.; Rinna, A. Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med* **30:**1-12; 2009.
[153] Pompella, A.; Corti, A.; Paolicchi, A.; Giommarelli, C.; Zunino, F. Gamma-glutamyltransferase, redox regulation and cancer drug resistance. *Curr Opin Pharmacol* **7:**360-366; 2007.
[154] Geiszt, M.; Lekstrom, K.; Brenner, S.; Hewitt, S. M.; Dana, R.; Malech, H. L.; Leto, T. L. NAD(P)H oxidase 1, a product of differentiated colon epithelial cells, can partially replace glycoprotein 91phox in the regulated production of superoxide by phagocytes. *J Immunol* **171:**299-306; 2003.

[155] Takeya, R.; Ueno, N.; Kami, K.; Taura, M.; Kohjima, M.; Izaki, T.; Nunoi, H.; Sumimoto, H. Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem* **278:**25234-25246; 2003.
[156] Liu, Y.; Fiskum, G.; Schubert, D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* **80:**780-787; 2002.
[157] Trachootham, D.; Alexandre, J.; Huang, P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* **8:**579-591; 2009.
[158] Soltoff, S. P. Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. *Trends Pharmacol Sci* **28:**453-458; 2007.
[159] Park, E. J.; Lim, J. H.; Nam, S. I.; Park, J. W.; Kwon, T. K. Rottlerin induces heme oxygenase-1 (HO-1) up-regulation through reactive oxygen species (ROS) dependent and PKC delta-independent pathway in human colon cancer HT29 cells. *Biochimie* **92:**110-115; 2010.
[160] Soltoff, S. P. Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J Biol Chem* **276:**37986-37992; 2001.
[161] Hers, I.; Tavare, J. M.; Denton, R. M. The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity. *FEBS Lett* **460:**433-436; 1999.
[162] Standaert, M. L.; Bandyopadhyay, G.; Antwi, E. K.; Farese, R. V. RO 31-8220 activates c-Jun N-terminal kinase and glycogen synthase in rat adipocytes and L6 myotubes. Comparison to actions of insulin. *Endocrinology* **140:**2145-2151; 1999.
[163] Lim, J. H.; Park, J. W.; Kim, S. H.; Choi, Y. H.; Choi, K. S.; Kwon, T. K. Rottlerin induces pro-apoptotic endoplasmic reticulum stress through the protein kinase C-delta-independent pathway in human colon cancer cells. *Apoptosis* **13:**1378-1385; 2008.

ISBN 978-82-7589-287-2